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Comparison of site-dependent degradation of peptide drugs within the gut of rats and rabbits

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Abstract—Intestinal luminal degradation of neurotensin and acetylneurotensin-(8–13) within the gut of rats and rabbits was compared using brush-border membranes. Patterns of differential proteolysis of these two peptides within the intestine were similar within the same species and between the species. In both rats and rabbits, jejunal brush-border membranes had the highest proteolytic activities degrading neurotensin and acetylneurotensin-(8–13), and caecal or ileocaecal brush-border membranes had the lowest activities. In both species, patterns of site-dependent degradation of neurotensin and acetylneurotensin-(8–13) agreed with the distribution profiles of endopeptidase-24.11 and angiotensin-converting enzyme within the gut. The distal intestine of rats and rabbits has the lowest activities degrading these two compounds. The results demonstrate that distribution of peptidases within the gut will affect site-dependent degradation and absorption of peptide drugs.

Oral delivery is the long-term goal for peptide drugs. To achieve the goal it is essential to understand how the cellular features of intestinal enterocytes affect intestinal availability of peptide drugs. Brush-border membrane peptidases constitute the major barrier of intestinal absorptive cells (enterocytes) which will limit oral absorption of peptide drugs (Bai & Amidon 1992). Differential distribution of angiotensin-converting enzyme (ACE) and dipeptidylpeptidase IV (DPP IV) along the intestine in rats, DPP IV in man, and aminopeptidase M in rats, rabbits and in man has been reported (Auricchio et al 1978; Sterchi 1981; Miura et al 1983; Triadou et al 1983). Moreover, the regional differences within the intestine in activities of ACE and endopeptidase-24.11 were similar in rabbits and rats (Bai 1993a, b). Degradation of peptide drugs by brush-border membrane peptidases should follow the principle of amino acid sequences of peptide drugs and substrate specificity of peptidases. It is expected that intestinal distribution of key brush-border membrane peptidases, which initiate proteolysis of peptide drugs, will affect site-dependent intestinal availability of this class of drugs.

Neurotensin and acetylneurotensin-(8–13) have important pharmacological effects. Neurotensin (pGlu-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu), a neuropeptide widely distributed in the brain and gut (Turner 1987), stimulates secretion of pancreatic bicarbonate and inhibits gastric acid secretion (Blackburn et al 1980). Moreover, this compound could be used for combating hypertension and hyperthermia (Blackburn et al 1980). Acetylneurotensin-(8–13) (Ac-Arg-Arg-Pro-Tyr-Ile-Leu) is the shortest analogue of neurotensin which retains its full pharmacological effects (Granier et al 1982). The fate of these two peptides permeating across the brush-border membrane of enterocytes is unknown. According to substrate specificity of brush-border membrane peptidases, these two peptides will not be substrates for aminopeptidase M, DPP IV, aminopeptidase P, or carboxypeptidase P (Bai & Amidon 1992). However, they would be substrates for endopeptidase-24.11 and ACE since they have hydrophobic amino acids and the C-terminal leucine (Turner 1987; Bai & Amidon 1992). Moreover, neurotensin would also be a substrate for brush-border membrane endopeptidase-2 (Kenny & Ingram 1987). The specific aim of this study is to use neurotensin and acetylneurotensin-(8–13) as model peptide drugs to illustrate how distribution of brush-border membrane peptidases of enterocytes affects site-dependent degradation of peptide drugs in the intestine of rats and rabbits. The rat and rabbit are used as the animal models since these two species and man have certain similar physiological intestinal properties. For example, the transfer of substances from the intestinal lumen to the blood is dependent inversely on their molecule sizes in rats, rabbits, and man (Loehry et al 1973; Donovan et al 1990). Intestinal distribution of aminopeptidase A and DPP IV is similar in the rabbit and in man while the trend of DPP IV activity in the rat and human intestine is somewhat similar (Auricchio et al 1978; Sterchi 1981; Miura et al 1983).

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Materials and methods

Materials. Neurotensin, acetylneurotensin-(8–13), and pentobarbitone were obtained from Sigma Chemical Co. (St Louis, MO, USA). Bovine γ-globulin and dye reagent for the protein assay were obtained from Bio-Rad-Lab (Richmond, CA, USA). Acetonitrile was of HPLC grade. All other chemical reagents and buffer components were of analytical grade.

Preparation of brush-border membranes. Young male New Zealand White rabbits, 6–7 lbs, and young male Sprague-Dawley rats, 300 g, were used. The small intestine was divided into various segments. The first 25 and 8 cm of the rabbit and rat small intestine, respectively, were used as the duodenum, the next 60 and 35 cm as the jejunum, the last 60 and 25 cm of the small intestine proximal to the ileocaecal junction as the ileum, and the segment between the jejunum and the ileum as the jejuno-ileal junction. The caecum was anatomically different from the small intestine and was clearly identified. The ileocaecal junction instead of the caecum was used for the rabbit. The intestinal mucosa of each segment was scraped off, suspended in a hypotonic solution (50 mM mannitol in 2 mM pH 7·5 HEPES/Tris buffer), and then homogenized using a blender and a glass/Teflon Potter homogenizer. Mucosal homogenates were thus obtained, and brush-border membranes were prepared as reported previously (Kessler et al 1978; Bai 1993a). Briefly, $CaCl_2$ (1 M) was added to the homogenates to achieve a final concentration of 10 mM and then the mixture was centrifuged at 3000 *g* for 15 min. Supernatant was collected and centrifuged at 27 000 *g* for 30 min to obtain pellets. Pellets were resuspended in a 150 mM NaCl, 10 mM Tris/HCl (pH 7·5) solution and homogenized with a glass/Teflon Potter homogenizer. Then centrifugation at 3000 and 27 000 *g* was repeated to obtain pure brush-border membranes. Protein concentrations were determined using the Bradford method with γ-globulin as the protein standard (Bai 1993a). Activity of alkaline phosphatase was determined to assess purity of brush-border membranes.

Proteolysis of peptides. 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 (Barelli et al 1988; Bai 1993a). Proteolysis of neurotensin and acetylneurotensin-(8–13) was performed at room temperature (21°C) and the reaction was terminated using 10% trichloroacetic acid.

Assay. The HPLC system consisted of an SIL autoinjector, LC-

600 pump, SPD-6A UV spectrophotometric detector, and CR 601 recorder (Shimadzu Corporation, Kyoto, Japan). Samples of enzyme substrates were assayed using a C8 Beckman column (5 μ, 4·6 mm × 15 cm) and mobile phases of 0·01 M NaH_2PO_4 (pH 3) and acetonitrile in a volume ratio either of 80:20 or 85:15. A detection wavelength of 220 nm was used. Neurotensin and acetylneurotensin-(8–13) were assayed using a C18 Beckman column (5 μ, 4·6 mm × 15 cm) and the mobile phase of 0·005% trifluoroacetic acid and acetonitrile in a volume ratio of 75:25. Neurotensin was eluted at 6 min at a flow rate of 1·5 mL min^{-1}, and acetylneurotensin-(8–13) was eluted at 6 min at a flow rate of 1 mL min^{-1}.

Results

Neurotensin and acetylneurotensin-(8–13) were chemically stable in the experimental conditions. Proteolysis of neurotensin and acetylneurotensin-(8–13) by rat and rabbit intestinal brush-border membranes is summarized in Table 1. Degradation of neurotensin by brush-border membranes of the rabbit intestine had the following rank order: jejunum > jejuno-ileal junction > ileum ~ duodenum > ileocaecal junction. That of acetylneurotensin-(8–13) was as follows: jejunum > duodenum ~ jejuno-ileal junction ~ ileum > ileocaecal junction. Degradation of neurotensin and acetylneurotensin-(8–13) by intestinal brush-border membranes of the rat showed the following regional differences: jejunum > duodenum ~ jejuno-ileal junction > ileum > caecum. In both rats and rabbits, the jejunum had the highest endopeptidase-24.11 and ACE activities while the caecum or ileocaecal junction had the lowest activities (Table 2). Clearly, the patterns of site-dependent degradation of neurotensin and acetylneurotensin-(8–13) within the gut corresponded to the distribution profiles of endopeptidase-24.11 and ACE.

Table 1. Degradation of neurotensin and acetylneurotensin-(8 13) by brush-border membranes prepared from the intestine of the rat and rabbit.

	Proteolysis rate (μmol min^{-1} (g protein) $^{-1}$)			
	Neurotensin		Acetylneurotensin-(8 13)	
Segment	Rat	Rabbit	Rat	Rabbit
Duodenum	5·30 ± 0·23	6·15 ± 0·65	2·60 ± 0·17	5·92 ± 0·20
Jejunum	9·30 ± 0·13	9·16 ± 0·53	5·49 ± 0·28	7·56 ± 0·61
Jejuno-ileal junction	5·01 ± 0·18	7·29 ± 0·16	2·69 ± 0·32	5·78 ± 0·69
Ileum	2·48 ± 0·17	6·45 ± 0·75	1·49 ± 0·11	5·42 ± 0·69
Ileocaecal junction[a]	0·67 ± 0·01	3·90 ± 0·01	0·28 ± 0·07	2·16 ± 0·37

Data are expressed as the mean ± s.e., [a] the caecum was used for the rat studies.

Table 2. Activities of endopeptidase-24.11 and ACE along the intestine of the rat and rabbit.

	Activity (μmol min^{-1} (g protein)$^{-1}$)			
	Endopeptidase-24.11		ACE	
Segment	Rat	Rabbit	Rat	Rabbit
Duodenum	1·17 ± 0·07	0·99 ± 0·05	2·08 ± 0·08	87·7 ± 4·60
Jejunum	3·21 ± 0·11	1·54 ± 0·05	6·49 ± 0·29	148·3 ± 17·0
Jejuno-ileal junction	1·15 ± 0·03	0·79 ± 0·04	1·81 ± 0·33	83·6 ± 13·2
Ileum	1·05 ± 0·05	0·73 ± 0·07	0·83 ± 0·11	17·9 ± 5·40
Ileocaecal junction[a]	0·41 ± 0·04	0·61 ± 0·02	0·12 ± 0·01	9·7 ± 0·59

Data are expressed as the mean ± s.e., [a] the caecum was used for the rat studies.

Discussion

In both rats and rabbits, proteolysis of neurotensin and acetylneurotensin-(8–13) by intestinal brush-border membranes showed regional differences within the gut. In both species, the jejunum had the highest activity against these two peptides while the caecum or ileocaecal junction had the lowest activity. Profiles of differential proteolysis of neurotensin and acetylneurotensin-(8–13) by brush-border membranes from various intestinal sites of rats and rabbits were similar between the species and within the species. Site-dependent proteolysis of neurotensin and acetylneurotensin-(8–13) by the rabbit intestine did not completely resemble distribution profiles of endopeptidase-24.11 and ACE along the rabbit intestine, nor did that of these compounds by rat intestine. Differential proteolysis of neurotensin and acetylneurotensin-(8–13) by rabbit intestinal brush-border membrane followed more closely the distribution profile of endopeptidase-24.11 than that of ACE. Results of the rat intestine indicate that profiles of differential proteolysis of these two compounds followed more closely that of ACE. This seems to suggest that endopeptidase-24.11 played a more significant role than ACE in degradation of these two peptides by rabbit intestinal brush-border membranes while ACE was more important in the rat intestine. Comparison of results from rats and rabbits indicates that distribution of brush-border membrane peptidases within the gut affects site-dependent degradation and absorption. In both species, endopeptidase-24.11 and ACE are key peptidases which will limit absorption of neurotensin and acetylneurotensin-(8 13).

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Dark effect of 8-methoxypsoralen on human erythrocytes

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Abstract—Furanocoumarin 8-methoxypsoralen (8-MOP) (1–100 $\mu g\ mL^{-1}$) in the dark showed a protective affect against hypotonic haemolysis of the erythrocyte membrane. However, the effect against heat-induced haemolysis was dependent on the concentration of 8-MOP; lower concentrations of 8-MOP showed an inhibiting effect, whereas higher concentrations caused acceleration of haemolysis. 8-MOP was not able to induce haemolysis in isotonic solution at 20 or 37 C. Reaction of erythrocytes with 8-MOP in the dark resulted in a shrinkage of the cells and alterations of their shapes. We conclude that modification of erythrocyte membrane by 8-MOP proceeds via reaction with membrane lipids and proteins. This indicates that the effect on the cell membrane plays an important role in the mechanism of the action of 8-MOP on the cells.

Furanocoumarin 8-methoxypsoralen (8-MOP) is used in combination with UV-A (PUVA) in the treatment of various skin diseases such as psoriasis, vitiligo, a disease of accelerated epidermal cell proliferation, and leucoderma (Dall'Acqua & Caffieri 1988). The photochemotherapeutic effects of furanocoumarins for the skin are thought to arise from their ability to form cyclobutane-type adducts with pyrimidine bases of nucleic acids after UV radiation. Recent investigations have shown that membrane constituents may be a biological target in the photosensitizing action of furanocoumarins. Damage to the membrane can be induced either by an oxygen-dependent mechanism (lipid peroxidation, formation of cross-links in ghost proteins) or an oxygen-independent mechanism, which is due to a direct photoreaction between furanocoumarins and unsaturated fatty acids (Midden 1988; Dall'Acqua & Caffieri 1988; Averbeck 1989; Dall'Acqua & Martelli 1991). The effect of furanocoumarins on the cell membrane without photoactivation is less known. It has been shown that various furocoumarins are able to cause haemolysis of red blood cells in the dark at relatively high concentrations ($4{\cdot}6 \times 10^{-4}$ M) (Vedaldi et al 1988). Laskin et al (1985) detected specific binding of 8-MOP to the surface and cytoplasm of five human cell lines and Walther et al (1990) showed a time-dependent, reversible reduction of rosette-forming lymphocytes after a single in-vivo administration of 8-MOP. In our previous papers we reported an increased proliferation of phytohaemagglutinin-stimulated lymphocytes in the presence of 8-MOP (Gawron et al 1990; Górski et al 1991). The present studies carried out on human erythrocytes, give further evidence on the action of 8-MOP on the cell membrane in the dark.

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Materials and methods

Chemicals. 8-Methoxypsoralen (8-MOP) (Sigma, USA) dissolved in dimethylsulphoxide (DMSO) (Merck, Germany) was the stock solution (10 or 20 mg mL^{-1}) used in the studies. The solutions were always kept in the dark.

Haemolysis in isotonic solution. Erythrocytes were obtained from peripheral blood taken by venipuncture. Sodium citrate was used to prevent clotting. Packed red blood cells were washed three times with phosphate-buffered saline (154 mM NaCl, 10 mM sodium phosphate, pH 7·4) and resuspended in the same saline solution at 50% v/v. Fifty microlitres of erythrocyte suspension was added to 2·5 mL 8-MOP solution (1–100 $\mu g\ mL^{-1}$) in phosphate-buffered saline in a siliconized glass tube. The control sample contained DMSO at the same concentration as that of 8-MOP. The highest concentration of DMSO was 0·5%. Each experiment was performed in triplicate. After 60 min of incubation (20 or 37°C) the reaction mixtures were centrifuged for 5 min at 1800 rev min^{-1} and the absorbance of the supernatant was measured at 540 nm. The relative haemolysis was determined by comparison with a sample showing 100% haemolysis.

All experimental procedures with 8-MOP were carried out in red light. Incubation took place in the dark.

Six independent experiments were performed with erythrocytes obtained from different blood donors.

Heat-induced haemolysis. The inhibition of heat-induced haemolysis was carried out as for haemolysis in isotonic solution. The erythrocytes were incubated with 8-MOP solutions in isotonic phosphate-buffered saline at 54°C for 30 min in a water bath in the dark.

Haemolysis in hypotonic solution. The extent of protection of erythrocytes was measured after 30-min incubation of erythro-