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Further Characterization and Evidence for a Precursor in the Formation of Plasma Antinatriferic Factor (40371)

K. A. GRUBER AND V. M. BUCKALEW, JR.

Departments of Physiology and Pharmacology and Medicine, Bowman Gray School of Medicine, Winston-Salem, North Carolina 27103

Considerable evidence supports the existence of a humoral natriuretic factor which modulates the renal response to extracellular fluid volume (ECFV) expansion (1). Reports from this laboratory have demonstrated a factor in plasma of ECFV expanded (VE) dogs which is natriuretic in rats and inhibits sodium transport (antinatriferic activity) in the toad urinary bladder (2, 3), a biological analogue of the distal renal tubule. A similar factor has been reported by other laboratories in plasma of VE humans (4), in urine of VE dogs (5), and in renal tissue of VE rats (6).

We now wish to report the partial purification of this substance by high pressure liquid chromatography and the development of a chemical assay for it. The data suggest the factor is a low molecular weight, acidic peptide and provide evidence of its formation from a precursor molecule.

Materials and methods. Blood samples (150 ml) were obtained from the jugular vein of hydropenic (H) and VE dogs as previously described (3). Blood was collected in heparinized syringes and handled according to two different protocols.

Group I. Blood was centrifuged at 2500 rpm at 4° for 20 min, the plasma was aspirated and stored at −4° until processing by column chromatography. The time interval between collection and processing varied from several days to 2 months. Eighteen to twenty milliliters of plasma were eluted on a Biogel P-2 (medium) column, 2 × 95 cm, with 1.0 *M* acetic acid as eluant. Ten milliliter fractions were collected automatically and monitored for uv absorbance at 280 nm, and for electrical resistance to detect the salt peak. The fraction eluting immediately after the salt peak (Fraction IV) was lyophilized and stored at −70° for high pressure liquid chromatography. Biogel Fraction IV was redissolved in 300 μl of .05 *M* HCl and separated on a high pressure Partisil SCX (cation-exchange) column (Whatman Inc., Clifton, NJ) under a protocol previously described (7). Four minute fractions (~1 ml) were collected in a fraction collector.

Peptides in the column effluent were detected by a discontinuous stream-splitting valve coupled to a fluorescamine detection system, as previously reported (8). The valve loops were calibrated to provide one percent of the column effluent for detection, while 99% was diverted to a fraction collector. Column effluent fractions comprising each peak seen on the recorder were pooled and freeze dried. The residue was redissolved in amphibian Ringer and assayed for antinatriferic activity (AA) as previously described (3). AA is reported as percent decrease in short circuit current (SCC).

Group II. In Group II, blood from each dog was split into equal, paired samples and processed by two different methods. In Group II (a) (rapid processing) blood was collected in iced, heparinized syringes containing 50 nmoles bacitracin (an enzyme inhibitor), and centrifuged at 10,000 rpm at 4° for 5 min. The plasma was quickly aspirated, acidified to pH 5.0 with 10% acetic acid, and placed in a boiling water bath for 20 min. The total elapsed time from drawing of blood to placement in the water bath was 15 min. In Group II (b) (slow processing), blood was drawn without bacitracin and centrifuged at 2500 rpm for twenty minutes at 4°. The plasma was aspirated and allowed to sit at room temperature for approximately 30 min, then acidified and boiled as in Group II (a). In Group II (b), the elapsed time from drawing of blood to boiling was approximately 60 minutes. Then the extract was centrifuged and the supernatant removed and stored at −70°. Twenty-five mililiter supernatant samples were eluted on Biogel P-2 as described above for Group I. Biogel P-2 Fraction IV was then lyophilized and processed on Partisil SCX as described above for Group I.

In most instances, bio-assays were per-

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formed on randomly selected bladders. However, in Group II samples, two of the six pairs of assays were performed on paired hemibladders from the same toad and one pair of assays on the same bladder section.

Ten percent of each SCX fraction was used for the reverse-phase peptide analysis of Gruber *et al.* (9). The sample (200 μl) was diluted with 300 μl of 0.05 *M* sodium phosphate buffer (pH 7), reacted with fluorescamine, and the peptide-fluorophors separated on a Partisil ODS-2 column (Whatman Inc., Clifton, NJ). The peptide-fluorophors were eluted with a 5–30% acetone:water gradient.

Results. AA in plasma extracts of VE dogs was consistently found in a post salt u-v absorbing peak (peak IV) on Biogel P-2 chromatography in Group I samples as previously reported (2). Partisil SCX chromatography of Biogel Fraction IV resulted in the appearance of several fluorescamine-reactive peaks V (Fig. 1). No consistent difference could be observed in the chromatograms of VE and H plasma. These peaks did not contain intrinsic fluorescence (at 390 nm excitation - 475 nm emission). This was shown by turning off the fluorescamine pump in the preparative monitoring system and observing the absence of any peaks on the recorder. AA was only found in the void volume peak (Fraction I) of the VE extracts (Fig. 1 and Table I). There was negligible AA in the fraction (II) immediately after the void volume (Table I), nor did any other column fraction contain AA. Since Guggenheim *et al.* (10) reported that ammonia has AA, all samples were analyzed for ammonia. Our "ammonia titration" curve on the toad bladder shows a plateau of AA at −14% between 0.5 m*M* and 1.5 m*M*. In Group I and II samples, ammonia concentrations were uniformly less than 0.4 m*M*, causing trivial degree of AA in our assay.

Aliquots of the Partisil SCX void volume fractions were reacted with fluorescamine at pH 7, and the resulting peptide-fluorophors separated on a Partisil ODS (reverse-phase) column. The pH used for the reaction has been shown to maximize the fluorescence of peptides, while minimizing the fluorescence of amino acids (11). A group of peaks, consistently found after the void volume peak in VE samples, was used as a marker permitting a blind assay for AA. The fluorescence of

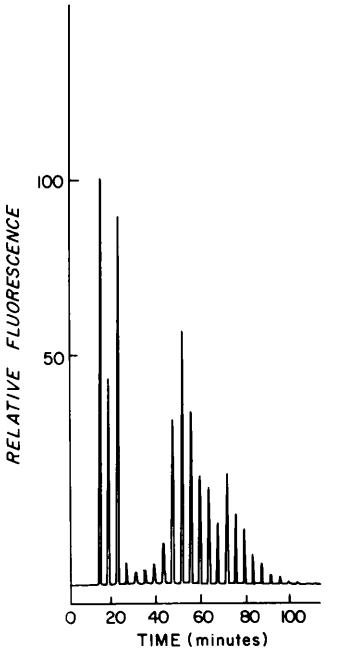

FIG. 1. Partisil SCX chromatogram of Biogel P-2 Fraction IV from an ECFV expanded dog. AA is found in the first two sampling periods (void volume). Each line in the discontinuous tracing represents the fluorescence in one test tube in the fraction collector.

Group I		
Sample #	Fraction 1 AA	Fraction 2 AA
	%	%
	Expanded	
1	−27	−16
2	−21	−11
3	−23	+9
4	−34	+19
5	−23	—
6	−26	−2
mean	−26	0
SE	1.89	6.40
	Hydropenic	
1	−10	
2	+31	
3	−9	
4	−27	
5	−5	
mean	−4	
SE	9.53	
P[b]	<.02	

[a] Fraction 1 = void volume of HPLC column. Fraction 2 = fraction immediately after void volume.

[b] Significance of difference between volume expanded and hydropenic groups.

these peaks was due entirely to their reaction with fluorescamine. The reverse-phase chemical assay correlated with the toad bladder assay in 80% of the samples tested.

To determine whether enzymatic degradation reduced the yield of our factor, we collected a series of plasma samples to which bacitracin had been added. To our surprise, this resulted in a decrease in activity in the VE samples, and reverse-phase chromatograms which resembled H samples.

Accordingly, a study was performed in which plasma samples from VE dogs were divided into paired aliquots (Group II a and b). Bacitracin was added to one-half of the sample, which was processed rapidly with immersion in boiling water to stop further enzymatic activity and to precipitate proteins. The other half was processed more slowly without bacitracin, and boiled after 60 minutes. The conditions under which the slowly processed samples were handled approximated those by which the samples in Group I were handled, with the exception that Group I was not boiled.

The results, seen in Table II, clearly show that the rapidly processed samples have significantly less AA than their slowly processed mate. It is interesting to note that sample 6, which gave the lowest AA, was obtained from the dog with the lowest sodium excretion.

Figure 2 shows a typical reverse-phase chromatogram of a Group II plasma extract. The heights of Peaks I and II have a significant correlation with the SCC of Group II (a and b) samples (Fig. 3). H samples did not contain these peaks, as would be expected since they did not possess biological activity.

TABLE II. GROUP II EXPERIMENTS EFFECT OF PROCESSING TIME ON ANTINATRIFERIC ACTIVITY OF VOLUME EXPANDED SAMPLES.

	Antinatriferic activity			
Sample #	Rapid	Slow	Δ	%[a]
	%	%	%	
1	−10	−22	−12	120
2	−24	−30	−6	25
3	−13	−28	−15	115
4[b]	−15	−25	−10	67
5[c]	−16	−30	−14	88
6[c]	−7	−13	−6	86
mean	−14	−25	−10	83
SE	2.39	2.65	1.59	14
P[d]			<.01	

[a] Difference beween rapid and slow samples.
[b] Paired assay performed on same bladder.
[c] Paired assay performed on hemibladders from same toad.
[d] Significance of difference between paired samples by paired *t* test.

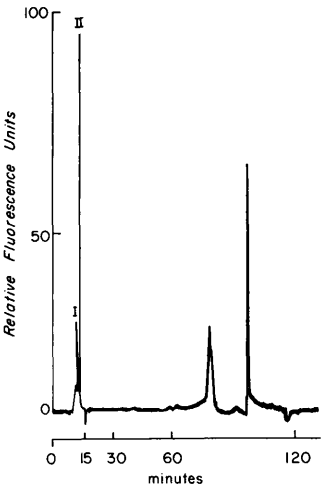

FIG. 2. Reverse-phase chromatogram of a Group II sample. Note peaks I and II.

Discussion. An earlier report from this laboratory demonstrated AA in the post salt fraction of plasma from ECFV expanded dogs eluted on Biogel P-2 in 1 *M* acetic acid (12). The present study shows further purification of the antinatriferic factor in this fraction by high pressure liquid chromatography. The mobility on Biogel P-2 suggests the factor is a low molecular weight molecule. It is excluded from a cation-exchange resin, appears to react with a reagent (fluorescamine) specific for amino groups at a pH which allows only peptides to develop maximal fluorescence, and is formed by enzymatic action. These data suggest that the antinatriferic factor is an acidic peptide of low molecular weight (~500). These results are in accord with the reported characterization of an antinatriferic factor isolated from urine of uremic patients (13).

Reports have indicated the presence of two natriuretic factors in urine of ECFV expanded subjects (1). One factor, which causes natriuresis in rats after a 20-min delay, appears to have a larger molecular weight than a second factor which produces an immediate natriuresis. The low molecular weight factor is antinatriferic, while the higher molecular

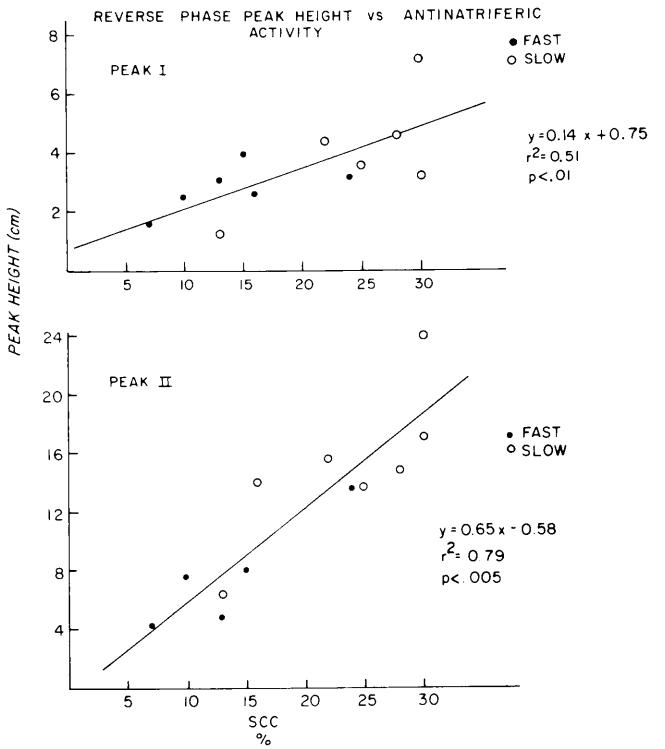

FIG. 3. The plot of height of peaks I and II (from Group II samples) against the SCC of each sample. Note the striking correlation.

weight factor is not, as has been shown in unpublished studies from this laboratory, and others (14). Speculation is that the higher molecular weight factor could be a precursor of the lower molecular weight factor (15).

The correlation of 2 small molecular weight peptides (on reverse-phase chromatography) with SCC in Group II samples (Fig. 3) suggests that they may be the breakdown products of a precursor molecule. This precursor may be the natriuretic, non-antinatriferic factor previously described (14). These peptides may be responsible for antinatriferic activity. Because of the high correlation between antinatriferic activity and peak height, it may be possible to chemically assay for antinatriferic activity in plasma extracts using the height of Peaks I and/or II.

Our results show that rapid processing of plasma samples reduces the recovery of the antinatriferic factor. This finding may provide an explanation for previous conflicting reports on the presence of antinatriferic factor in plasma (1). Our data is the first direct evidence that "natriuretic hormone" is a cascading system. Confirmation of this hypothesis will require isolation and characterization of the precursor substance and its *in vitro* conversion by enzymatic digestion to an effector substance.

Summary. Antinatriferic factor was isolated from VE dog plasma on high pressure liquid chromatography. The use of an enzyme inhibitor while collecting plasma reduced the presence of this factor. A reverse-phase chromatography peptide map revealed 2 peptides whose presence was directly correlated with antinatriferic activity. The results suggest that antinatriferic factor is a small acidic peptide, formed from a precursor molecule. Reverse-phase chromatography may prove to be a chemical assay for antinatriferic factor.

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