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TWO NEW HORMONES: PROHORMONE ATRIAL NATRIURETIC PEPTIDES 1-30 AND 31-67 CIRCULATE IN MAN

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SUMMARY: Two peptides consisting of amino acids 1-30 and 31-67 of the N-terminal end of the prohormone of atrial natriuretic factor (pro ANF) which vasodilate aortas in vitro, lower blood pressure in vivo, and have natriuretic properties were found to circulate in 54 normal human volunteers. The mean circulating concentration of pro ANF 1-30 was 1861 ± 87 pg/ml (SEM) while pro ANF 31-67 mean concentration was 1478 ± 71 pg/ml versus a level of 67 ± 3 pg/ml for atrial natriuretic factor (ANF). In chronic renal failure their mean concentrations increased to 40,484 ± 6,929 pg/ml (SEM), 108,566 ± 16,888 pg/ml, and 348 ± 81 pg/ml for pro ANFs 1-30 and 31-67 and ANF respectively. Since pro ANF 1-30 and pro ANF 31-67 circulate in man and have physiologic effects they meet the criteria of two new hormones.

Atrial natriuretic factor (ANF), the 28 amino acid carboxy-terminal end of the 126 amino acid prohormone has natriuretic, diuretic, and potent vasodilator properties (1-4). Recently we have found that peptides from the N-terminal end of this prohormone, i.e. amino acids 1-30 (pro ANF 1-30) and amino acids 31-67 (pro ANF 31-67) (Fig. 1) also have potent vasodilatory properties equal to ANF (5). These peptides vasodilatory effects were associated with a four to five fold increase in cyclic GMP which was found to be due to activation of particulate guanylate cyclase [E.C. 4.6.1.2] (5). Pro ANFs 1-30 and pro ANF 31-67 also enhance the guanylate cyclase-cyclic GMP system in the kidney similar to ANF (6).

A hormone by definition needs to be produced in one organ (or gland), circulate in the bloodstream, and then have physiologic effects in another organ or tissue. Although pro ANFs 1-30 and 31-67 are synthesized in the heart and have effects in other tissues (kidney and aorta) (5,6), whether they circulate or not has not been determined. The present study was designed to investigate whether or not pro ANFs 1-30 and 31-67 circulate

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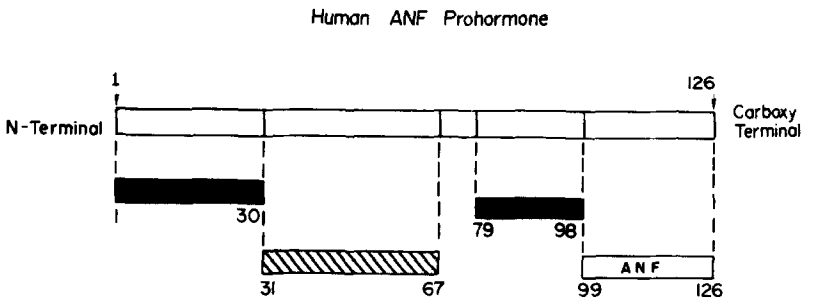

Figure 1. Human prohormone of atrial natriuretic factor. The numbers of the various amino acids correspond to the peptide fragments described in the text. Number one is the first amino acid at the amino (N)-terminal end of the prohormone.

normally in man. Both peptides were found to circulate in 54 normal volunteers. In addition to circulating in normal individuals these peptides increased markedly in 15 persons with chronic renal failure.

MATERIALS AND METHODS

Fifty-four normal volunteers, ages 22-49, all normotensive with their blood pressures below 138/88 were studied. These 35 men and 19 women had heart rates ranging from 60 to 92 beats per minute with respirations between 12 to 14 per minute. None of these volunteers had any known disease. Importantly, none of these subjects had any abnormality of salt and water metabolism. None of the 54 volunteers were on any medication except that seven of the 19 women were on birth control pills. (The results are subdivided below for women on birth control pills.)

Fifteen male patients with chronic renal failure and varying in age from 28 to 75 years were also studied. The severity of their renal failure as defined by their serum creatinine and blood urea nitrogen concentrations is listed in Table 1 with their circulating concentrations of pro ANF 1-30, pro ANF 31-67, and ANF. The respiratory rates for the 15 chronic renal failure patients were between 10 to 16 respirations per minute with heart rates ranging from 66 to 92 beats per minute. Their blood pressure ranged from 104/70 mmHg to 170/100 mmHg (Table 1).

Radioimmunoassays to measure the circulating concentration of pro ANF 1-30, pro ANF 31-67 and ANF (amino acids 99-126) were developed utilizing synthetic ^{125}I-labeled peptides and antisera purchased from Peninsula Laboratories (Belmont, CA). We evaluated the respective antisera and found that the antisera to pro ANF 1-30 had no detectable cross-reactivity with either ANF or pro ANF 31-67. The antisera to pro ANF 31-67, likewise, is very specific with no detectable cross-reactivity with either ANF or pro ANF 1-30. Neither antisera had any cross-reactivity with vasopressin, somatostatin or oxytocin (7).

To test these peptides for purity they were subjected to high pressure liquid chromatography utilizing a C-18 column. The flow rate was 1 ml/min with 0.1 percent trifluroacetate (TFA) solvent in pump A and 60 percent acetonitrile in 0.1 percent TFA in pump B. A gradient of 0 to 60 percent B was achieved in 40 minutes. The UV deflection was set at 210 nanometers and 1 µg/ml of each peptide was injected. Three separate and distinct peaks were found at 32 percent, 48.5 percent, and 39 percent acetonitrile respectively for ANF, pro ANF 1-30, and pro ANF 31-67.

To determine if these peptides circulate in man, samples from normal and chronic renal failure patients were collected in 5 ml EDTA tubes and immediately centrifuged at 3000 xg for 15 minutes. Following

centrifugation the plasma was extracted with 100 percent ethanol (1:1 dilution), vortexed, and allowed to stand at 4°C for 30 minutes similar to the method previously described to extract somatostatin for assay (8). Following 30 minutes at 4°C, the samples were centrifuged at 3000 xg for 15 minutes and the supernatants taken to dryness via controlled nitrogen flow. The samples were then ready for radioimmunoassay.

For each radioimmunoassay, the extracted plasma was first reconstituted in 100 µl of 0.1 M phosphate buffer (pH 7.4) containing 0.05 M NaCl, 0.1 percent bovine serum albumin, 0.1 percent Triton X-100, and 0.01 percent 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 hours. Then 100 µl of the I^{125}-labeled peptides (10,000 cpm) were added, mixed, and incubated for 18 hours at 4°C. The precipitation of the antibody bound tracer was accomplished by adding 100 µl of goat antirabbit globulin after the above 18 hour period and incubating this mixture for 2 hours at room temperature. Each tube was then centrifuged at 3000 x g for 20 minutes. 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 ANFs 1-30, 31-67, and ANF were 4.8 percent, 5.3 percent, and 5.7 percent respectively. The interassay coefficient of variation was 8 percent for both pro ANF 1-30 and 31-67 while ANFs interassay variation was 6.9 percent.

Recovery was examined by adding synthetic unlabeled pro ANF 1-30 and pro ANF 31-67 at 100, 200, and 400 pg/ml to pooled plasma. Recovery for pro ANF 1-30 was 83.5 ± 13.2 (S.D.) percent while pro ANF 31-67 recovery was 100.9 ± 8.9 percent. Recovery of ANF was 92 ± 11 (S.D.) percent.

RESULTS

The mean circulating concentrations of immunoreactive (ir) pro ANF 1-30, ir pro ANF 31-67 and ir ANF are shown in Fig. 2. The circulating concentrations of these peptides were similar in both women and men (Fig. 2). The mean concentration of ir pro ANF 1-30 in 35 men was 1869 ± 117 pg/ml (SEM) while in 19 women its concentration was 1847 ± 127 pg/ml. The concentration of ir-pro ANF 31-67 in these 35 men was 1459 ± 94 pg/ml (SEM) while its concentration in these 19 women was 1400 ± 105 pg/ml. There were only 3 persons among these 54 volunteers who smoked cigarettes. Cigarette smoking did not effect the level of these 2 circulating peptides as the mean concentration of ir-pro ANF 1-30 was 1841 ± 120 pg/ml while ir-pro ANF 31-67 was 1461 ± 116 pg/ml in these smokers. Seven of the women were on birth control pills. Their ir-pro ANF 1-30 and ir pro ANF 31-67 concentrations averaged 1750 ± 139 pg/ml (SEM) and 1274 ± 118 pg/ml versus 1903 ± 186 pg/ml and 1473 ± 151 pg/ml respectively for the 12 women not taking exogenous conjugated estrogens. The average ANF concentration for women on birth control pills was 62 ± 5 pg/ml (SEM) versus 68 ± 4 pg/ml for those not on birth control pills. The difference in concentration of each of these circulating peptides for the women taking birth control pills compared to those women not taking birth control pills was not significant when examined by the Student's t test for unpaired values.

The circulating concentrations of both pro ANF 1-30 and pro ANF 31-67 were both significantly higher than the concentration of ANF which averaged

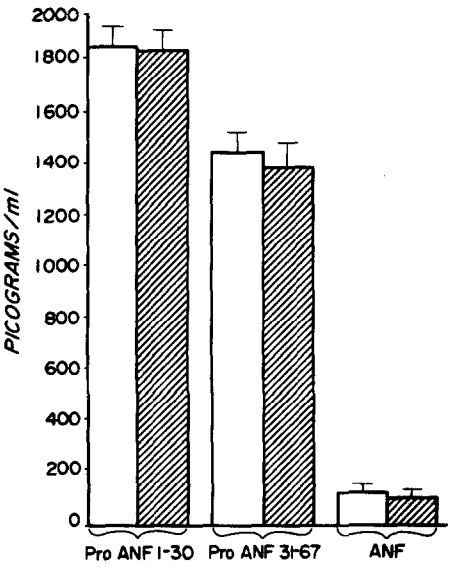

Figure 2. Circulating concentrations of human prohormone atrial natriuretic factors 1-30, 31-67 and 99-126 (ANF) in man. The concentrations illustrated are the means ± standard errors of the means for 54 normal individuals that were assayed in triplicate. The values for men of these peptides is depicted by the open bars while their circulating concentrations in women is illustrated with the diagonal line bars. The concentrations of both pro ANF 1-30 and pro ANF 31-67 were significantly higher ($p < 0.001$) than ANF when analyzed by the Student's t test for unpaired values.

67 ± 3 pg/ml (SEM) in these 54 volunteers. To determine if chronic renal failure has any effect on the circulating levels of these peptides, we studied 15 male patients with chronic renal failure. As seen in Table 1, the circulating levels of pro ANF 1-30 and pro ANF 31-67 markedly increased in renal failure. Pro ANF 1-30 increased 20 fold while pro ANF 31-67 increased over 70 fold (Table 1). The ANF levels in these patients increased approximately 5 fold (Table 1).

DISCUSSION

The present investigation demonstrates that pro ANF 1-30 and pro ANF 31-67 which vasodilate aortas (5) circulate normally in man at much higher concentrations than ANF. The reason that these 2 peptides circulate at much higher concentrations than ANF is unknown but may relate to how long each peptide circulates. Atrial natriuretic factor only circulates for 3 minutes (9) while in preliminary studies in rats we have found that pro ANFs 1-30 and 31-67 circulate for more than an hour. In renal failure, pro ANF 31-67 increased over 70 fold while pro ANF 1-30 increased 20 fold suggesting that pro ANF 31-67 may circulate longer than pro ANF 1-30 since its concentration increased more in renal failure. It may be, however, that the breakdown of 31-67 is exclusively in the kidney while pro ANF 1-30

TABLE 1

PRO ANFS 1-30 AND 31-67 CIRCULATING CONCENTRATIONS IN CHRONIC RENAL FAILURE

Subjects	Pro ANF* 1-30 (pg/ml)	Pro ANF 31-67 (pg/ml)	ANF (pg/ml)	BUN (mg/dl)	Creatinine (mg/dl)	BP (mmHg)	Heart Rate (bpm)
1	25,490	96,450	319	57	10.5	160/80	76
2	101,430	110,720	700	47	11.3	118/70	68
3	66,400	256,150	1232	35	7.9	140/90	92
4	20,510	47,610	243	60	17.7	104/70	80
5	13,170	79,940	231	67	12.2	140/72	72
6	8,180	28,410	127	39	6.7	170/100	80
7	12,870	45,730	101	90	17.1	124/80	66
8	29,500	100,870	300	76	17.3	120/80	84
9	60,280	165,610	513	70	11.1	140/80	80
10	37,850	61,840	133	27	4.7	178/102	86
11	36,750	109,780	271	90	13.4	150/70	80
12	32,490	98,920	404	58	8.7	138/80	88
13	74,500	231,520	410	25	9.6	170/100	76
14	32,220	103,740	121	53	17.7	170/70	88
15	11,400	42,240	251	66	9.9	140/80	70
Mean	40,484	108,566	348	57	11.7	144/82	79
SEM	6,929	16,888	81				

*Pro ANF = prohormone atrial natriuretic factor; ANF = atrial natriuretic factor; BUN = blood urea nitrogen; BP = blood pressure; SEM = Standard error of the mean.

is metabolized in other tissues as well, resulting in lower concentrations in chronic renal failure. We are currently determining exactly how long each of these peptides circulate.

Since pro ANF 1-30 and pro ANF 31-67 circulate normally in man and have effects in different tissues from which they are released (5,6) they are actually hormones. There are, thus, at least 3 hormones contained in the 126 amino acid prohormone similar to the prohormone for proopiomelanocortin which contains several hormones within its amino acid sequence (10,11). It is important to note that amino acids 79-98 (i.e. pro ANF 79-98 of Fig. 1) also vasodilates pre-constricted aortas (5). We have not as yet developed a radioimmonoassay for pro ANF 79-98 since this peptide does not have a tyrosine in it to label with iodine, but our data (5,6) would suggest that

79-98 may also be a hormone if it circulates. Thus, 4 or more hormones may be contained in this 126 amino acid prohormone. The above 4 peptides are what one might expect to be formed by proteases in the circulation. Atrial natriuretic factor is formed by proteolytic separation of the final 28 amino acids at the carboxy terminal end of this 126 amino acid prohormone (Fig. 1). Both trypsin and thrombin can break the arginine-serine bond between amino acids 98-99 to form ANF (12). Both trypsin and thrombin may also break a similar bond between amino acids 67-68 (arginine-aspartic acid) to form peptide fragment 1-67. In addition to trypsin and thrombin there is a large family of aspartic proteases [E.C. 4.23] in the circulation (13-17). Three peptides that could be formed by aspartic proteases attacking the aspartic acid bonds in this prohormone would consist of amino acids 1-30, 31-67, and 79-98 (Fig. 1).

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