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DEGRADATION OF BRAIN NATRIURETIC PEPTIDE BY NEUTRAL ENDOPEPTIDASE: SPECIES SPECIFIC SITES OF PROTEOLYSIS DETERMINED BY MASS SPECTROMETRY

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Brain natriuretic peptide (BNP) from 3 different species was cleaved by neutral endopeptidase (NEP) and the products separated by HPLC. The newly formed products were identified by fast atom bombardment or nebulizer-assisted electrospray mass spectrometry to elucidate the sites of proteolysis. Porcine BNP was cleaved at the Arg^{8}-Leu^{9} and Ser^{14}-Leu^{15} bonds. Rat BNP was cleaved at the Arg^{23}-Leu^{24} and Arg^{30}-Leu^{31} bonds. Human BNP was cleaved at the Pro^{2}-Lys^{3}, Met^{4}-Val^{5} and Arg^{17}-Leu^{18} bonds. The Cys-Phe bond which is present in all species of BNP is not cleaved by NEP.

Atrial natriuretic peptide (ANP) was first discovered as a circulating natriuretic and depressor peptide which is released from the heart in response to volume expansion (1). ANP has been found to be significantly elevated in animal models of heart failure (2) as well as patients with severe heart failure (3) thus implicating this peptide with the pathophysiology of this disease. Several investigators have found that neutral endopeptidase (EC 3.4.24.11) will cleave and inactivate ANP by a specific proteolytic event occuring at the Cys^{7}-Phe^{8} bond in the ring structure of ANP (4). This ring-opened form of ANP is biologically inactive and exists in human circulation as 20 -30% of the total immunoreactive ANP (5). Inhibitors of neutral endopeptidase (NEP) have been shown to potentiate exogenously administered ANP by elevating Na^{+} and cGMP excretion and depressor effects. Additionally, inhibitors of NEP have been shown to potentiate endogenous ANP effects in animal models of heart failure by elevating circulating ANP concentrations and increasing Na^{+} and cGMP excretion (6). More recently, the administration of NEP inhibitors to heart failure patients has resulted in renal and cardiovascular effects similar to that of low dose ANP infusion (7).

Subsequent to the purification and identification of ANP, Sudoh et al. (8) discovered a natriuretic peptide from porcine brain that is homologous to ANP and named it brain natriuretic peptide (BNP). Other investigators have purified the peptide from human and rat brain or have obtained the cDNA encoding BNP (9, 10). Sequence comparison has revealed that BNP has diverged significantly between

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species and has retained only 12 invariant amino acids with respect to ANP. ANP is highly conserved between species and has permitted only an Ile/Met substitution at position 12 in human ANP. Brain natriuretic peptide has been found to be present in circulation and also synthesized in the heart (11). Additionally, a specific BNP/guanylate cyclase receptor has been cloned and sequenced which is highly homologous to the ANP/guanylate cyclase receptor (12). These data suggest that BNP is a circulating hormone with its own receptor system similar to ANP. More recently, circulating BNP has been found to be significantly elevated in patients with heart failure (13). Since the Cys-Phe position is conserved in all species of BNP examined to date, it was of interest to determine whether neutral endopeptidase cleaves BNP at the Cys-Phe position as well. Three species of BNP were examined in this study and NEP did not cleave at the Cys-Phe bond in any case. NEP prefers Arg-Leu, Arg-Ile or Ser-Leu sites in BNP.

Methods

Neutral endopeptidase was solubilized and purified from rat kidney-membrane fractions using a modification of the procedure described by Almenoff and Orlowski (14). NEP activity was measured using Glutaryl-Ala-Ala-Phe-2 naphthylamide (Glut-Ala-Ala-Phe-2NA) as the substrate according to the procedure of Orlowski and Wilk (15). Porcine, rat BNP were obtained from Peninsula Laboratories, Belmont, CA and human BNP was obtained from Peptides International, Louisville, KY.

Proteolysis of BNP was measured by incubating BNP with neutral endopeptidase in a final volume of 0.05 ml containing 300 μM BNP, 17 μg/ml NEP, 50 mM sodium phosphate, pH 7.4 and 0.1 M NaCl. After the indicated times, incubations were stopped by the addition of TFA and acetonitrile. HPLC separation of products was performed on a Waters HPLC system using a C_4 RP column (Vydac) employing a 0.1% TFA (A) + 0.1% TFA in acetonitrile (B) gradient (10% A to 50% B in 25 min) unless otherwise indicated. Absorbance was monitored at 215 nm and selected fractions were collected and dried under N_2 for mass spectral analysis.

Molecular weights of rat and porcine BNP as well as related proteolysis products were determined by fast atom bombardment (FAB) mass spectrometry using a VG ZAB-HF mass spectrometer with an accelerating voltage of 5 to 6 kV and equipped with an Ion Tech fast atom gun . Samples were dissolved in a matrix of dithiothreitol: dithioerythritol (3:1) in 1.0% trifluoroacetic acid and desorbed by Xenon atoms from the FAB gun operated at 8 kV. The positive ions produced by FAB were acquired at an instrument resolution between 1,000 and 4,000 ($M/\Delta M$), depending on the signal intensity and molecular weight of the analyte. Average molecular weights were obtained in instances where low ion intensity precluded the measurement of monoisotopic weights.

Molecular weights of human BNP and related proteolysis products were determined by nebulizer-assisted electrospray mass spectrometry (16,17) otherwise known as ion spray mass spectrometry (18). Collected peaks from HPLC separations were dissolved in a solution of methanol:H_2O (1:1) containing 10 mM ammonium acetate and 0.1% formic acid. The dissolved sample was taken up in a 0.05 ml syringe and infused into the ionizing chamber at an infusion rate of 0.003 ml/min using a Harvard Aparatus syringe pump, model number 22. Spectra were acquired on a Sciex API III triple quadrupole mass spectrometer. The electrospray voltage was +4800. Zero grade air was used as the nebulization gas at a pressure of 40 psi.

Assignment of the peptide sequence to the mass of the ion measured was done using the Mac Pro Mass program that searched the parent sequence for a contiguous amino acid sequence that corresponded to the correct mass for each ion identified.

Results

Overlays of the HPLC chromatograms demonstrating the degradation of porcine and rat BNP by neutral endopeptidase are shown in Figure 1. Porcine BNP hydrolysis by NEP occurred at a rate of 150 nmol/min/mg based on a reduction in peak height for BNP (Figure 1A). Two new peaks were evident after 60 minutes of incubation with NEP. They had retention times of 15 min (peak A) and 16.5 min (peak B), while the parent peak (porcine BNP) had a retention time of 18.5 min (peak C). Rat BNP hydrolysis by NEP proceeded at approximately the same rate as porcine BNP and the results are shown in Figure 1B. Three new peaks were formed after hydrolysis for 60 minutes and had retention times of 14.3 min (peak A), 16.1 min (peak B), and 18.1 min (peak C). The parent peak (rat BNP) had a retention time of 19.8 min (peak D). The newly formed peptide peaks described above were evaluated by FAB mass spectrometry to elucidate the sites of proteolysis by NEP. Table 1 summarizes the experimental and calculated mass of each peak and the peptide assignment. Peak A derived from porcine BNP had an $(M+H)^+$ ion at m/z 2245.49 Da which was assigned

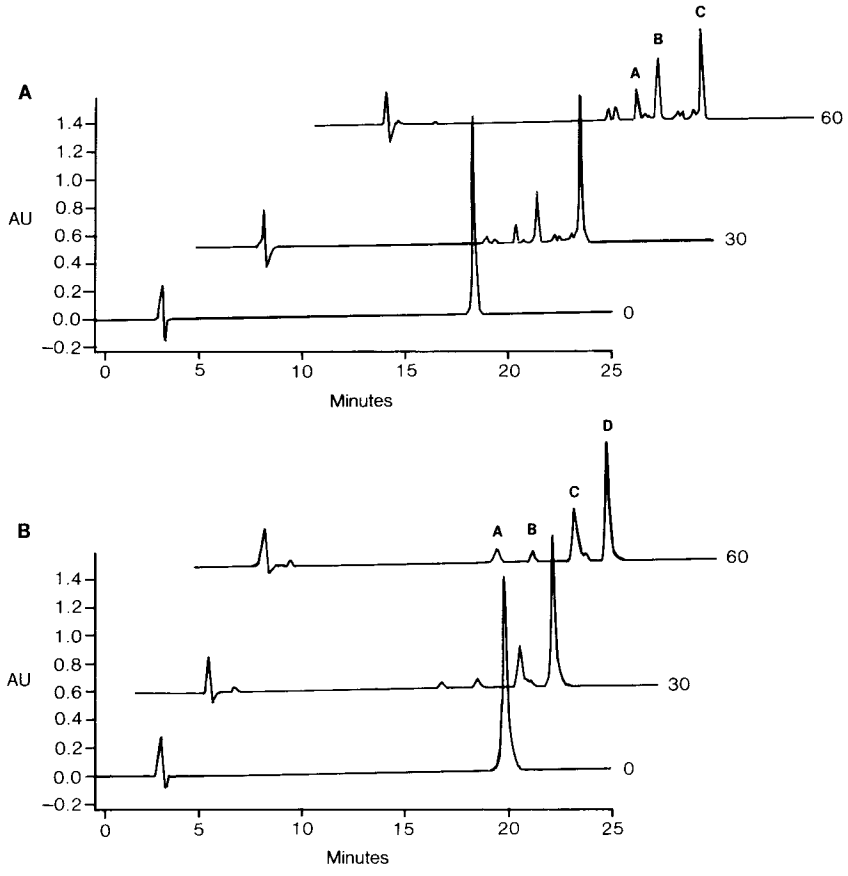

Figure 1. Degradation of porcine and rat BNP by neutral endopeptidase. Porcine BNP (panel A) and rat BNP (panel B) were digested with NEP for the times indicated at the end of each HPLC trace as described in Methods. The peaks labelled in the 60 min digestion were collected for FAB mass spectrometry.

Table 1. Mass assignments for hydrolysis products of porcine and rat BNP by neutral endopeptidase. Peaks shown below correspond to the peaks isolated from the HPLC separation of proteolysis products shown in Figure 1. All mass determinations were made by FAB mass spectrometry as described in the Methods section and are presented as monoisotopic molecular weights unless otherwise indicated. DTT was used to reduce disulfide bonds where indicated.

	Experimental	Calculated	Assignment
Porcine BNP			
Peak A	2245.49	2245.11	[Asp^{1}-Arg^{8}]-[Leu^{15}-Tyr^{26}]
(+DTT)	897.50	897.40	Asp^{1}-Arg^{8}
(+DTT)	1350.70	1350.73	Leu^{15}-Tyr^{26}
Peak B	2886.50	2886.46	[Asp^{1}-Tyr^{26}] + 18D
(+DTT)	1538.6	1538.75	Asp^{1}-Ser^{14}
(+DTT)	1350.7	1350.73	Leu^{15}-Tyr^{26}
Peak C	2868.5	2868.45	Asp^{1}-Tyr^{26}
Rat BNP			
Peak A	3211.3*	3211.68	[Asn^{1}-Arg^{30}] + 18D
Peak B	3193.3*	3193.58	Asn^{1}-Arg^{30}
Peak C	3471.8*	3472.02	Asn^{1}-Phe^{32} + 18D
(+DTT)	2479.8	2479.23	Asn^{1}-Arg^{23}
(+DTT)	993.4	993.52	Leu^{24}-Phe^{32}
Peak D	3453.4*	3453.73	Asn^{1}-Phe^{32}

* Average molecular weight.

to [Asp^{1}-Arg^{8}]-[Leu^{15}-Tyr^{26}]. This corresponded to porcine BNP with the hexapeptide, Leu^{9}-Ser^{14}, deleted from the ring. This assignment was confirmed by reducing the disulfide bond in peak A by DTT treatment. This yielded $(M+H)^+$ ions at m/z 897.50 Da and m/z 1350.70 Da which corresponded to Asp^{1}-Arg^{8} and Leu^{15}-Tyr^{26}, respectively. The sum of the mass of these two ions was equal to the mass of the parent ion plus 2 Da, which is expected after reduction of the disulfide bond by addition of one hydrogen to each cysteine residue. Peak B yielded an ion having an $(M+H)^+$ ion at m/z 2886.50 Da which corresponded to porcine BNP plus 18 Da, indicating that hydrolysis within the ring must have occurred with the net addition of one H_2O molecule to the parent structure. Reduction of peak B with DTT yielded 2 $(M+H)^+$ ions at m/z 1538.6 Da and 1350.7 Da, corresponding to Asp^{1}-Ser^{14} and Leu^{15}-Tyr^{26}, respectively. This indicates that hydrolysis of the Ser ^{14}-Leu^{15} bond has occurred. Peak C had an $(M+H)^+$ ion at m/z 2868.5 Da which was porcine BNP, the substrate. Taken together, these results indicated that NEP cleaved porcine BNP primarily at the Ser^{14}-Leu^{15} bond with a secondary proteolytic event at the Arg^{8}-Leu^{9} bond. Peak A obtained from the proteolysis of rat BNP had an $(M+H)^+$ ion at m/z 3211.3 Da which was assigned to Asn^{1}-Arg^{30} plus 18 Da. Peak B had an $(M+H)^+$ ion at m/z 3193.58 Da which corresponded to Asn^{1}-Arg^{30}. These two results indicated that cleavage at the Arg^{30}-Leu^{31} bond has occurred and that the species in peak A

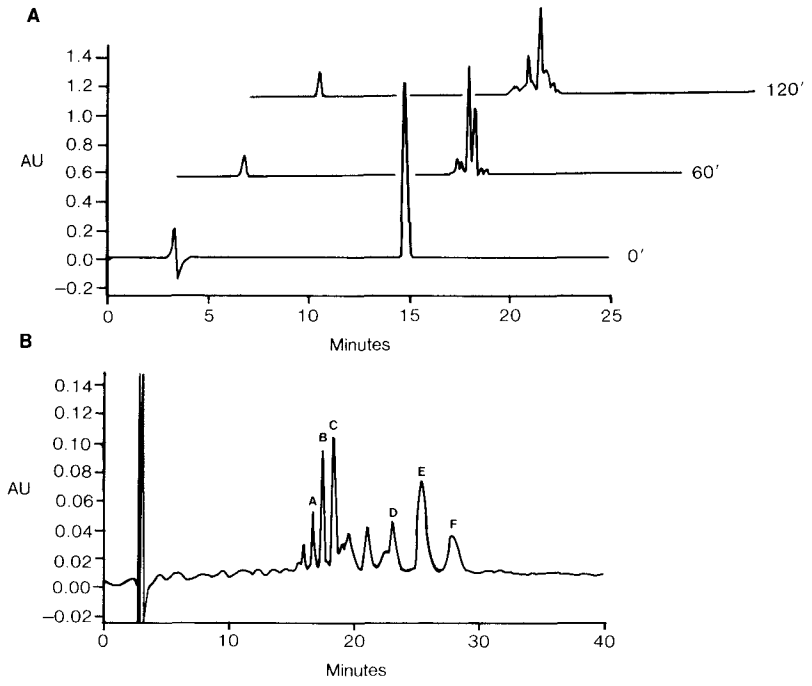

Figure 2. Degradation of human BNP by neutral endopeptidase. The separation of products was attempted with the linear gradient conditions used for porcine and rat BNP (panel A). A modified gradient (90% A:10% B to 81% A:19% B in 10 min followed by 81% A:19% B isocratic for 20 min) was used improve the separation of products, as seen in panel B. Peaks labelled in panel B were obtained after digestion of human BNP for 60 min as described in Methods and were collected for ion spray mass spectrometry.

has been cleaved within the ring. Peak C had an $(M+H)^+$ ion at m/z 3471.8 Da which corresponds to Asn^1-Phe^{32} plus 18 Da. After reduction of the disulfide bond with DTT, 2 $(M+H)^+$ ions at m/z 2479.23 Da and m/z 993.52 Da were obtained which corresponded to Asn^1-Arg^{23} and Leu^{24}-Phe^{32}, respectively. This result indicated that the Arg^{23}-Leu^{24} bond was the primary site of proteolysis within the ring. Peak D had an an $(M+H)^+$ ion at m/z 3453.4 Da which corresponded to rat BNP, the substrate. Human BNP was degraded by NEP with a rate of hydrolysis similar to that observed for porcine and rat BNP. When the products of hydrolysis were separated by HPLC using the gradient conditions established for porcine and rat BNP, the products did not separate sufficiently to permit analysis by mass spectrometry (Figure 2A). The separation conditions were modified by utilizing a gradient/isocratic method such that six distinct peaks (labelled A-F) were evident and could be collected for analysis. All of the peptide products were analyzed by ion spray mass spectrometry which is more sensitive than FAB mass spectrometry because the ionization procedure yields ions with multiple positive charges that can be more readily detected. Peak A contained a single component with ions having m/z ratios at 760.58, $(M+4H)^{4+}$, and 1014.00, $(M+3H)^{3+}$, which gave an average mass of 3038.68 Da and corresponded to Val^5-His^{32} + 18 Da. Since peak A was the only peak to have a single component and was

Table 2. Mass assignments for hydrolysis products of human BNP by neutral endopeptidase. Peaks shown below correspond to the peaks isolated from the HPLC separation of proteolysis products shown in Figure 2B. All mass determinations were made by ion spray mass spectrometry as described in the Methods section. Average molecular weights are shown for all determinations. DTT was used to reduce disulfide bonds where indicated.

	Experimental	Calculated	Assignment
Peak A	3038.68	3038.54	[Val^{5}-His^{32}] + 18 D
(+DTT)	1599.40	1599.89	Ile^{18}-His^{32}
(+DTT)	1440.23	1440.68	Val^{5}-Arg^{17}
Peak C and B	3297.82	3297.92	[Lys^{3}-His^{32}] + 18 D
	3038.68	3038.55	[Val^{5}-His^{32}] + 18 D
Peak D	3482.20	3482.11	[Ser^{1}-His^{32}] + 18 D
	3020.50	3020.54	Val^{5}-His^{32}
Peak E	3279.40	3279.92	Lys^{3}-His^{32}
Peak F	3464.20	3464.10	Ser^{1}-His^{32}

hydrolysed at some position within the ring, this material was reduced with DTT and analyzed. One component had ions with m/z at 534.2, $(M+3H)^{3+}$, and 800.6, $(M+2H)^{2+}$, which gave an average mass of 1599.40 Da. The other component had ions with m/z at 481.14, $(M+3H)^{3+}$, and 721.02, $(M+2H)^{2+}$, which gave an average mass of 1440.23 Da. The assignment for these ions was Ile^{18}-His^{32} and Val^{5}-Arg^{17}, respectively. These data are summarized in Table 2 and indicated that proteolysis within the ring had occurred at the Arg^{17}-Ile^{18} site as well as cleavage at the Met^{4}-Val^{5} position. Peaks B and C contained the same two components. One series of ions occurred at the following m/z ratios: 550.52, $(M+6H)^{6+}$; 660.50, $(M+5H)^{5+}$, 825.52, $(M+4H)^{4+}$ and 1100.58, $(M+3H)^{3+}$. This gave an average mass of 3297.82 Da and corresponded to Lys^{3}-His^{32} + 18 Da. Another series of ions occurred at the following m/z ratios: 760.58, $(M+4H)^{4+}$ and 1014.00 $(M+3H)^{3+}$. This gave an average mass of 3038.68 Da and corresponded to Val^{5}-His^{32} + 18 Da. These data indicate that proteolysis has occurred at the Pro^{2}-Lys^{3} position and the Met^{4}-Val^{5} position in addition to cleavage within the ring which was probably the Arg^{17}-Ile^{18} site. Peak D also contained two components having the following m/z ratios: 617.4, $(M+5H)^{5+}$; 871.45, $(M+4H)^{4+}$, and 1162.0, $(M+3H)^{3+}$. This gave an average mass of 3482.2 Da which corresponded to Ser^{1}-His^{32} + 18 Da. Another series of ions had the following m/z ratios: 605.0, $(M+5H)^{5+}$ and 756.1, $(M+4H)^{4+}$. This gave an average mass of 3020.5 Da which corresponded to Val^{5}-His^{32}. These results indicated that cleavage within the ring had occurred in the parent peptide before any cleavage at the amino terminus had occurred. Presumably, this cleavage within the ring occurred at the Arg^{17}-Ile^{18} site. The second component represented the parent peptide with the 4 amino terminal amino acids removed and no proteolysis within the ring. Peak E

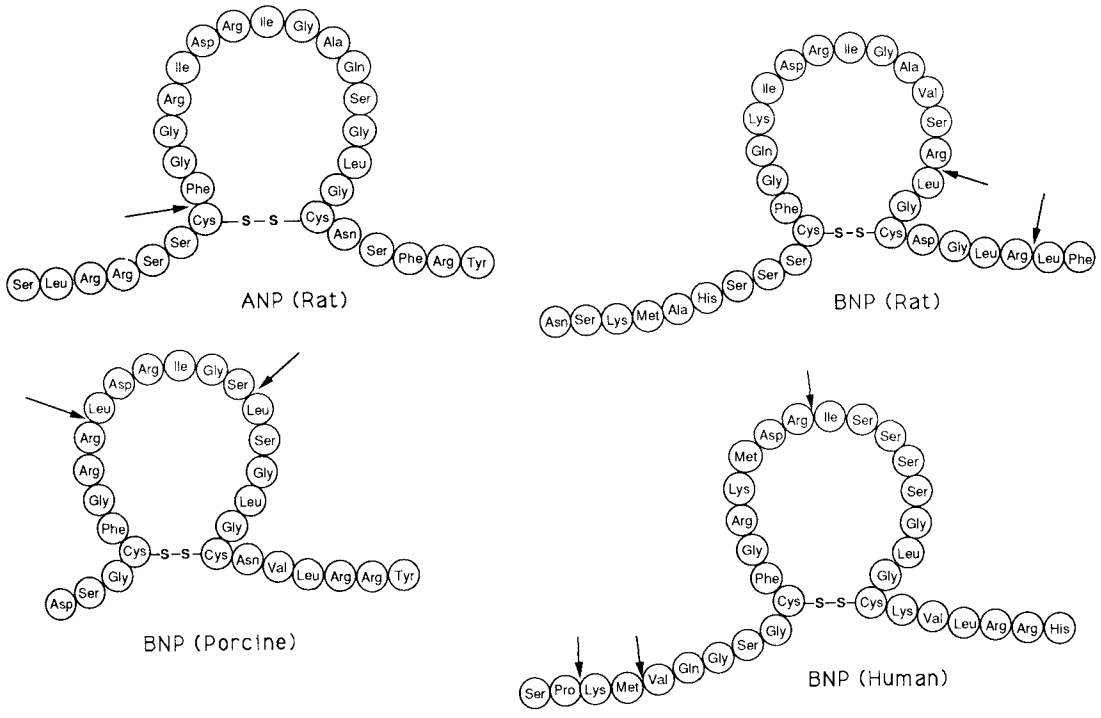

Figure 3. The sites of proteolysis by neutral endopeptidase for natriuretic peptides.

contained a single component with ions having m/z ratios at: 656.96, $(M+5H)^{5+}$; 820.88, $(M+4H)^{4+}$, and 1094.5, $(M+3H)^{3+}$. This gave an average mass of 3279.4 Da which corresponded to Lys^{3}-His^{32}. This result indicated that cleavage at the Pro^{2}-Lys^{3} position occurred with no other proteolytic events in human BNP. Peak F was a single component with ions occurring at the following ratios: 578.4, $(M+6H)^{6+}$; 693.86, $(M+5H)^{5+}$; 867.00, $(M+4H)^{4+}$, and 1155.70 $(M+3H)^{3+}$. This gave an average mass of 3464.2 Da, corresponding to intact human BNP, the substrate. Figure 3 illustrates the above results.

Discussion

Unique sites of proteolysis by neutral endopeptidase for 3 species of BNP has been determined. Porcine BNP was shown to be cleaved at the Arg^{8}-Leu^{9} and Ser^{14}-Leu^{15} bonds resulting in a deletion of the Leu^{9}-Ser^{14} hexapeptide from the ring structure of the parent peptide. This result is in agreement with the report by Vogt-Schaden et al. (19) that porcine BNP is cleaved primarily at the Arg^{8}-Leu^{9} and Ser^{14}-Leu^{15} bonds by NEP. They used HPLC to separate proteolysis products and determined the structure of the resulting peptides by Edman degradation. In the present study, mass spectrometry was used to identify the peptide products and obtain the same result. NEP cleaved rat BNP at the Arg^{23}-Leu^{24} and Arg^{30}-Leu^{31} bonds which is not surprising since the report of Orlowski and Wilk (15) showed that NEP prefers substrates having Arg-Leu at the scissile bond. The only other Arg-Leu bond

in the series of ANP or BNP studied to date is in porcine BNP which is also cleaved by NEP indicating that Arg-Leu is a preferred site of proteolysis. Human BNP was cleaved within the ring structure at the Arg^{17}-Ile^{18} bond which is a conservative substitution for Arg-Leu which is favored. It is interesting to note that rat and human ANP as well as the 3 species of BNP all have the homologous Arg-Ile bond conserved but NEP does not cleave ANP or rat and porcine BNP at this position. Arg-Ile cleavage by thermolysin, a bacterial metalloprotease with homology to NEP, has been reported in truncated C-receptor analogs that do not contain any Arg-Leu bonds (20). The cleavage of human BNP at the Pro^{2}-Lys^{3} bond is difficult to explain since NEP is considered to require an hydrophobic amino acid at the P_1' position of substrates (15). In this regard, cleavage at the Met^{4}-Val^{5} position can be more readily explained. The Cys-Phe bond that is cleaved in rat and human ANP by NEP is conserved in all 3 species of BNP but is not cleaved by this protease. It appears that Arg-Leu is the favored site of cleavage in BNP and that Arg-Ile and Ser-Leu are secondary proteolysis sites. The Cys-Phe bond is probably not cleaved in BNP because of the availability of preferred sites in these peptides.

This study utilized mass spectrometry to identify the proteolysis products of BNP by NEP. This technique gives a very accurate measurement of the mass of a given analyte and in this study the experimental values agreed within 0.2 daltons of the calculated value. Since the parent sequence of the peptide being studied is known, the structure of the degradation product can readily be determined after a computer search for a contiguous amino acid sequence that will correspond to the experimentally determined mass. The results obtained for porcine BNP hydrolysis by BNP agree with the observation made by Vogt-Schaden et al. (16) who used Edman degradation to determine the same results. Also, Ackerman et al. (20) successfully used mass spectrometry to observe the proteolysis of C-receptor ligands by thermolysin. Mass spectrometry is a powerful tool that can detect multiple components of peptide peaks that would otherwise give ambiguous results by classical sequencing procedures.

In conclusion, NEP has been shown to degrade BNP at unique sites in a species-specific manner. It will be important to consider the possibility that NEP inhibitors may potentiate endogenous BNP levels as well as ANP levels in subsequent in vivo and clinical studies.

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