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Inactivation of atrial natriuretic factor by the renal brush border

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Atrial natriuretic factor (ANF), a 28-amino-acid peptide secreted from the mammalian heart, is known to be cleared rapidly from the circulation. In vitro and in vivo studies implicate the kidney as an important site for clearance and subsequent degradation of atrial natriuretic factor. We have observed that atrial natriuretic factor is inactivated rapidly by rabbit kidney brush-border membranes. The rate of degradation of ANF measured by the loss of bioactivity followed a similar time-course to the decrease in peptide peak area measured by high-performance liquid chromatography. Interestingly, inactivation of ANF produced only a single major degradation product, which was isolated and purified. Sequence analysis revealed that the product had the same sequence of amino acids as ANF with the Cys-7–Phe-8 bond cleaved and the disulfide bridge between Cys-7 and Cys-23 remaining intact. As the renal brush border contains an abundance of proteolytic activities, it is surprising that this peptide is cleaved primarily at a single peptide bond.

Introduction

Atrial natriuretic factors are peptides which have been isolated from mammalian cardiac atria and cause natriuresis, diuresis and vasorelaxation [1,2]. When these peptides are exogenously administered, they have a very short half-life, on the order of a few minutes [3–5]. While much effort has focused on the mode of action of atrial natriuretic factor, little is known about its metabolism. However, evidence is accumulating that the kidney may be a major site for clearance of ANF from the circulation. Compared to liver, lung, heart and plasma, the kidney had the highest ANF-degrading activity [3]. When ANF is administered to dogs, 80% of the material survives passage through the lungs, while only 20% of bioactive material is detected after passage through the kidney [6]. A study involving normal and anephric rats indicated that the kidney plays a major role in the clearance of ANF from the plasma and that proteolytic degradation, rather than excretion, accounts for a large proportion of its clearance from the circulation [4]. While it has been suggested that kallikrein, a serine protease, may inactivate atrial natriuretic peptides [7,8], others have found no evidence for this [9].

A number of peptide hormones, such as angiotensin, glucagon and LHRH, are believed to be inactivated by proteolytic degradation at the brush border of the renal proximal tubule [10], which is rich in hydrolases [11]. Such conclusions were drawn from experiments employing in vitro techniques, such as incubation of the peptide with a renal brush-border membrane preparation. Using a similar approach, we report here that ANF is

Abbreviation: ANF, atrial natriuretic factor.

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inactivated by renal brush-border membranes and that, in contrast to previous results with atriopeptin III [12], the peptide is cleaved at a single peptide bond.

Experimental Procedures

Preparation of rabbit kidney brush-border membranes

The following procedure was performed at 4 °C. Rabbit kidneys were obtained fresh and were perfused with buffer A (5 mM Tris (pH 7.5)/0.25 M sucrose/3 mM $MgCl_2$/1 mM EDTA) to remove the blood. The kidneys were minced, homogenized in 5 vol. of ice-cold buffer A with a Brinkmann Polytron and filtered through two layers of cheesecloth. The filtrate was centrifuged at $5000 \times g$ for 20 min, the pellet was discarded and the supernatant was centrifuged at $15000 \times g$ for 20 min. The supernatant was retained and then centrifuged at $100000 \times g$ for 90 min. The pellet was resuspended in 50 mM Tris (pH 7.5) and the suspension was centrifuged at $100000 \times g$ for 90 min. The washed pellet was resuspended in 50 mM Tris (pH 7.5) to give a final protein concentration of 4 mg/ml using the Bradford protein assay [13]. The membrane preparation was assayed for aminopeptidase and alkaline phosphatase activities, marker enzymes for the kidney brush border, according to the methods described by Kramers and Robinson [14]. The final membrane preparation had specific activities of 2892 $\pm$ 64 nmol/min per mg protein for alkaline phosphatase and 1104 $\pm$ 35 nmol/min per mg protein for aminopeptidase, and were enriched 8-fold and 11-fold, respectively, compared to the homogenate [15].

Assay for ANF degradation

The reaction mixtures contained 40 μM ANF, 50 mM Tris (pH 7.5) and brush-border membranes (10 μg) in a final volume of 0.5 ml. All incubations were at 25 °C. The reaction was terminated by removing the membranes by filtration on Gelman GA-6 filters. The filtrates were immediately frozen on dry ice and were stable for 1 week when stored at −80 °C. Samples containing the peptide degradation products were resolved by C_{18} reverse-phase HPLC. The frozen samples were thawed immediately before injection (0.025 ml aliquot) onto a Vydac C_{18} column (4.6 mm i.d. × 25 cm; 5 μm/300 A) equilibrated with water containing 0.05% trifluoroacetic acid. Peptide fragments were separated on a linear gradient of 0–35% acetonitrile also containing 0.05% trifluoroacetic acid developed for 26 min at a flow rate of 1 ml/min. Both the gradient and the flow rate (1 ml/min) were maintained by a Waters HPLC system consisting of two Model 510 pumps connected to an Automated Gradient Controller. Column effluent was monitored at 220 nm by a Waters Model 481 spectrophotometer, and the amount of atrial peptide material and peptide fragments were measured by peak area via a Hewlett-Packard Model 3392A integrator. Separation and isolation of the peptide fragments was achieved using similar chromatography conditions, except that a larger Vydac C_{18} column (10 mm i.d. × 25 cm; 5 μm/300 A) was used at a flow rate of 2 ml/min.

Peptide sequencing and amino-acid analysis

Automated Edman degradation chemistry was used to determine the protein sequence analysis data. An Applied Biosystems Model 470A gas-phase sequencer was employed for the degradations [16] with respective phenylthiohydantoin–amino-acid derivatives identified by HPLC analysis as modified by Hunkapiller et al. [17]. Compositional analysis data were collected from 6 M HCl hydrolysates (vapor phase, 110 °C, 24 h) using ninhydrin-based analysis performed on a Beckman 6300 High Performance Analyzer.

Assay for ANF bioactivity

The biological activity of ANF was assayed by measuring the vasorelaxation of norepinephrine-contracted rabbit aorta by the method of Currie et al. [2].

Results and Discussion

The ANF used in this study was the 28-amino-acid peptide, which is the form released from the heart into the circulation [18,19]. ANF was incubated with rabbit kidney brush-border membranes for various time intervals and the reaction terminated by filtration, as described in Experi-

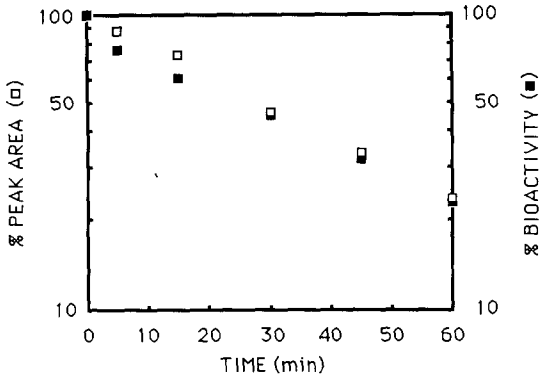

Fig. 1. Rate of degradation of ANF by renal brush-border membranes. Time-course of the decrease in integrated peak area of ANF, measured at 220 nm of column effluent from a reverse-phase HPLC column, is denoted by (□). Time-course of the loss of ANF vasorelaxant activity is represented by (■).

mental Procedures. The filtrates were analyzed for ANF content by HPLC, and for ANF bioactivity using a rabbit aorta bioassay. ANF was rapidly degraded by renal brush-border memrbanes and, as shown in Fig. 1, the decrease in ANF content was concomitant with the loss of ANF bioactivity.

Fig. 2 shows consecutive HPLC profiles following the time-course of ANF degradation. Surprisingly, for a peptide of 28 amino acids, there was only one major degradation product (labelled peak 1) generated during the incubation. Similar results have been obtained with three preparations of brush-border membranes. The product represented by peak 1 was isolated, purified and sequenced. The peptide peak corresponding to the starting ANF was similarly isolated, purified and sequenced to confirm that no other degradation product coeluted. Sequence analysis revealed that the peak 1 product was ANF containing a single cleavage at the Cys-7–Phe-8 bond, with the disulfide bridge between Cys-7 and Cys-23 remaining intact (Fig. 3). Under similar incubation conditions, we have shown previously that the 24-amino-acid atrial natriuretic peptide, atriopeptin III, is also rapidly degraded by the same brush-border membrane preparation but, in this case, at least three cleavage sites were observed [12]. Considering the renal brush-border membrane contains high levels of aminopeptidase activity [11], it is striking that no other bonds in ANF were cleaved significantly except the Cys–Phe bond. As atriopeptin III and ANF differ only by the absence or presence, respectively, of the tetrapeptide Ser-Leu-Arg-Arg at the amino terminus, these results imply that this sequence of residues offers protection against aminopeptidase digestion. That ANF and atriopeptin III are proteolytically cleaved prior to phenylalanine(s) is significant: the kidney contains an endopeptidase which cleaves peptide bonds on the amino side of hydrophobic residues [20–23]. Experiments to isolate and identify the brush-border enzyme responsible for ANF inactivation are currently in progress.

It is well established that proteins and polypeptides are filtered from the bloodstream by the kidneys and are subsequently absorbed by endocytosis in the proximal tubule, where they are hydrolyzed by lysosomal enzymes [10]. Small peptides, however, appear to be proteolytically degraded at the luminal memrbane and the result-

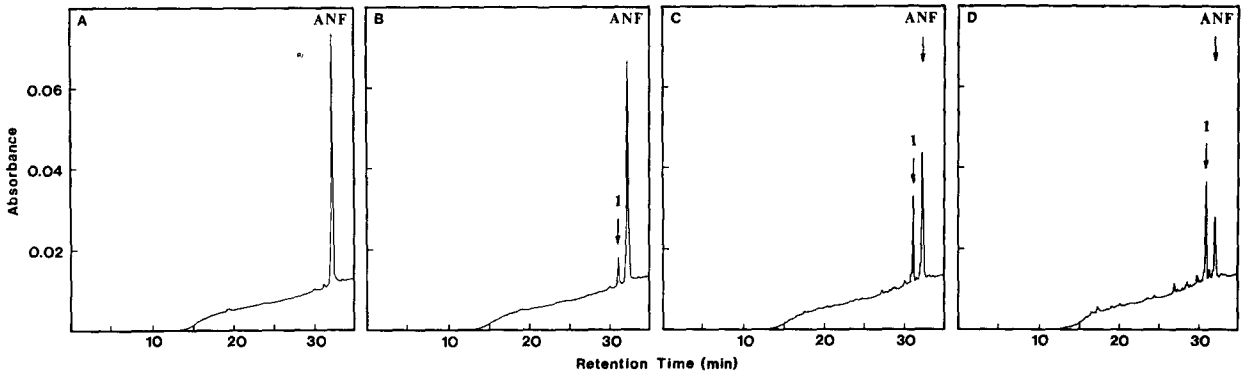

Fig. 2. Reverse-phase HPLC profiles of the time-course of ANF degradation by renal brush-border membranes with (A) no incubation and after incubating for: (B) 5 min; (C) 30 min; (D) 60 min. Absorbance of the column effluent was measured at 220 nm.

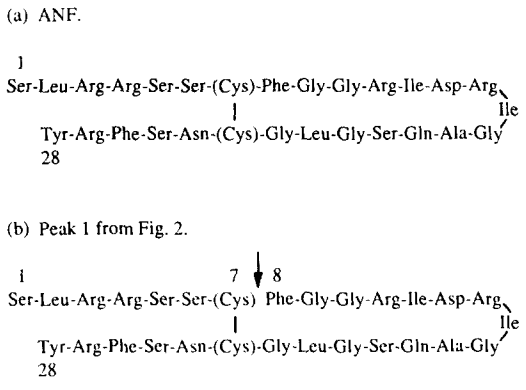

Fig. 3. Sequence analysis of (a) ANF and (b) its degradation product (peak 1 from Fig. 2) after a 45 min incubation with renal brush-border membranes. The position of the peptide bond which is cleaved is denoted by the arrow.

ing peptide fragments are reabsorbed. These metabolites may then undergo further hydrolysis intracellularly. As our results predict, ANF, being a relatively small peptide, would be hydrolyzed at the surface of the brush-border membrane of the renal proximal tubule. To date, there has been no convincing evidence that ANF is excreted intact in the urine [4].

In conclusion, our results provide an insight into a possible mode of ANF metabolism involving the brush-border membrane of the kidney which may be relevant to the clearance of the peptide in vivo. The data obtained may be useful in the design of degradation-resistant ANF analogs which might have a longer duration of action.

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