J. Enzyme Inhibition, 1993, Vol. 7, pp. 257-263
Reprints available directly from the publisher
Photocopying permitted by license only

DIFFERENTIAL CLEAVAGE OF URODILATIN AND ATRIAL NATRIURETIC FACTOR BY THROMBIN AND PROTEASE 3.4.24.11

CAROL BERRY, YUMI SAKANE, RADHIKA RAMANNAN, CHRISTINE KRULAN, JOSEPH BALWIERCZAK and RAJENDRA GHAI*

Research Department, Pharmaceuticals Division, Ciba-Geigy Corporation, Summit, New Jersey 07901, U.S.A.

(Received 21 June 1993)

Human urodilatin (residues 95–126) and atrial natriuretic factor (residues 99–126, based on ANF prohormone sequence) were incubated separately with three proteases, thrombin, angiotensin converting enzyme (ACE), and neutral endopeptidase 3.4.24.11 (NEP). Thrombin cleaved urodilatin on the carboxyl side of arginine[98] to yield ANF but under the same conditions did not cleave h-ANF. Neither urodilatin nor ANF was cleaved by ACE. ANF was rapidly degraded by NEP resulting in a major product cleaved between amino acid residues Cys[105] and Phe[106]. Urodilatin was also cleaved by NEP and the amino acid sequencing of the cleaved product revealed the site of cleavage to be the same Cys[105]-Phe[106] site as for ANF with a second cleavage site at Gly[118]-Leu[119]. However, cleavage of urodilatin by NEP proceeded much more slowly when compared to ANF. A comparison of the affinities of ANF and urodilatin for purified NEP from rabbit kidney revealed K_m values of 11.7 and 3.1 μM, respectively. The turnover rates (k_{cat}/K_m) for urodilatin and h-ANF with NEP were 4.6 and 37.3 min^{-1} μM^{-1}, respectively. Thus, urodilatin is much less efficiently hydrolyzed by purified NEP than is ANF. The four residue extension at the N-terminus of urodilatin may be important for protection against rapid biological inactivation.

KEY WORDS: Urodilatin, h-ANF, neutral endopeptidase 3.4.24.11, cleavage site.

INTRODUCTION

Urodilatin is a 32 amino acid member of the ANF family that is synthesized in the kidney.[1,2] Urodilatin is identical to h-ANF and differs only in an N-terminal extension of four amino acids, Thr-Ala-Pro-Arg.[3] Urodilatin has not been detected in plasma but is the major form of ANF found in the urine.[1,2,4] When compared to ANF, urodilatin has similar vasorelaxant properties albeit more potent renal effects.[4–7] ANF has been shown to be hydrolyzed and inactivated by NEP, the major ANF degrading enzyme in the kidney.[8] Urodilatin, however, has been reported to be resistant to hydrolysis by kidney NEP.[7,11] In the present study, we wished to evaluate the reported lack of activity of NEP on urodilatin and to determine whether other proteases play a role in the enzymatic inactivation of urodilatin. This report describes the hydrolysis of urodilatin by three proteases and identifies the site of cleavage of urodilatin by NEP.

* Correspondence

MATERIALS AND METHODS

Synthetic human urodilatin, atrial natriuretic factor, thrombin and angiotensin converting enzyme were obtained from Sigma. Trifluoroacetic acid (HPLC grade) was obtained from Pierce Chemical Company. Acetonitrile (HPLC grade) was purchased from EM Science (Cherry Hill, NJ). All other chemicals were of analytical reagent grade.

Preparation of Purified Rabbit Kidney Cortex NEP

NEP was purified from rabbit kidney cortex as described by Sonnenberg *et al.*[8]

NEP Hydrolysis of Urodilatin and h-ANF

Enzymatic activity was determined in duplicates by measuring the disappearance of the substrate (h-ANF or urodilatin) using a 3 min RP-HPLC separation. An aliquot (0.1 μg) of the purified NEP in 50 mM Tris HCl buffer, pH 7.4, was pre-incubated for 10 min and the reaction was initiated by the addition of 10 μM h-ANF or urodilatin in a total reaction volume of 125 μl. Incubation was allowed to continue at 37°C for 10 or 30 min (for ANF or urodilatin, respectively). The reaction was terminated by the addition of 12.5 μl of 1.1% TFA and separated by reverse phase HPLC using a C4 cartridge in a 5 min isocratic separation. Twenty five percent of buffer B (0.1% TFA in 80% acetonitrile) was used. Buffer A was 0.1% TFA in water. HPLC was performed on a Waters 840 system equipped with a model 712 WISP and two HPLC pumps. A lambda 482 Spectrophotometer (Waters, Milford, MA) was used to monitor UV absorbance. One unit of activity is defined as the hydrolysis of 1 nmol of h-ANF or urodilatin per min at 37°C and pH 7.4.

Preparation and Separation of Urodilatin Fragments

Urodilatin fragments were prepared by incubating urodilatin with purified rabbit NEP in a ratio of 30:1 for 60 min. The products were separated using a microbondapak C_{18} column (Waters, Milford, MA) and a separation system described by Sonnenberg *et al.*[8]. Two fractions were isolated. Fraction I was the hydrolysis product of NEP; it consisted of a major peak and a shoulder which could not be further separated. Fraction II was intact urodilatin. The two fractions were collected and lyophylized.

Thrombin Hydrolysis of Urodilatin and h-ANF

Similar conditions as for NEP were used, except that 0.031 μg of thrombin was used and the incubation time for both substrates was 10 min. In some experiments urodilatin was first incubated with thrombin as described. Thrombin was then inactivated by incubating for 10 min in boiling water and cooling to room temperature. NEP was then added to the reaction mixture and the incubation continued at 37°C. Conditions for separation and identification of products of thrombin hydrolysis and thrombin plus NEP hydrolysis were the same as described for NEP.

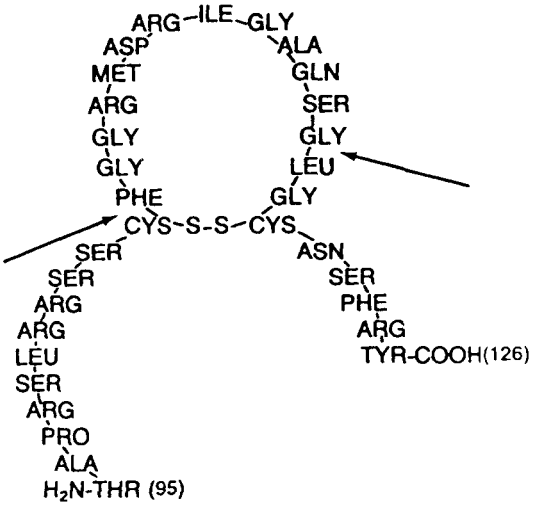

FIGURE 1 Structure of urodilatin (h-ANF 95-126). Arrows indicate sites of cleavage by NEP.

ACE Hydrolysis of Urodilatin and h-ANF

Urodilatin and h-ANF were incubated with 5 μg of ACE in 0.1M potassium phosphate buffer, pH 8.3, containing 0.3M NaCl for 30 or 60 min. Identification of products was carried out as described for NEP.

Determination of Protein

Protein assays for the purified enzyme were carried out in duplicates according to the method of Lowry *et al.*[12]

Kinetic Studies

K_m values were determined by least squares analysis of linear transformations calculated according to the method Lineweaver-Burk.[9,12]. K_{cat} values were calculated according to the method of Segel.[13]

Amino Acid Analyses and Amino-terminal Sequencing

Amino acid analyses were performed using phenylisothiocyanate derivatization; amino-acid sequencing was performed using phenylthiohydantoin derivatization. Derivatives were separated and quantified by HPLC as described by Koehn.[15]

RESULTS

The structure of urodilatin is shown in Figure 1. Thrombin is a sulfhydryl-containing protease that cleaves between Arg-Ser peptide bonds. Urodilatin, but not h-ANF,

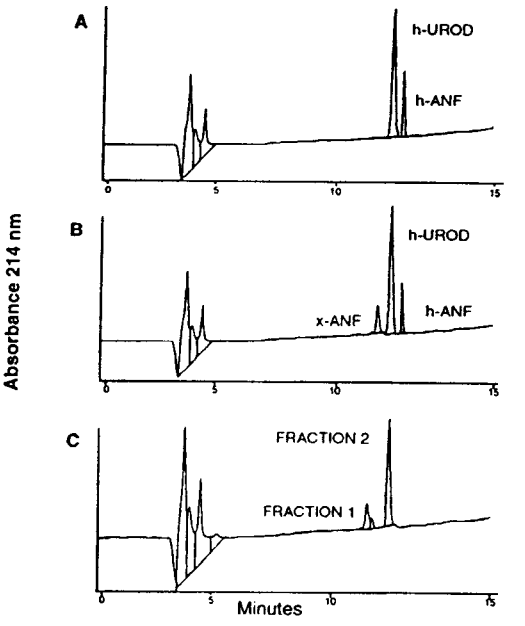

FIGURE 2 High performance liquid chromatography of urodilatin and products from digestion of urodilatin by, (A) thrombin, (B) thrombin and NEP, (C) NEP.

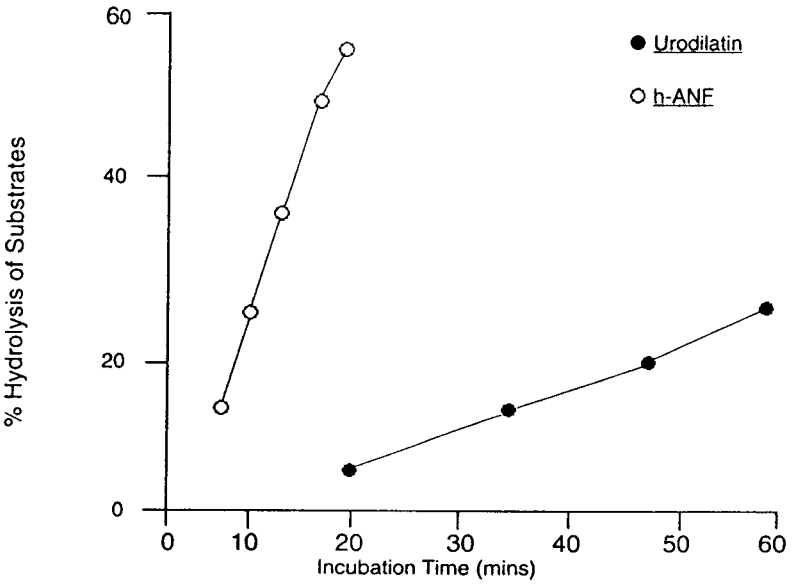

FIGURE 3 Rates of hydrolysis of urodilatin and h-ANF by NEP. Results represent the mean of three experiments.

TABLE I
Hydrolysis of urodilatin and h-ANF by NEP and thrombin

		Specific Activity (μmol/min/mg)		
Peptide	(n)	NEP	(n)	Thrombin
Urodilatin	(6)	0.27 ± 0.01	(5)	0.414 ● 0.017
h-ANF	(6)	1.54 ± 0.07	(5)	0

Mean values ± SE are given. n indicates number of determinations.

was cleaved by thrombin between residues Arg^{98}-Ser^{99}. The product of cleavage of urodilatin by thrombin had a similar retention time to h-ANF (Figure 2A) and was further cleaved by NEP to yield X-ANF,[8] the product of cleavage of h-ANF by NEP (Figure 2B). Neither urodilatin no h-ANF was hydrolyzed by ACE. NEP is a metalloprotease that inactivates numerous hormones by cleavage at the amino-side of hydrophobic amino acids. It has been identified as the major ANF-degrading enzyme in the kidney.[8] NEP cleaves the cysteine-phenylalanine bond (Cys^{105}-Phe^{106}) in ANF.[15] Urodilatin has been reported to be resistant to hydrolysis by NEP.[11] Incubation of urodilatin with purified rabbit kidney NEP for 60 minutes resulted in the generation of two major products (fraction I) which could be separated from intact urodilatin (fraction II) by HPLC (Figure 2C). Fraction I consisted of a major peak with a shoulder which could not be separated by HPLC. As shown in Figure 3, the rate of hydrolysis was much faster for h-ANF than for urodilatin. We therefore determined the affinity of urodilatin and h-ANF using purified NEP. A comparison of the specific activities of thrombin and NEP using urodilatin and h-ANF as substrates is shown in Table 1. Table 2 shows the kinetics of NEP hydrolysis of urodilatin and h-ANF. The K_m values for urodilatin and h-ANF hydrolysis with NEP were 3.1 and 11.7 μM, respectively. The k_{cat} and k_{cat}/K_m for urodilatin were 12.8 min^{-1} and 4.1 min^{-1} μM^{-1}; and for h-ANF were 437 min^{-1} and 37.3 min^{-1} μM^{-1}. Thus, NEP hydrolyzes h-ANF more efficiently than urodilatin.

TABLE II
Kinetics of rabbit NEP hydrolysis of urodilatin and h-ANF

Substrate	(n)	K_m μM	k_{cat} min^{-1}	k_{cat}/K_m min^{-1} μM^{-1}
h-ANF	(4)	11.7 ± 0.2	437 ± 39	37.3 ± 3.0
Urodilatin	(5)	3.1 ± 0.5	12.8 ± 1.1	4.1 ± 0.6

Mean values ± SE are given. n indicates number of determinations.

TABLE III
Sequence analysis of fractions I and II

	Cycle Number									
	1	2	3	4	5	6	7	8	9	10
Fraction I	Thr	Ala	Pro	Arg	Ser	Leu	Arg	Arg	Ser	Ser
	Phe	Gly	Gly	Arg	Met	Asp	Arg	Ile	Gly	Ala
	Leu	Gly	Cys							
Fraction II	Thr	Ala	Pro	Arg	Ser	Leu	Arg	Arg	Ser	Ser

In order to identify the site of cleavage of urodilatin by NEP, the hydrolysis products (fraction I) were separated from substrate (fraction II) by HPLC (Figure 1C) collected, pooled and lyophilized. Fractions I and II were re-purified under the same conditions, lyophilized and subjected to amino acid analysis. The amino acid analyses showed the same amino acid composition for both fractions. Fractions I and II were each subjected to amino terminal sequence analyses. The first 10 cycles are shown in Table 3. In the first cycle, three amino terminal residues (threonine, phenylalanine and leucine) were detected in fraction I while only one amino terminal residue (threonine) was detected for fraction II. Subsequent cycles for fraction I confirmed the presence of at least three amino terminal residues. Urodilatin, like ANF, has a disulfide bond between 2 cysteine residues at positions 105 and 121. Our results are consistent with the interpretation that one site of cleavage of urodilatin by NEP is at Cys^{105}-Phe^{106}, identical to the ANF cleavage site.[8] Based on the sequencing data we have identified a second site of cleavage at Gly^{118}-Leu^{119}.

DISCUSSION

The kidney is thought to play a major role in the degradation of many peptidic hormones since the brush border of the proximal tubule epithelial cells contains ectoenzymes facing the luminal medium.[16] These enzymes include NEP and ACE as well as aminopeptidase N, aminopeptidase A, dipeptidyl peptidase IV and aminopeptidase W.[14] Two of these proteases found in the kidney were compared for their ability to hydrolyze urodilatin and h-ANF. Thrombin was included in the study because of its specificity to cleave the Arg-Ser bond outside the ring structure.

Urodilatin, but not h-ANF, is cleaved by thrombin. The product of thrombin hydrolysis of urodilatin is ANF. It is known that ANF is not cleaved by ACE.[14] In the present study ACE did not cleave either urodilatin or ANF.

It has been reported that NEP exhibits almost no activity on urodilatin.[11] Gagleman[11] observed only minor hydrolysis of urodilatin during a 40 minute incubation with NEP while ANF incubated under the same conditions was completely degraded. The results reported here indicate that urodilatin is cleaved by NEP albeit less efficiently

than h-ANF. The site of cleavage by NEP in both peptides is at the Cys^{105}-Phe^{106} bond inside the ring. This cleavage inactivates h-ANF.[16] It is most likely that cleavage at the same bond also inactivates urodilatin. A second cleavage site in urodilatin by NEP has been identified at Gly^{118}-Leu^{119}.

Urodilatin is the major form of ANF secreted in the urine; h-ANF is not found in the urine except after inhibition of NEP.[2,19] This difference can be explained by the degree of susceptibility of these two peptides to hydrolysis by NEP, the major ANF degrading enzyme found in the kidney.

Urodilatin has also been reported to be inactivated by phosphorylation at the Ser^{104} position and to regain activity during de-phosphorylation with acid phosphatase.[18] The interplay of urodilatin release, function and inactivation with h-ANF levels and function has not been investigated. Also, the relative contributions of excretion and cleavage by NEP (and other kidney enzymes) to the inactivation of urodilatin remain to be determined.

References

1. Saxenhoefer H., Raselli A., Weidmann P., Forsmann W-G, Bub, A., Ferrari, P. and Show G. (1990) *Am. J. Physiol.*, **259** (Renal Fluid Electrolyte Physiol. 28) F832.
2. Feller, S.M., Gagelmann, M. and Forsmann, W.G., (1989) *TIPS*, **10**, 93.
3. Schulze-Knappe, P., Forsmann, K., Herbst, F., Hock, D., Pipkorn, R. and Forsmann W.G. (1988) *Klin Wochenschr.*, **66**, 752.
4. Drummer, C., Fieldler F., Konig, A. and Gerzer, R. (1991) *J. Am. Soc. Nephrol.*, **1**, 1109.
5. Hildebrandt, D., Mizelle, H.L., Brands, M.W. and Hall, J.E. (1992) *Am. J. Physiol.*, **262** (Regulatory Integrative Comp. Physiol 31) R395.
6. Hollister, A.S. and Inagami, T. (1991) *Am. J. Hypertension*, **4**, 850.
7. Goetz K.L. (1991) *Am. J. Physiol.*, **261** (Renal Fluid Electrolyte Physiol. 30) F921.
8. Sonnenberg, J.L., Sakane, Y., Jeng, A.Y., Koehn, J.A., Ansell, J.A., Wennogle, L.P. and Ghai, R.D. (1987) *Peptides*, **9**, 173.
9. Schulz R., Sakane, Y., Berry, C. and Ghai, R. (1991) *J. Enz. Inhibit.*, **4**, 347.
10. Kreiter, P.A. and Trapani, A.J., (1989) *Drug Met. Disp.*, **17**, 14.
11. Gagleman, M., Hock, D. and Forsmann, W.-G. (1988) *FEBS Lett.*, **233**, 249.
12. Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) *J. Biol. Chem.*, **193**, 265.
13. Segel, I.H. (1975) Biochemical Calculations, 2nd Edition. New York, John Wiley and Sons, Inc.
14. Vanneste, Y., Pauwels, S., Lambotte L. and Deschodt-Lankman, M. (1990) *Biochem. Biophys. Res. Commun.*, **173**, 265.
15. Koehne J.A., Norman, J.A., Jones, B.A., LeSueur, L., Sakane, Y. and Ghai, R.D. (1987) *J. Biol. Chem.*, **262**, 11623.
16. Ghai R.D., Webb, R.L., Sonnenberg, J.L., Sakane Y. and Ghai, G. (1991) *J. Enz. Inhibit.*, **4**, 267.
17. Sole, J., Bauer, K., Solcova, A., Dohlemann, C., Strom, T.M. and Weil, J. (1991) *Life Sci.*, **48**, 2451.
18. Dorner, T., Gaglemann, M., Feller, S., Herbst, F. and Forsmann, W.G. (1989) *Biochem. Biophys. Res. Commun.*, **163**, 830.
19. Seymour, A.A. (1991), *Cardiovascular Drug Rev.*, **9**, 285.