Clinical Chemistry 52:1
82–87 (2006)

Proteomics and
Protein Markers

Dipeptidyl-Peptidase IV Converts Intact B-Type Natriuretic Peptide into Its *des*-SerPro Form

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Background: **Analysis of plasma B-type natriuretic peptide (BNP) has suggested the in vivo formation of a truncated form, BNP (3–32), also called *des*-SerPro-BNP. The objectives of this study were to investigate (*a*) whether BNP and other natriuretic peptides are truncated by dipeptidyl-peptidase IV (DPP IV/CD26; EC 3.4.14.5) and (*b*) whether this truncation affects the susceptibility to cleavage by neutral endopeptidase (NEP; EC 3.4.24.11).**

Methods: **Human BNP (1–32), A-type natriuretic peptide 1–28 (ANP 1–28), and related peptides were incubated with purified DPP IV and with human plasma. In addition, BNP (1–32), BNP (3–32), and ANP (1–28) were subjected to hydrolysis by NEP. Cleavage products were analyzed by mass spectrometry.**

Results: **BNP (1–32) was cleaved by purified DPP IV with a specificity constant of 0.37×10^6 L · mol^{-1} · s^{-1}. The DPP IV activity in EDTA-plasma was able to truncate BNP (1–32) ex vivo. Addition of Vildagliptin, a specific DPP IV inhibitor, prevented this truncation in a concentration-dependent manner. Under in vitro circumstances in which ANP was hydrolyzed extensively, BNP (1–32) and BNP (3–32) were very resistant to NEP-mediated cleavage.**

Conclusions: **DPP IV cleaves BNP (1–32) with an efficiency higher than or comparable to several known in vivo substrates of the enzyme. Even after loss of the amino-terminal dipeptide, BNP remains highly resistant to cleavage by NEP.**

The heart secretes 2 natriuretic peptides, A-type natriuretic peptide (atrial natriuretic peptide; ANP)[4] and B-type natriuretic peptide (brain natriuretic peptide; BNP), which are involved in the regulation of body fluid homeostasis and vascular tone *(1)*.

Atrial and ventricular cardiomyocytes produce proANP (1–126) and proBNP (1–108), respectively, which are subsequently cleaved into an amino-terminal part [NT-proANP (1–98) and NT-proBNP (1–76)] and the biologically active ANP (1–28) and BNP (1–32). It has been reported that several proANP fragments [e.g., proANP (1–30) and proANP (79–98)] also circulate in plasma and may play a role as natriuretic hormones. Myocyte stretch and volume overload both stimulate the release of ANP and BNP as well as their amino-terminal counterparts NT-proANP and NT-proBNP. Accordingly, high circulating concentrations of the processed peptides are found in symptomatic as well as asymptomatic patients with impaired cardiac function. Several excellent reviews have been published on the use of BNP as a marker for diagnosis and prognosis of heart failure *(2, 3)*. Measurement of plasma BNP enables the discrimination of cardiac from noncardiac causes of dyspnea with high accuracy. BNP concentrations are increased according to the New York Heart Association (NYHA) classification scale, and recent work showed the clinical utility of BNP in risk stratification and heart failure therapy. In addition, recombinant human BNP (Nesiritide) has been used as a treatment strategy in the management of heart failure [reviewed by de Denus et al. *(4)*].

The physiologic effects of BNP (1–32) and ANP (1–28) are caused by binding to natriuretic peptide receptor type A, which is coupled to cGMP *(5, 6)*.

The biology of natriuretic peptides in general and the degradation and elimination mechanisms in particular are

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Received July 13, 2005; accepted October 10, 2005.
Previously published online at DOI: 10.1373/clinchem.2005.057638

[4] Nonstandard abbreviations: ANP and BNP, A- and B-type natriuretic peptide, respectively; NT-proANP and NT-proBNP, amino-terminal portions of ANP and BNP, respectively; NEP, neutral endopeptidase; DPP IV, dipeptidyl-peptidase IV; and TFA, trifluoroacetic acid.

only partly understood *(5)*. In addition to elimination by renal excretion and by binding to natriuretic peptide receptor type C *(7)*, natriuretic peptides are also cleared through proteolysis by peptidases, the most closely studied being neutral endopeptidase (NEP; EC 3.4.24.11), a zinc metalloproteinase. Many reviews on BNP mention NEP-mediated inactivation as a clearance mechanism, but at the same time several authors have reported that BNP seems relatively resistant to NEP degradation compared with ANP *(8)*. As a consequence, the issue of NEP involvement in BNP clearance remains controversial *(9–13)*.

Shimizu et al. *(14)* found that, on incubation of human BNP (1–32) in whole blood, 2 amino acid residues (Ser-Pro) were released from the amino terminus. Goetze *(5)* suggested that endogenous BNP (1–32) may undergo amino-terminal trimming by an "amino-dipeptidase". The identity of the enzyme responsible for this truncation has remained unknown, however. The exopeptidase dipeptidyl-peptidase IV (DPP IV/CD26; EC 3.4.14.5), a cell-surface protease that occurs in a soluble form in plasma, cleaves many bioactive peptides of medical importance. It preferentially cleaves dipeptides from the amino terminus of peptides with a proline or alanine in the second position. The substrate specificity of DPP IV is not that strict, however; cleavage after a penultimate Ser, Gly, Thr, Val, and Leu also has been observed. Moreover, vasoactive intestinal peptide and pituitary adenylate cyclase-activating peptide, both of which have a serine in this position, are known DPP IV substrates *(15)*.

BNP (1–32) and proANP (1–30) carry a proline in the penultimate position. ANP (1–28) has a leucine and proANP (79–98) has a serine in this position, but these are not the preferred amino acids for DPP IV.

The first aim of this study was to ascertain whether BNP (1–32), ANP (1–28), proANP (1–30), and proANP (79–98) are truncated by DPP IV and to determine the kinetic data of the observed cleavages. We also investigated the specificity of the truncations by use of Vildagliptin, a DPP IV-specific inhibitor (NVP-LAF237) developed as a therapeutic agent for the treatment of type 2 diabetes (currently in phase III clinical trials) *(16–18)*. The second aim was to investigate whether the DPP IV-mediated loss of the amino-terminal dipeptide changes the susceptibility of human BNP to cleavage by human NEP.

Materials and Methods

BNP (1–32), proANP (1–30), and proANP (79–98) were obtained from Bachem. Tris, ANP (1–28), and EDTA were obtained from Sigma; acetonitrile was from Acros Organics. Vildagliptin was custom synthesized by GLSynthesis Inc. (Worcester, MA). EDTA-anticoagulated plasma specimens were obtained from apparently healthy persons (n = 5) at the University Hospital of Antwerp (Belgium).

Soluble DPP IV was purified from human seminal fluid as described previously *(19)*. The specific activity of the preparation was 35 U/mg. One unit (U) of DPP IV activity is the amount of enzyme required to catalyze the conversion of 1 micromole of substrate per minute in the presence of 0.5 mmol/L Gly-Pro-*p*-nitroanilide and 50 mmol/L Tris buffer (pH 8.3) at 37 °C.

Recombinant human NEP was obtained from R&D Systems.

To study the decay curves of the natriuretic peptide substrates BNP (1–32), ANP (1–28), proANP (1–30), and proANP (79–98), we mixed 70 μL of DPP IV with 70 μL of a 10 μmol/L substrate solution and incubated the mixture at 37 °C in the presence of 50 mmol/L Tris-HCl (pH 7.5) containing 1 mmol/L EDTA (n = 3). The final DPP IV activity was 29 U/L.

At certain time intervals, 20-μL samples were withdrawn and quenched with 5 μL of a 10 mL/L solution of trifluoroacetic acid (TFA). C18 ZipTips (Millipore Corp.) were used to desalt the samples. Stepwise elution was performed with 20 μL of 300 mL/L and 10 μL of 500 mL/L acetonitrile in 1 mL/L acetic acid. The combined eluates were introduced in an Esquire ESI Ion Trap mass spectrometer (Bruker) by use of a syringe pump at 3 μL/min, and the composition was determined. The instrument was used in a normal range, normal resolution setting, optimized on an *m/z* value near the most abundant ion of the intact peptide. The spectra were deconvoluted, and the concentrations of the intact and truncated peptides were calculated from their relative abundances. The concentration of intact peptide was plotted against time.

To determine whether the relative abundance was a good measure of the relative concentration of BNP (1–32), it was necessary to establish that the signal was proportional to the concentration and that this proportionality factor was the same for the intact and truncated peptides. We therefore incubated 5 μmol/L BNP (1–32) at 37 °C with 29 U/L DPP IV or 50 mmol/L Tris buffer (pH 8.3) in a total volume of 50 μL. After 1 h of incubation, the reaction was stopped by addition of 12.5 μL of a 10 mL/L solution of TFA. Equal volumes of peptide dilutions that had been incubated with either an excess of DPP IV or the incubation buffer were mixed and desalted. The ratio of the truncated over intact form was determined and compared with the theoretical ratio.

Concentrations of BNP (1–32) from 5 to 100 μmol/L were incubated with DPP IV at 37 °C for 2–8 min (10%–50% conversion). The amount of converted substrate was calculated from the relative abundances of the intact and cleaved forms. The mean rate of conversion was plotted vs the mean substrate concentration at the chosen time interval *(20)*, and the results were directly fitted to the Michaelis–Menten equation.

To study the truncation of BNP (1–32) in human EDTA-plasma, we added 5 μL of a 100 μmol/L solution of BNP (1–32) to 90 μL of EDTA-plasma from 5 different individuals with or without 1 μmol/L Vildagliptin. The samples were incubated at room temperature (22 °C). After 1 h, 2.5 mL/L TFA was added, and the samples

were centrifuged twice for 5 min at 13 000*g* (Microfuge® 18 Centrifuge; Beckman Coulter). The resulting supernatants were desalted and analyzed as described above.

To study the susceptibility of BNP (1–32) and BNP (3–32) to NEP-mediated hydrolysis, we incubated 35 μL of 20 μmol/L BNP (1–32) for 1 h at 37 °C with 35 μL of DPP IV in 100 mmol/L Tris-HCl buffer to obtain BNP (3–32). The final DPP IV activity was 29 U/L. In a control vial, DPP IV was omitted and the BNP (1–32) remained intact during the incubation. To each of the vials, we added 100 ng of recombinant human NEP. After 1, 5, 10, 20, 30, 45, and 60 min, we withdrew 20-μL samples and stopped the reaction by addition of EDTA at room temperature (final concentration, 10 mmol/L). After 6 min, we added 6 mmol/L dithiothreitol and left the mixture at room temperature for 10 min. Finally, we added TFA to a final concentration of 2 mL/L. Samples were desalted and analyzed as described above. A similar type of experiment was performed with ANP as a substrate.

Results

Shown in Fig. 1 are the time courses of BNP (1–32), ANP (1–28), proANP (1–30), and proANP (79–98) degradation by purified natural human DPP IV. The DPP IV activity in this experiment was similar to the physiologic concentrations in human plasma. The truncation of BNP (1–32) was much faster than the truncation of proANP (1–30). Under the conditions used, ANP (1–28) and proANP (79–98) were not degraded by DPP IV after 60 min of incubation.

Fitting the data on concentration dependency with the Michaelis–Menten equation (Fig. 2) yielded a mean (SD) K_m of 35 (4) μmol/L and a k_{cat} of 13 (0.6) s^{-1} (n = 3). The resulting specificity constant k_{cat}/K_m was 0.37 (0.05) × 10^6 $L \cdot mol^{-1} \cdot s^{-1}$.

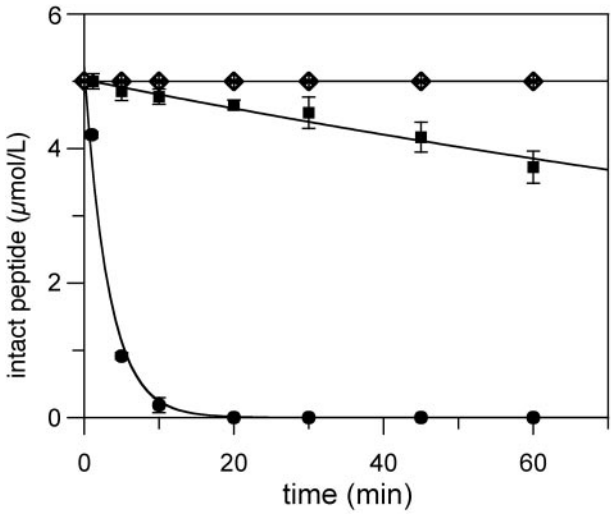

Fig. 1. Time course of truncation of natriuretic peptides by purified human DPP IV.

●, 5 μmol/L BNP (1–32); ∗, 5 μmol/L ANP (1–28); ■, 5 μmol/L proANP (1–30); ◇, 5 μmol/L proANP (79–98). *Error bars*, SE of 3 independent measurements.

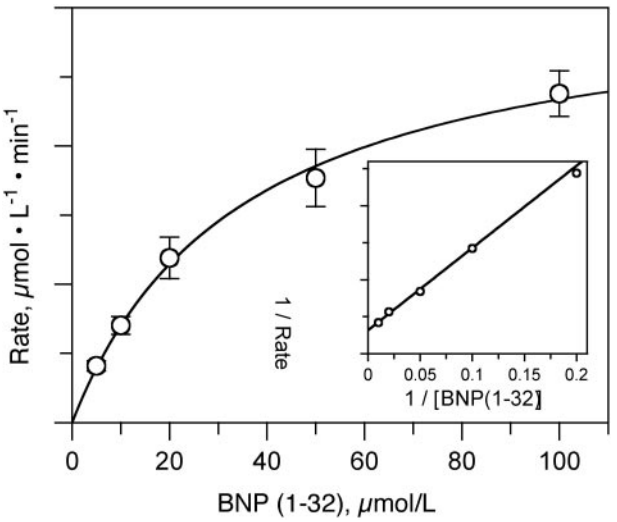

Fig. 2. Catalytic characteristics of BNP (1–32) truncation by DPP IV.

Michaelis–Menten plot; (*inset*), Lineweaver–Burk plot. The *error bars* represent the SE of 3 independent measurements.

Vildagliptin inhibited the cleavage of BNP (1–32) in a concentration-dependent manner (Fig. 3). The inhibitor concentrations used in our experiments were at the least 20-fold lower than the c_{max} of the inhibitor in patients with type 2 diabetes treated with Vildagliptin *(17)*. We used the data in Fig. 3 to estimate an IC_{50} for Vildagliptin; the calculated IC_{50} was 32 (4) nmol/L, similar to the reported value *(18)*.

When BNP (1–32) was added exogenously to human EDTA-plasma at room temperature, >50% was degraded to *des*-SerPro-BNP during incubation for 1 h. This truncation was prevented by addition of Vildagliptin, indicating that the endogenous DPP IV present in plasma is able to truncate BNP (1–32). Under identical conditions, incuba-

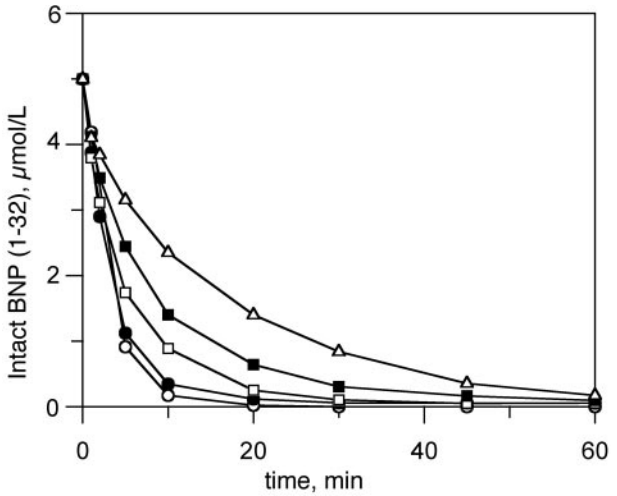

Fig. 3. Inhibition of DPP IV-catalyzed BNP (1–32) truncation.

Decay of BNP (1–32) with 29 U/L DPP IV in the presence of increasing concentrations of Vildagliptin: △, 100 nmol/L; ■, 50 nmol/L; □, 25 nmol/L; ●, 10 nmol/L; ○, 0 nmol/L.

tion of BNP (1–32) with 20 U/L purified DPP IV in phosphate-buffered saline (155 mmol/L NaCl, 1 mmol/L KH_2PO_4, 3 mmol/L $Na_2HPO_4 \cdot 7\ H_2O$, pH 7.4) also led to partial, although slightly faster degradation (data not shown).

We incubated BNP (1–32) with NEP. After 1 h, the NEP was inactivated by addition of EDTA. To reduce the disulfide bridge and open the ring structure, we added dithiothreitol. To investigate whether loss of the amino-terminal dipeptide of BNP would influence the cleavage by NEP, we also incubated BNP (3–32) with NEP (Fig. 4). After 10 min of incubation with NEP, trace amounts of BNP (5–32) were detected, and further degradation occurred at Arg17-Ile18. By the end of the 60-min incubation period, fragment BNP (21–32) had appeared. The intact peptide still dominated the mass spectrum after 1 h of incubation. The cleaved forms were identified in both the truncated and the intact BNP sample. The truncation of BNP (3–32) was not significantly faster than the truncation of BNP (1–32).

We performed the same type of experiment with ANP (1–28) as the NEP substrate. Already after 1 min of incubation, peaks appeared that identified the ring opening [ANP (8–28)], and after 5 min, the intact peptide had

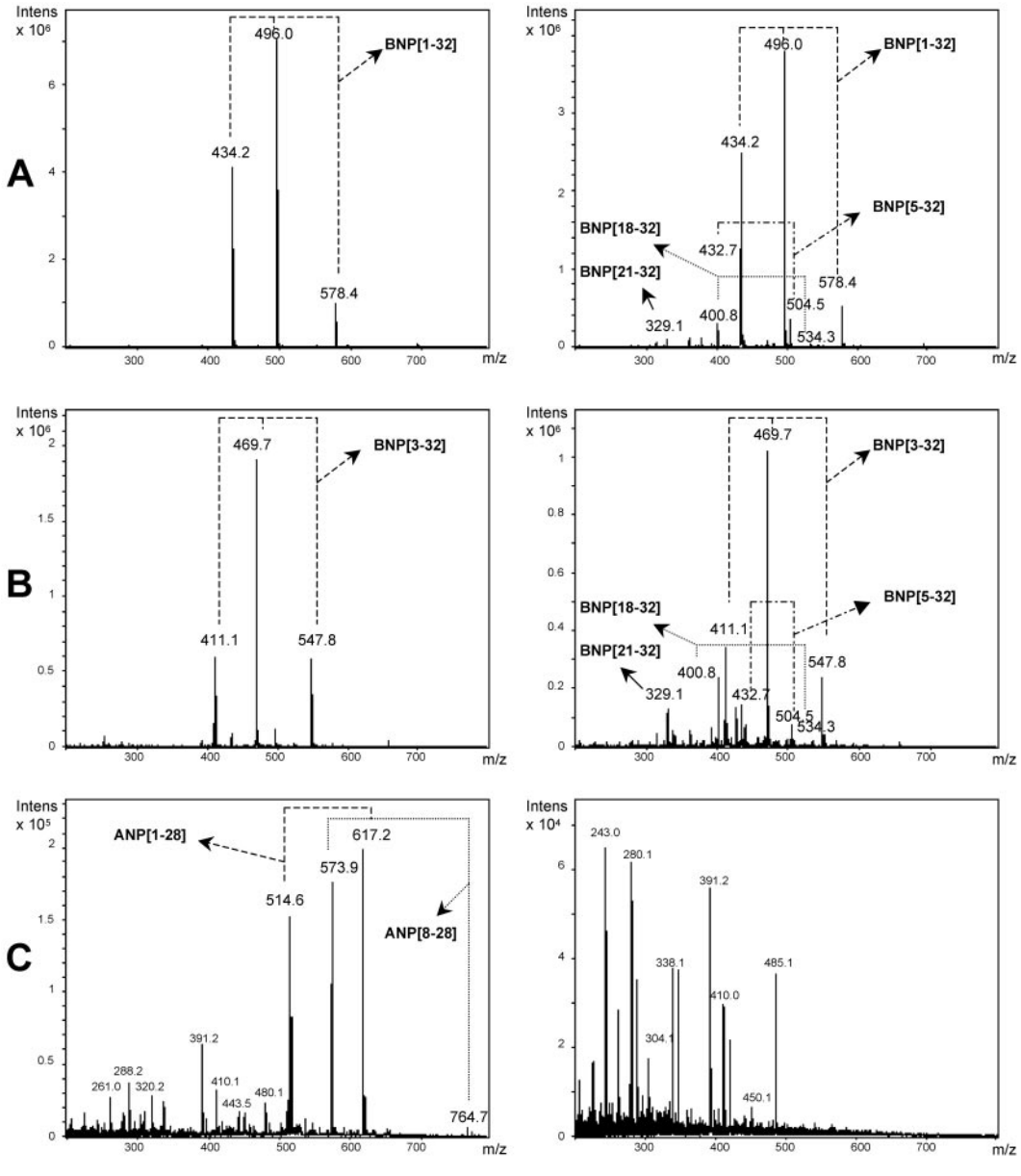

Fig. 4. Mass spectrometric analysis of NEP-mediated cleavage of (*A*) BNP (1–32), (*B*) BNP (3–32), and (*C*) ANP (1–28).

disappeared from the spectrum. After 20 min, ANP was completely degraded into very small fragments.

Discussion

In this study, we showed that BNP (1–32) is a very good substrate for DPP IV. The peptide was rapidly cleaved by purified DPP IV at 37 °C. Incubation of the same peptide concentration in EDTA-plasma at room temperature led to the appearance of BNP (3–32). In both experiments, the observed amino-terminal cleavage was mediated by DPP IV, as is supported by the fact that Vildagliptin, a specific DPP IV inhibitor, abolished the observed truncation. BNP (1–32) had a higher specificity constant than glucagon-like peptide-1, glucose-dependent insulinotropic polypeptide, and several chemokines, peptides for which the in vivo relevance of truncation by DPP IV has been well documented *(15)*.

Whether the cleavage of BNP (1–32) by DPP IV has physiologic implications remains to be elucidated. Little is known about the importance of the amino-terminal region of BNP (1–32) for receptor binding. In 1997, Schoenfeld et al. *(21)* constructed phage display libraries of mutant human BNP. During panning experiments, no receptor selectivity mutations were found in the tail sequences of the human BNP. The proline in the amino-terminal penultimate position of BNP is conserved among several species (such as pig, dog, sheep, and Arabian camel). The amino-terminal part of BNP might also influence the stability of the peptide or its clearance. For example, urodilatin, which is homologous to ANP except for an amino-terminal extension by 4 amino acid residues, is inert against specific proteolysis by NEP *(22)*.

A thorough knowledge of the amino-terminal cleavage forms may also be critical when choosing epitopes for antibody production and immunoassay design *(5)*. This was also discussed very recently in a special report in this journal by Apple et al. *(23)*.

Rawlins et al. *(24)* have shown that 3 commercially available automated immunoassays for the determination of BNP can recognize BNP (1–32) as well as BNP (3–32). However, the Access 2 BNP immunoassay (Biosite) gave a 13% lower molar immunoreactivity for BNP (3–32) compared with BNP (1–32). The ADVIA Centaur BNP immunoassay (Bayer Diagnostics) showed the same molar immunoreactivity for both forms of BNP (1–32), and the AxSYM BNP method (Abbott Diagnostics) displayed an 12% higher molar immunoreactivity for BNP (3–32) than for BNP (1–32). The authors state that differences in results between methods for specific samples could be attributable in part to degradation of BNP (1–32) in these samples. They emphasized that to understand the large intermethod differences, it is necessary to characterize endogenous BNP (1–32) fragments, to study the degradation in EDTA-plasma, and to identify the exact epitopes recognized by all immunoassays. DPP IV, which cleaves the amino terminus, can significantly reduce the biomarker immunoreactivity when antibodies whose BNP binding characteristics are influenced by the loss of the amino-terminal dipeptide are used.

The literature available today is not conclusive on the role of NEP in the degradation of BNP. Cleavage by NEP is clearly species dependent *(8–11)*. It has also been observed that the amino-terminal region of the peptides plays an important role in the susceptibility to hydrolysis by NEP. Vogt-Schaden et al. *(25)* observed that the Cys-Phe bond in human ANP, but not the corresponding Cys-Phe bond in porcine BNP, was cleaved by NEP, and they related this observation to the difference in the amino termini of the peptides. In our experiments, we found clear NEP-mediated degradation of ANP (1–28). Human BNP, however, was degraded only very slowly by the purified human NEP. The loss of the amino-terminal dipeptide did not change BNP resistance to NEP-mediated degradation. Our findings correspond to the observations of Kenny et al. *(11)*, who used human BNP (1–32) and porcine NEP. They found an initial attack outside the ring at Met4-Val5 followed by hydrolysis at Arg17-Ile18 *(11)*. Our data indicate that loss of the amino-terminal dipeptide does not change the resistance of human BNP to human NEP-mediated degradation.

In conclusion, we report here, to the best of our knowledge, for the first time experimental evidence of the truncation of BNP (1–32) by both purified human DPP IV and by the endogenous DPP IV present in human plasma. This cleavage occurs fairly efficient and correlates with previous findings that BNP (3–32) is found in human plasma.

This work was supported by the Fund for Scientific Research Flanders-Belgium (F.W.O.-Vlaanderen).

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