Metabolism of Acetylneurotensin(8–13) by Proteolytic Activities of Intestinal Enterocytes

Jane P. F. Bai[1,2] and Li-Ling Chang[1]

Received March 1, 1994; accepted August 7, 1994

KEY WORDS: acetylneurotensin(8—13); neurotensin peptides; proteolysis; intestinal enterocytes.

INTRODUCTION

Recently, oral dDAVP (1-deamino-8-D-arginine vasopressin) with bioavailability of 1% has been marketed (1), indicating oral peptides with a single digit bioavailability still have therapeutic usefulness. To achieve clinically useful oral bioavailability, degradation by enterocyte proteolytic activities needs to be minimized. Acetyl-neurotensin 8–13 (Ac-Arg-Arg-Pro-Tyr-Ile-Leu) is the shortest fragment of neurotensin (pGlu-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu) with full phamacological activity (2). Neurotensin is present in central and peripheral nervous systems and in the intestine where it is mainly distributed in the jejuno-ileal region (4,5). It stimulates secretion of pancreatic bicarbonate, and inhibits gastric emptying and gastric acid secretion (6,7). Moreover, it induces vasodilation, increases vascular permeability; and is also suggested for treating hypertension and hyperthermia (8).

According to the substrate specificity of brush-border membrane peptidases and amino acid sequences of peptides, neurotensin is cleaved to endopeptidase-2, endopeptidase-24.11, and angiotensin converting enzyme (ACE) while acetylneurotensin(8–13) is cleaved by the latter two enzymes (9, 10). Though the significance of cytosolic enzymes in metabolically limiting absorption of peptide drugs is unknown, it is expected that cytosolic activity should not be ignored if peptide drugs are absorbed by passive diffusion through the brush-border membrane and cytosol or the drugs are not absorbed via specific pathways bypassing the cytosol. This study characterizes brush-border membrane and cytosolic metabolism of acetylneurotensin(8–13) and the effect of enzyme inhibition on its intestinal absorption, hoping to provide essential information for improving its enzymatic stability and intestinal absorption.

MATERIALS AND METHODS

Materials

Acetylneurotensin-(8–13), benzyloxycarbonyl-Gly-Pro-MCA, thiorphan, and pentobarbital were obtained from Sigma Chemical Co. (St. Louis, MO). Captopril was obtained as a gift from Dr. S. J. Lucania (Bristol-Myers Squibb, NJ). Bovine γ-globulin and dye reagent for the protein assay were obtained from Bio-Rad Lab. (Richmond, CA). Acetonitrile was of HPLC grade. All other chemical reagents and buffer components were of analytical grade.

Hydrolysis by Brush-Border Membranes and Cytosol

Thirteen young male Sprague-Dawley rats (300 g) were used as the animal model. The first 8 cm of rat small intestine was used as the duodenum, the next 35 cm as the jejunum, the last 25 cm proximal to the ileocecal junction as the ileum, and the segment between the jejunum and the ileum as the jejunoileal junction. Brush-border membranes of individual segment or the whole intestine were prepared using the method of Kessler et al., 1978, as published previously (11, 12). Briefly, the intestinal mucosae of each segment were scraped off, suspended in a hypotonic solution (50 mM mannitol in the 2 mM pH 7.5 Hepes/Tris buffer), and then homogenized using a blender and a glass/Teflon potter homogenizer. $CaCl_2$ (1 M) was added to the homogenates to achieve a final concentration of 10 mM and then the mixture was centrifuged at 3,000 g for 15 min. The supernatant was collected and then centrifuged at 27,000 g for 30 min to obtain pellets.

Pellets were resuspended in a Tris/HCl (10 mM, pH 6.8 at 37°C), NaCl (150 mM) solution and homogenized with a glass/Teflon potter homogenizer. Then centrifugations at 3000 X g and 27,000 X g were repeated to obtain pure brush-border membranes. Protein concentrations were determined using the Bradford method and γ-globulin as the protein standard (13). Activity of alkaline phosphatase was determined to assess the purity of brush border membranes (14). On the average, brush-border membranes were 20 (±0.7) times enriched. In a separate preparataion, intestinal homogenate was subjected to centrifugation at 100,000 X g at 4°C for 1 hr to obtain cytosol (15).

A 300-μl incubation mixture consisted of 10 mM Tris/HCl buffer (pH 7.5), 150 mM NaCl, 0.02 mM substrate, and 0.03–0.006 mg brush-border membrane proteins or cytosolic proteins (12, 16). Proteolysis of acetylneurotensin-(8–13) was performed at room temperature and reaction was terminated using 0.1 N HCl. In inhibitor studies, experimental conditions remained the same except that inhibitors were added.

In-Situ Single Pass Perfusion

Surgical and cannulation procedures of in-situ single pass perfusion on rats were as described previously (17). The perfusion solution consisted of pH 4.5 citrate buffer, acetylneurotensin(8–13) (0.02 mM), captopril(30 μM), thiorphan (30 μM), chymostatin (0.005 mg/ml), and syobean trypsin inhibitor(0.005 mg/ml). Osmolality was adjusted using NaCl, and trace amount of PEG-4000 and 14C-PEG-4000 were used as the water flux marker. Membrane permeability was estimated as published previously (17).

HPLC Assay

The HPLC system consisted of SIL autoinjector, LC-600 pump, SPD-6A UV spectrophotometric detector, and CR 601 recorder (Shimadzu Corportaion, Kyoto, Japan). Acetylneurotensin-(8–13) was assayed using a C18 Beck-

[1] College of Pharmacy, University of Minnesota, Minneapolis, Minnesota 55455.

[2] To whom correspondence should be addressed.

0724-8741/95/0100-0164$07.50/0

man column (5 μ, 4.6 mm X 15 cm) and a gradient system. Mobile phase A was 0.05% trifluoroacetic acid (TFA), and mobile phase B was 95% ACN and 5% TFA. For metabolism by brush-border membrane, the gradient system was from 18% B to 35% B in 30 min at a flow rate of 1 ml/min, and then was maintained at 35% for 10 min. For cytosolic metabolism, the gradient system was from 8% B to 35% B in 40 min at a flow rate of 1 ml/min.

Amino Acid Analysis

Metabolites were collected, dried using a rotary evaporation apparatus, hydrolyzed at 110°C overnight, and then subject to a Beckman amino acid analyzer.

RESULTS AND DISCUSSION

Degradation of acetylneurotensin-(8–13) by intestinal brush-border showed a profile similar to the distribution of endopeptidase-24.11 and ACE (Table 1), having the order of jejunum>jejunoileal junction≥duodenum>ileum>caecum. Its degradation by jejunal brush-border was selectively eliminated by 30 μM thiorphan (100%) and reduced by 30 μM captopril (32%). The Ki of thiorphan against endopeptidase is 4.7 nM while that of captopril against ACE 1.7 nM (18). According to the specificity of enzymes and structure of acetylneurotensin-(8–13), it is proposed that this peptide compound is subjected to only endopeptidase-24.11 and ACE in brush border membrane, where there are nine major brush-border peptidases (9, 10, 19), with two peptide bonds sensitive to endopeptidase-24.11 and one of these sensitive to ACE. Complete inhibition by thiorphan and partial inhibition by captopril confirms that endopeptidase-24.11 and ACE were both involved; however, endopeptidase-24.11 is the major responsible enzyme.

Shown by gradient HPLC, there were two major initial metabolites produced from metabolism by brush-border membrane, one eluted at 5.1 min and the other at 12 min; acetylneurotensin eluted at 24 min (Fig. 1). The peaks of these two metabolites first increased and then decreased with time, suggesting that they were initial metabolites which were further degraded to shorter secondary metabolites. In the presence of 30 μM captopril, the peak of acetylneurotensin(8–13) continued to decrease with time while its metabolites increased, confirming incomplete inhibition. However, in the presence of 30 μM thiorphan, no metabolites werre detectable while the peak of acetylneurotensin(8–13) remained the same, indicating that inhibition was complete. Amino acid analysis yielded an amino acid ratio of Arg:Tyr:Pro = 2:1:1 for the 5.1 min metabolite, Ile-Leu = 1:1 for the 12 min metabolite, and Arg:Tyr:Leu:Ile:Pro = 2:1:1:1:1 for the peak at 24 min. Since the amino acid analyzer does not detect acetyl group, the metabolite of Arg-Arg-Pro-Tyr is assumed to be acetyl-Arg-Arg-Pro-Tyr. Therefore, the two major acetylneurotensin metabolites by brush-border peptidases were acetyl-Arg-Arg-Pro-Tyr and Ile-Leu. In summary, proteolysis of acetylneurotensin(8–13) in brush-border membrane is mainly due to cleavage by endopeptidase-24.11 at Tyr-Ile.

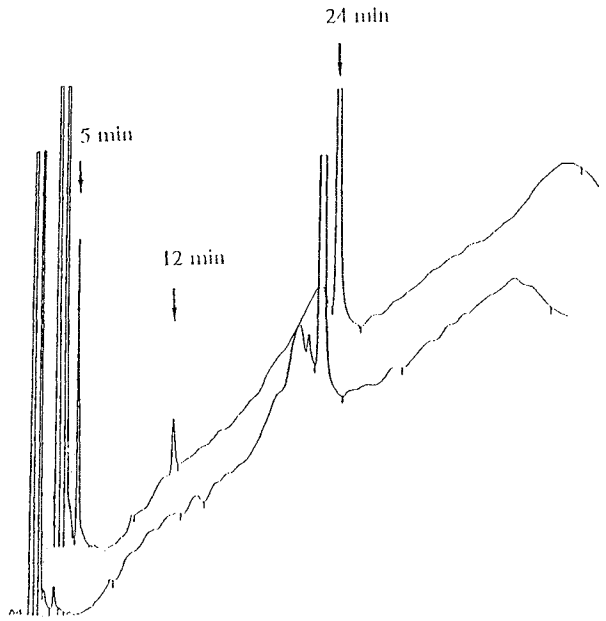

Figure 1. Chromatograms of acetylneurotensin(8–13) metabolism by jejunal brush-border membrane with the bottom one being after 15 sec incubation and the one shifted to the right after 10 min incubation. The gradient system, consisting mobile phas A: 0.05% trifluoroacetic acid (TFA); mobile phase B: ACN:0.05% TFA = 95:5, was from 18% B to 35% B in 30 min at a flow rate of 1 ml/min, and then was maintained at 35% for 10 min.

Cytosolic degradation of acetylneurotensin(8–13) with a rate of 0.68 μmole/min g protein was 2 to 8 times slower than that by brush-border membrane. Though the literature suggests that there are only di-/tripeptidases in the cytosol of enterocytes (19), its degradation by cytosolic enzymes indi-

Table 1. Comparison of Differential Degradation of Acetylneurotensin (8–13) by Intestinal Brush-Border Membrane and Distribution of Endopeptidase-24.11 and ACE

Segment	Proteolysis rate (μmol/min/g tissue)		
	Acetylneurotensin (8–13)	Endopeptidase-24.11	ACE
Duodenum	2.3 ± 0.2	1.1 ± 0.1	1.8 ± 0.1
Jejunum	9.1 ± 0.5	5.3 ± 0.2	10.8 ± 0.5
Jejunoileal junction	5.9 ± 0.7	2.6 ± 0.2	4.0 ± 0.7
Ileum	2.7 ± 0.2	2.0 ± 0.2	1.4 ± 0.2
Caecum	0.2 ± 0.1	0.2 ± 0.0	0.1 ± 0.0

Note: The results represent the results of three experiments (Mean ± S.E.)
ACE: angiotensin converting enzyme.

cated that the presence of other cytosolic enzyme(s) capable of degrading larger peptides. The cytosolic metabolism yielded three major metabolites detected by gradient HPLC (Fig. 2). Amino acid analysis revealed that the peaks at 6, 8, and 26 min were Arg-Arg-Pro, Leu, and Arg-Arg-Pro-Tyr-Ile. Again, both Arg-Arg-Pro and Arg-Arg-Pro-Tyr-Ile should be Ac-Arg-Arg-Pro and Ac-Arg-Arg-Pro-Tyr-Ile. The peak at 16 min corresponded to an unknown peptide from the cytosol. The results suggested that acetylneurotensin (8–13) was cleaved at Pro-Tyr and Ile-Leu by cytosolic enzymes, with the major cleavage at Pro-Tyr. Inhibition of cytosolic metabolism of acetylneurotensin(8–13) by various inhibitors was examined: 60% by benzyloxycarbonyl-Gly-Pro-MCA, a specific substrate of post-proline cleaving enzyme, at a concentration of one half of its saturated solubility in Tris buffer (22); 83% by 0.2 mM 1,10-phenanthroline, a metal chelator; 80% by 30 mM diisopropylfluoride, a serine protease inhibitor; and 100% by coadministration of them. The gradient HPLC revealed that the peak at 6 min reduced in the presence of 1,10-phenanthroline, diisopropylfluoridate, and benzyloxycarbonyl-Gly-Pro-MCA. The cleavage of Pro-Tyr bond being simultaneous sensitive to the metal chelator and serine protease inhibitor suggests that more than one enzyme is involved. One is likely to be the post-proline cleaving enzyme (E.C.21.16), a serine protease, and the other, neurotensin endopeptidase (E.C.3.4.24.16), sensitive to metal chelators (20,21,22). Both enzymes have been suggested to be present in the intestine (22, 23). The former specifically cleave the peptide bond at the C-terminal end of proline residue while the latter at the N- or C-terminal end of aromatic acid residues (20,21). The cleavage of Ile-Leu is due to unknown carboxypeptidase(s).

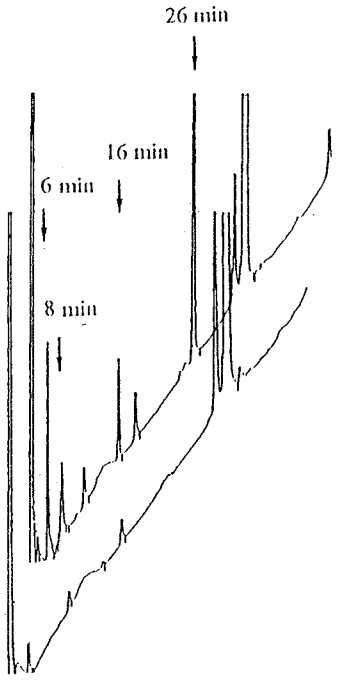

Figure 2. Chromatograms of acetylneurotensin(8–13) metabolism by intestinal cytosol with the bottom one being after 0 min and the one shifted to the right after 30 min incubation. The gradient system, consisting mobile phas A:0.05% trifluoroacetic acid (TFA); mobile phase B: ACN:0.05% TFA = 95:5, was from 8% B to 35% B in 40 min at a flow rate of 1 ml/min.

Table 2. The Fraction Absorbed and Dimensionless Effective Permeability (Peff*) of Acetylneurotensin (8–13) in Rat Jejunum at pH 4.5

Rat	Fraction absorbed (%/cm)	Peff*
1	4.2	2.8
2	3.6	3.9
3	4.6	4.8
4	2.7	3.8
5	2.2	3.0

Note: 1. No hydrolysis of acetylneurotensin (8–13) was observed within 15 min when it was incubated with buffer perfusate containing captopril (30 μM), thiorphan (30 μM), chymostatin (0.005 mg/ml) and soybean trypsin inhibitor (0.005 mg/ml).
2. No hydrolysis of acetylneurotensin (8–13) was observed within 30 min when it was incubated with brush-border membrane proteins (3 mg/ml) in the presence of captopril (30 μM) and thiorphan (30 μM) at 37°C.
3. N = 3.

Since the metabolism of acetylneurotensin(8–13) by brush-border membrane was completely inhibited, in-situ single perfusion was performed to evaluate its intestinal absorption. Several inhibitors, i.e., captopril (30 μM), thiorphan (30 μM), chymostatin (0.005 mg/ml), and soybean trypsin inhibitor (0.005 mg/ml), were included in the perfusion solution of acetylneurotensin(8–13) to warrant no lumenal degradation. A pH 4.5 citrate buffer was used for the perfusion solution to further minimize lumenal proteolytic activities. In the presence of captopril (30 μM) and thiorphan (30 μM), no hydrolysis of acetylneurotensin(8–13) by brush-border membrane was observed in 30 min. In the presence of all four inhibitors, this compound was not hydrolyzed in the blank perfusate-buffer perfused through the intestine. Under this condition, jejunal absorption of acetylneurotensin(8–13) was substantial with a fraction absorbed of 2.2 to 4.6%/cm. The water flux was within the desired limit (<1%) throughout the perfusion experiment. The dimensionless membrane permeability of acetylneurotensin(8–13) ranged from 2.8 to 4.8 (Table 2). Therefore, the intestinal absorption of acetylneurotensin (8–13) is expected to be extensive when lumenal and intracellular degradation is eliminated.

The results suggest that degradation of acetylneurotensin(8–13) by brush-border membrane is mainly due to cleavage at Tyr-Ile by endopeptidase-24.11 and that its degradation by cytosolic enzymes is at Pro-Tyr and Ile-Leu. If there is no specific mechanisms mediating intestinal absorption of acetylneurotensin (8–13) to bypass cytosolic proteolytic degradation, cytosolic proteolytic activities should be considered in oral delivery of peptidase drugs. Therefore, elimination of degradation by brush-border membrane and cytosolic enzymes at Tyr-Ile, Pro-Tyr, and Ile-Leu is necessary to minimize enzymatic degradation in order to achieve oral efficacy.

ACKNOWLEDGMENTS

This work was partially supported by the 1992 PMA

Starter Grant from the Pharmaceutical Manufacturers Association Foundation, Inc..

REFERENCES

1. "dDAVP" Pharm. J. May:649, 1993.
2. Granier, C., Rietschoten, J.V., Kitabgi, P., Poustis, C., Freyhet, P. Synthesis and characterization of neurotensin analogues for structure/activity relationship studies. *Eur. J. Biochem.* 124: 117–120, 1982.
3. Iversen LL, Overview: peptides in the nervous system in *Neuropeptides and their peptidases* (Tuner AJ ed)Ellis Horwood, Chichester, England, 1987. p.4.
4. Hammer RA, Leeman SE, Carraway R, Williams RH. Isolation of human intestinal neurotensin. *J. Biol. Chem.* 255:2476–2480, 1980.
5. Carraway RE, Leeman SE. Characterization of radioimmunoassayable neurotensin in the rat: Its differential distribution in the central nervous system, small intestine, and stomach. *J. Biol. Chem.* 251:7045–7052, 1976.
6. Blackburn, A.M., Fletcher, D.R., Adrian, T.E., Bloom, S.R. Neurotensin infusion in man: pharmacokinetics and effect on gastrointestinal and pituitary hormones. *J. Clin. Endocrinol Metab.* 51:1257–1261, 1980.
7. Fletcher, D.R., Blackburn, A.M., Adrian, T.E., Chadwick, V.S., Bloom, S.R. Effect of neurotensin on pancreatic function in man. *Life Sci.* 29:2157–2161, 1981.
8. Leeman SE, Carraway RE. Neurotensin: discovery, isolation, characterization, synthesis and possible physiological roles. *Anal.N.Y. Acad. Sci.* 1–16, 1982.
9. Skidgel RA, Engelbrecht S, Johnson AR, Erdos EG. Hydrolysis of substance P and neurotensin by converting enzyme and neutral endopeptidase. *Peptides* 5:769–776, 1984.
10. Kenny, A.J., Ingram J. Proteins of the kidney microvillar membrane:purification and properties of the phosphoramidon-insensitive endopeptidase ('endopeptidase-2') from rat kidney, *Biochem. J.* 245: 515–524, 1987.
11. Kessler, M., Acuto, O., Storelli, C., Murer, H., Muller, M., and Semenza, G. A modified procedure for the rapid preparation of efficiently transporting vesicles form small intestinal brush border membranes. *Biochim. Biophys. Acta* 506:136–154, 1978.
12. Bai, J.P.F. Distribution of brush-border membrane peptidases along the rabbit intestine: implication for oral delivery of peptide drugs. *Life Sci.* 52:941–947, 1993.
13. No information available.
14. Miura, S., Song, I.S., Morita, A., Erickson, R.H., and Kim, Y.S. Distribution and biosynthesis of aminopetidase N and dipeptidyl aminopeptidase IV in rat small intestine. *Biochim. Biophys. Acta.* 761: 66–75, 1983.
15. M. M. Bradford. A rapid and sensitive method for the quantitation of microgram quantities for protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72: 248–254, 1976.
16. Barelli, H., Vincent, J.P., Checler, F. Peripheral inactivation of neurotensin. *Eur. J. Biochem.* 175:481–489, 1988.
17. P.F. Bai, P. Subramanian, H.I. Mosberg, G.L. Amidon. Structural requirements for the intestinal mucosal-cell peptide transporter: the need for N-terminal α-amino group, *Pharm. Res.* 8(5):593–599, 1991.
18. Thorsett E.D. and Wyvratt M.J., Inhibition of zinc peptidases that hydrolyse neuropeptides. In: A. J. Turner (ed) Neuropeptides and their peptidases, Ellis Horwood, Chichester, England, P229–292, 1987.
19. Jane P. F. Bai and Amidon, G. L. Structural Specificity of Mucosal Cell transport and Metabolism of Amino Acids and Peptide Drugs: Implication for Oral Peptide Drug Delivery. *Pharm. Res.* 9(8):969–978, 1992.
20. S. Lundin, H.I. Bengtsson, H.G. Folkesson, B.R. Weström. Degradation of [mercaptopropionic acid[1], D-arginine[8]]-vasopressin (dDAVP) in pancreatic juice and intestinal mucosa homgenate. *Pharmacol. Toxicol.* 65:92–95, 1989.
21. T. Yoshimoto, K. Tsukumo, N. Takatsuka, D. Tsuru. An inhibitor for post-proline cleaving enzyme: distribution and partial purification from porcine pancreas. *J. Pharm. Dyn.* 5:734–740, 1982.
22. P. Dahms, R. Mentlein, Purification of the main somatostatin-degrading proteases from rat and pig brains, their action on other neuropeptides, and their identification as endopeptidase-24.15 and 24.16, *Eur. J. Biochem.* 208:145–154, 1992.
23. H. Barelli, J.P. Vincent, F. Checler, Peripheral inactivation of neurotensin: isolation and characterization of a metallopeptidase from rat ileum. *Eur. J. Biochem.* 175:481–489, 1988.