

Comparison of the Hydrolysis of the Three Types of Natriuretic Peptides by Human Kidney Neutral Endopeptidase 24.11

Yasuhiro Watanabe, Kenjiro Nakajima, Yoshimitsu Shimamori, and Yukio Fujimoto

Department of Clinical Biochemistry, Hokkaido Institute of Pharmaceutical Sciences, Katsuraoka-cho, Otaru-shi, Hokkaido 047-02, Japan

Received August 12, 1996; accepted March 5, 1997

The degradation of 3 human natriuretic peptides by human kidney neutral endopeptidase 24.11 has been investigated. The studies revealed that hANP-28 and hCNP-22 are the preferred substrates, whereas hBNP-32 is not. The enzyme has been known to inactivate hANP-28 from cleavage at the Cys-Phe bond at the beginning of its ring structure. Analysis of the cleavage sites of each peptide indicated that the initial cleavage site of hCNP-22 is analogous to that of hANP-28. The Cys-Phe bond of hBNP-32 was insensitive to this enzymatic cleavage. We speculate that the stability of hBNP-32 may result from the insusceptibility of its Cys-Phe bond at the beginning of the ring structure. © 1997 Academic Press

Neutral endopeptidase 24.11 (NEP)¹ has several other names which reflect its discovery. The enzyme was first discovered in brush border membranes of rabbit kidney tissue as an insulin B chain degrading enzyme (1) and then as an enkephalinase (2–4). It is a zinc containing metalloendopeptidase (5) and it cleaves substrates on the amino side of hydrophobic amino acids (1). NEP is particularly abundant in kidney brush border membranes (6). The natriuretic peptide family has potent diuretic, natriuretic, and

vasorelaxant properties and is composed of three distinct peptides. The three peptides contain a disulfide-linked loop consisting of 17 amino acids, the integrity of which is essential for their biological actions. The metabolic fate of hANP-28 in the plasma is well known. Peptide clearance via C-receptors (7) and enzymic degradation by NEP in the kidney (8) are thought to be responsible for its clearance from circulation. NEP hydrolyzes peptides via cleavage at the Cys-Phe bond at the beginning of their ring structure (9–11). Thus, numerous *in vivo* studies with NEP inhibitors were carried out to enhance the diuretic and natriuretic effects of natriuretic peptides (12,13). In this study, we compare the hydrolysis of three types of natriuretic peptides by human kidney NEP.

MATERIALS AND METHODS

Materials

Three types of natriuretic peptides (hANP-28, hBNP-32, and hCNP-22) and Suc-Ala-Ala-pNA, for measuring NEP activity, were purchased from The Peptide Institute, Inc. (Osaka). Porcine kidney aminopeptidase M was obtained from Pierce (Rockford, IL). All other reagents used were of the highest grade available from commercial sources. Human kidneys were obtained at autopsy from the Department of Pathology, Sapporo Medical College (Hokkaido, Japan) and exhibited no pathological abnormalities upon histological examination. Anti-DPP IV–Sepharose 4B was prepared as described previously (14).

¹ Abbreviations used: NEP, neutral endopeptidase 24.11; DPP IV, dipeptidyl peptidase IV; γ -GTP, γ -glutamyl transpeptidase; Suc-Ala-Ala-pNA, succinyl-alanyl-alanyl-alanine *p*-nitroanilide; hANP-28, atrial (A-type) natriuretic peptide (human, 1–28); hBNP-32, brain (B-type) natriuretic peptide (human, 1–32); hCNP-22, C-type natriuretic peptide (human, 1–22); SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TFA, trifluoroacetic acid.

Purification of NEP from Human Kidneys

NEP activity was monitored using Suc-Ala-Ala-Ala-pNA as the substrate according to the method outlined in our previous paper (15). Under assay conditions, NEP cleaves Suc-Ala-Ala-Ala-pNA into Suc-Ala-Ala and Ala-pNA, and then the resulting Ala-pNA is hydrolyzed into Ala and pNA by aminopeptidase M. One unit of enzyme activity is defined as the amount of enzyme that liberates 1 μ mol of pNA from Suc-Ala-Ala-Ala-pNA. Proteins were determined by measuring their absorbance at 280 nm or by the method described by Smith *et al.* (16) using a BCA protein assay kit (Pierce). The enzyme was purified from 200 g of frozen kidney tissue. Solubilized kidney membrane supernatants were prepared as described previously (15). Briefly, the membranes obtained were washed with a buffer containing 10 mM Tris-HCl, pH 8.0, and 1 M KCl, followed by centrifugation at 105,000g for 1 h. The KCl-washed membranes were further washed with a buffer containing 10 mM Tris-HCl, pH 8.0, and 1% (w/v) Brij 35. The Brij 35-washed membranes were collected by centrifugation as above, solubilized with 10 mM Tris-HCl, pH 8.0, containing 2% (w/v) Triton X-100, and then the solubilized supernatant was retained after centrifugation as above. Triton X-100-solubilized extracts were run through a DEAE-Toyopearl 650S column (2.5 $\times$ 10 cm) preequilibrated with 10 mM Tris-HCl, pH 8.0, containing 0.1% Triton X-100 (Buffer A). The column was washed thoroughly with the buffer, followed by a linear gradient of 0–0.5 M NaCl in Buffer A (600 ml in total). The fractions containing Suc-Ala-Ala-Ala-pNA hydrolytic activity were combined and concentrated by ultrafiltration, and then the concentrate was run through a Sephacryl S-300 HR column (2.5 $\times$ 100 cm) preequilibrated with Buffer A. The eluted fractions containing enzymatic activity were pooled and run through a DEAE-Cellulofine A-800 column (1.5 $\times$ 8 cm) preequilibrated with Buffer A. The column was washed with the buffer, followed by a linear gradient of 0–0.3 M NaCl in Buffer A (400 ml in total). During this step, the enzymes that emerged from the column were completely separated from aminopeptidase N, γ -GTP, and the other proteins in the kidney membranes. However, DPP IV, another protein common in kidney membranes, was incompletely separated. Accordingly, the enzyme preparation obtained was run through an anti-DPP IV-Sepharose 4B column (1.5 $\times$ 5 cm). Unbound proteins eluted with the buffer containing 10 mM Tris-HCl, pH 8.0, 0.15 M

NaCl, and 0.1% Triton X-100 were pooled as a final preparation.

Electrophoresis

SDS-PAGE was performed using 7.5% polyacrylamide gels using the method described by Laemmli (17).

Peptide Incubation and HPLC Analysis of the Cleavage Products

Hydrolysis of natriuretic peptides was measured by incubating each peptide with NEP in a final volume of 50 μ l containing 0.1 mM substrate, purified NEP, and 10 mM Tris-HCl, pH 8.0, at 37°C for the times indicated. After terminating the incubation by heating at 100°C for 2 min, reduction and alkylation were carried out to cleave internal disulfide bonds. The samples were then treated with dithiothreitol (final 10 mM) and incubated at 37°C for 2 h. Iodoacetamide was added (final 40 mM) and the incubation was continued for another 15 min. HPLC separation of the hydrolyzed products was performed in the Shimadzu LC10A HPLC system using TSKgel-ODS-80TMCT (Tosoh) employing a linear acetonitrile gradient in 0.1% TFA. Absorbance was monitored at 215 nm.

To determine the peptide cleavage sites, fractions corresponding to discrete peaks were collected and evaporated, and then each residue was hydrolyzed in 6 N HCl for 24 h at 110°C. The hydrolyzates were subjected to amino acid analysis using an amino acid analysis system (Shimadzu).

RESULTS AND DISCUSSION

NEP is a typical ectoenzyme, and thus it is anchored to the lipid bilayer through a hydrophobic domain near its N-terminus (18). Therefore, the entire protein can be extracted from membranes using detergents. We used 2% Triton X-100 to solubilize the enzyme. At the DEAE-cellulofine A-800 column chromatographic step, the NEP-containing fractions had no aminopeptidase M or γ -GTP activities, which are enzymes commonly found in kidney membranes, but some DPP IV activity, another abundant enzyme, was observed. It was not possible to separate it from the pooled fractions containing NEP. Therefore, we ran the sample through an anti-DPP IV-Sepharose 4B column and collected the eluted materials. Through this procedure, the DPP IV activity was removed and we had completed NEP purifica-

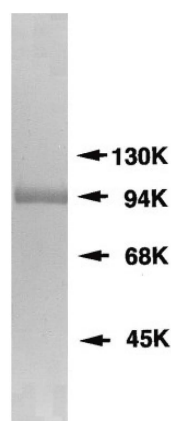


FIG. 1. SDS-polyacrylamide gel electrophoresis of the purified human NEP. About 5 μ g of each purified enzyme was applied to a 7.5% polyacrylamide gel. After running, the gel was stained for proteins with Coomassie brilliant blue G-250. The marker proteins for molecular weight determination were as follows: *Escherichia coli* β -galactosidase, M_r 130 kDa; phosphorylase a, M_r 94 kDa; bovine serum albumin, M_r 68 kDa; ovalbumin, M_r 45 kDa.

tion. After column chromatography, the specific activity of NEP was 4.85 U/mg protein for Suc-Ala-Ala-pNA. The apparent M_r of the enzyme was calculated to be 92 kDa (Fig. 1). Time-dependent degradation of the three natriuretic peptides by purified human NEP is shown in Fig. 2. Interestingly, hANP-28 and hCNP-22 were degraded at similar rates. Under the conditions we used, the half-lives of hANP-28 and hCNP-22 were 119 and 88 min, respectively. However, hBNP-32 was only slowly hy-

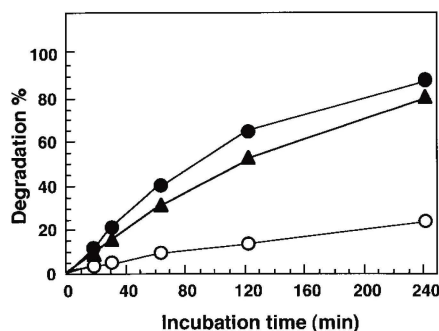


FIG. 2. Comparison of the rates at which NEP hydrolyzes to the three natriuretic peptides. Peptides were digested with purified human kidney NEP (40 ng) as described under Materials and Methods. At various time points, the reaction was stopped by heating, reduction, and alkylation, and the products were analyzed by HPLC. The percentage of degradation was calculated by the disappearance of the substrate peak. (▲) hANP-28; (○) hBNP-32; and (●) hCNP-22.

TABLE 1
Kinetic Parameters for Hydrolysis of Natriuretic Peptides by Human NEP

Substrate	K_m (mM)	V_{max} (pmol/min)	K_{cat} (min ⁻¹)	K_{cat}/K_m (min ⁻¹ mM ⁻¹)
hANP-28	28.3	38.5	145	5.12
hBNP-32	102	43.2	54.3	0.532
hCNP-22	12.4	66.1	97.4	7.85

Note. The values were calculated from the disappearance of the substrate peak after HPLC. The incubation conditions are described under Materials and Methods. The time of incubation and the range of substrate concentration were 60 min and 0.01–0.125 mM, respectively. K_{cat} was calculated based on a molecular weight of 92,000.

drolyzed by the enzyme, and about 80% of the substrate remained even after incubation for 240 min. The kinetic parameters of NEP toward the three natriuretic peptides were also determined (Table 1). As indicated, hANP-28 and hCNP-22 were hydrolyzed with similar catalytic efficiency. The V_{max} values were similar among the 3 peptides. However, owing to decreasing substrate affinity for hBNP-32, the K_{cat}/K_m value for hBNP-32 was significantly lower.

NEP is known to primarily hydrolyze peptide bonds of hydrophobic amino acid residues, such as Phe, Trp, Tyr, Leu, Ile, Val, Ala, and Met. Furthermore, we found that the enzyme required at least a tetrapeptide, and cleavage occurred between the second and the third hydrophobic amino acids of the tetrapeptide unit (19). NEP cleaves several biologically active peptides at specific sites (19). There are several potential sites at which NEP may cleave the three natriuretic peptides. In this study, we identified human NEP cleavage sites in the three natriuretic peptides. The peptide fragments produced by human NEP were separated by HPLC and the sequence of each peptide fragment was deduced from its amino acid composition (Fig. 3). Stephenson and Kenny cleaved hANP-28 with porcine kidney NEP and identified cleavage sites between Cys⁷-Phe⁸, Arg¹⁴-Ile¹⁵, Gly¹⁶-Ala¹⁷, and Ser²⁵-Phe²⁶ during incubation (8). Furthermore, Vanneste *et al.* found additional sites at Arg¹¹-Met¹², Gly²⁰-Leu²¹, and at an unusual bond between Arg⁴ and Ser⁵ with human kidney NEP (11). NEP is usually inert toward bonds adjacent to Ser residues because of its high hydrophilicity. In our study, the Arg⁴-Ser⁵ bond was also found to be hydrolyzed by human NEP, and our results from hANP-28 hydrolysis by human NEP agree with those observations made by Vanneste *et al.* (11).

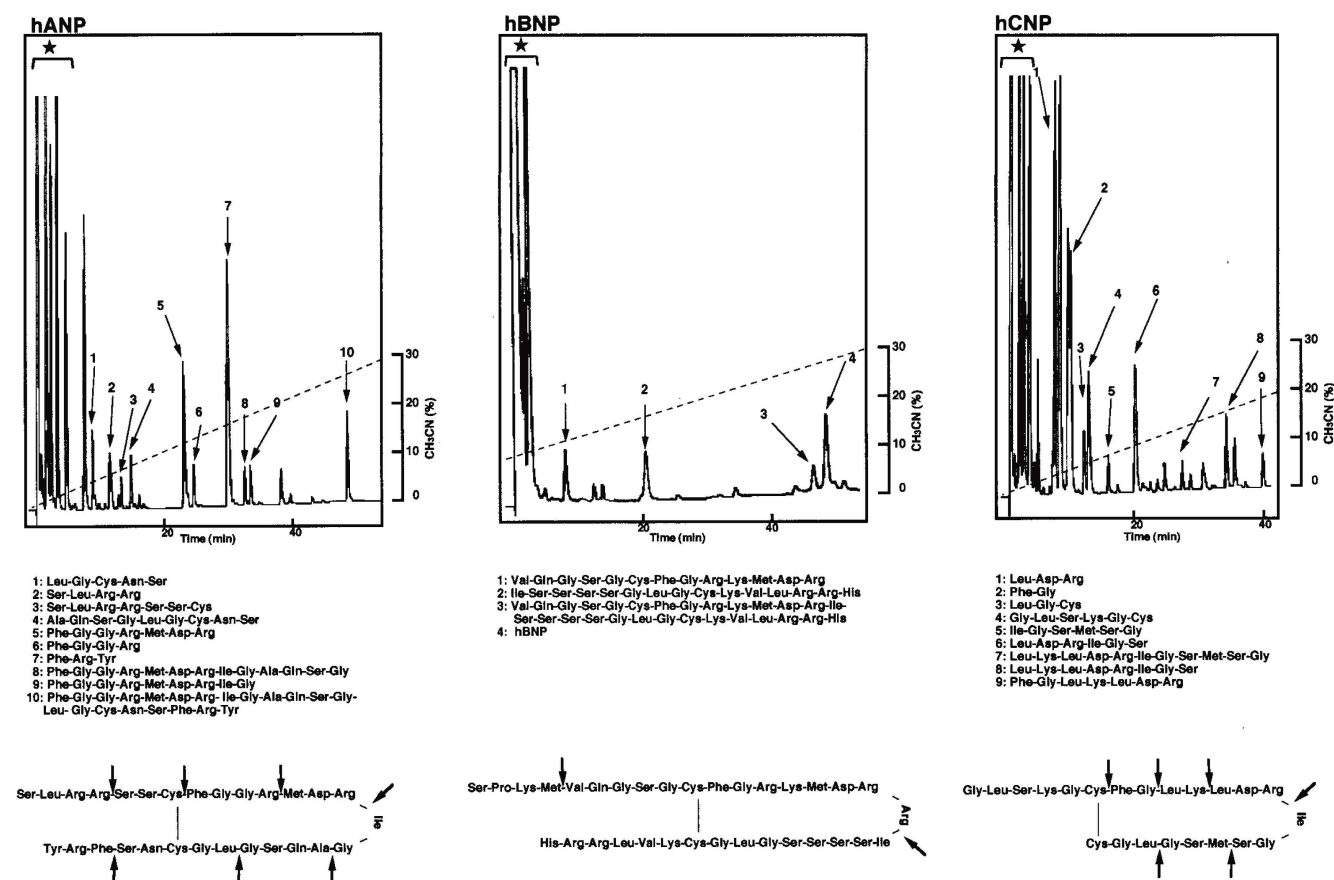


FIG. 3. Cleavage of the three natriuretic peptides by NEP. The reaction mixture contained 0.35 mM substrate, 10 mM Tris-HCl, pH 8.0, and purified NEP (300 ng for hANP-28 and hCNP-22, and 700 ng for hBNP-32) in a final volume of 50 μ l. Incubations were carried out at 37°C for 2 h, except hBNP-32, which was incubated for 4 h. Products from the reaction mixture were separated by HPLC in an acetonitrile gradient in 0.07% (v/v) TFA. Stars indicate nonpeptide-related peaks derived from the reagents used for reduction and alkylation. Arrows on the peptide structures indicate the sites of cleavage.

The cleavage sites of hBNP-32 have been reported by Norman *et al.* (20) with rat kidney enzyme and by Kenny *et al.* (21) with porcine kidney enzyme. In contrast to hANP-28, although hBNP-32 contains Cys-Phe at the beginning of the ring structure, the bond was not cleaved by human NEP. Our results indicate that hBNP-32 was cleaved at two sites, Met⁴-Val¹⁵ and Arg¹⁷-Ile¹⁸. This study is the first to determine of the NEP cleavage sites on hCNP-22. The fragments identified were derived from cleavages at Cys⁶-Phe⁷, Gly⁸-Leu⁹, Lys¹⁰-Leu¹¹, Arg¹³-Ile¹⁴, Ser¹⁶-Met¹⁷, and Gly¹⁹-Leu²⁰. Although some peaks were formed and then disappeared during the incubation period, the first peak to emerge after the limited incubation period was peak 4 (Gly-Leu-Ser-Lys-Gly-Cys). This suggests that like hANP-28, its primary cleavage site in hCNP-22 is between Cys⁶ and Phe⁷ at the beginning of the ring structure. The

ring structures of hANP-28 and hCNP-22 are essential for efficient hydrolysis by NEP. Reduction and alkylation of hANP-28 and hCNP-22 greatly decreased the degradation of the substrates (data not shown). Among the three natriuretic peptides, the hydrolysis rate of hBNP-32 by human NEP is significantly lower than those of hANP-28 and hCNP-22, and the Cys-Phe bond which is present in hBNP-32 is not cleaved.

NEP plays a role in the inactivation of BNP and ANP in the plasma; that is, NEP inhibitors increased plasma levels of BNP in humans (22) and potentiated the renal activity of BNP in laboratory animals (23). Mukoyama *et al.* reported that the time taken for half the plasma BNP to disappear was longer than for ANP and this was in part attributed to the lower binding affinity of BNP than ANP to clearance receptors (24). Our results show

that the low K_{cat}/K_m value of hBNP-32 for NEP may also contribute to the slow clearance of BNP from the plasma. The low affinity of hBNP-32 for NEP may explain the insusceptibility of the Cys-Phe bond to cleavage by NEP.

REFERENCES

1. Kerr MA, Kenny A. The purification and specificity of a neutral endopeptidase from rabbit kidney brush border. *Biochem J* **137**:477–488, 1974.
2. Malfroy B, Swerts JP, Guyon A, Roques BP, Schwartz JC. High affinity enkephalin-degrading peptidases in brain is increased after morphine. *Nature* **276**:523–526, 1978.
3. Almenoff J, Wilk S, Orlowski M. Membrane-bound pituitary metalloendopeptidase: Apparent identity to enkephalinase. *Biochem Biophys Res Commun* **102**:206–214, 1981.
4. Matsas R, Fulcher IS, Kenny AJ, Turner AJ. Substance P and (Leu)enkephalin are hydrolyzed by an enzyme in pig caudate synaptic membranes that is identical with the endopeptidase of kidney microvilli. *Proc Natl Acad Sci USA* **80**:3111–3114, 1983.
5. Kerr MA, Kenny AJ. The molecular weight and properties of a neutral metallo-endopeptidase from rabbit kidney brush border. *Biochem J* **137**:489–495, 1974.
6. Howell S, Murray H, Scott CS, Turner AJ, Kenny AJ. A highly sensitivity e.l.i.s.a. for endopeptidase-24.11, the common acute-lymphoblastic-leukaemia antigen (CALLA, CD-10), applicable to material of porcine and human origin. *Biochem J* **278**:417–421, 1991.
7. Maack T, Suzuki M, Almeida FA, Nussenzveig D, Scarborough RM, McEnroe GA, Lewicki JA. Physiological role of silent receptors of atrial natriuretic factor. *Sciences* **238**:675–678, 1987.
8. Stephenson SL, Kenny AJ. The hydrolysis of α -human natriuretic peptide by pig kidney microvillar membranes is inhibited by endopeptidase 24.11. *Biochem J* **243**:183–187, 1987.
9. Olins GM, Spear KL, Siegel NR, Reinhard EJ, Zurcher-Neely HA. Atrial peptide inactivation by rabbit-kidney brush-border membranes. *Eur J Biochem* **170**:431–434, 1987.
10. Kenny AJ, Stephenson SL. Role of endopeptidase 24.11. in the inactivation of atrial natriuretic peptide. *FEBS Lett* **232**:1–8, 1988.
11. Vanneste Y, Michel A, Dimaline R, Najdovski T, Deschodt-Lanckman M. Hydrolysis of α -human atrial natriuretic peptide *in vitro* by human kidney membranes and purified endopeptidase-24.11. *Biochem J* **254**:531–537, 1988.
12. Wilkins MR, Nunez DJ, Wharton J. The natriuretic peptide family: Turning hormones into drugs. *J Endocrinol* **137**:347–359, 1993.
13. Richards AM. The natriuretic peptides and hypertension. *J Int Med* **235**:543–560, 1994.
14. Egawa-Iwaki S, Watanabe Y, Fujimoto Y. Identification of a rat liver dipeptidyl aminopeptidase IV with a liver plasma membrane glycoprotein (gp110). *FEBS Lett* **286**:167–170, 1991.
15. Watanabe Y, Shimamori Y, Ozaki M, Uchida M, Fujimoto Y. Purification of membrane-bound neutral endopeptidase from rat kidney and its activation by polyamines. *J Biochem* **95**:1725–1732, 1984.
16. Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano MD, Fujimoto EK, Goeke NM, Olson BJ, Klenk DC. Measurement of protein using bicinchoninic acid. *Anal Biochem* **150**:76–85, 1985.
17. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**:680–685, 1970.
18. Shipp MA, Look AT. Hematopoietic differentiation antigens that are membrane-associated enzymes: Cutting is the key. *Blood* **82**:1052–1070, 1993.
19. Shimamori Y, Watanabe Y, Fujimoto Y. Specificity of a membrane-bound neutral endopeptidase from rat kidney. *Chem Pharm Bull* **34**:275–280, 1986.
20. Norman JA, Little D, Bolgar M, Donato G. Degradation of brain natriuretic peptide by neutral endopeptidase: Species specific sites of proteolysis determined by mass spectrometry. *Biochem Biophys Res Commun* **175**:22–30, 1991.
21. Kenny AJ, Bourne A, Ingram J. Hydrolysis of human and pig brain natriuretic peptides, urodilatin, C-type natriuretic peptide and some C-receptor ligands by endopeptidase-24.11. *Biochem J* **291**:83–88, 1993.
22. Lang CC, Motwani J, Coutie WJ, Struthers AD. Influence of candoxatril on plasma brain natriuretic peptide in heart failure. *Lancet* **338**:255, 1991.
23. Seymour AA, Asaad MM, Abboa-Offei BE, Rovnyak PL, Fennell S, Rogers WL. Potentiation of brain natriuretic peptides by SQ28,603, an inhibitor of neutral endopeptidase 3.4.21.11, in monkeys and rats. *J Pharmacol Exp Ther* **262**:60–70, 1992.
24. Mukoyama M, Nakao K, Hosoda K, Suga S, Saito Y, Ogawa Y, Shirakami G, Jougasaki M, Obata K, Yasue S, Kambayashi Y, Inoue K, Imura H. Brain natriuretic peptide as a novel cardiac hormone in humans. *J Clin Invest* **87**:1402–1412, 1991.