J. Pharm. Pharmacol. 1994, 46: 671–675
Received November 15, 1994
Accepted February 15, 1994

Subcellular Distribution of Proteolytic Activities Degrading Bioactive Peptides and Analogues in the Rat Small Intestinal and Colonic Enterocytes

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Abstract—The objective of this study was to compare, in rat small intestinal and colonic enterocytes, subcellular distributions of activities degrading the large peptides, neurotensin, acetylneurotensin (8-13), GRF(1-29)NH_2 (human growth hormone releasing factor fragment), (desNH_2Tyr^1,D-Ala^2,Ala^{15})-GRF(1-29)NH_2, insulin, and insulin B-chain. Proteolytic activities degrading individual peptides in the 10 000-*g* pellet, rich in intracellular organelles, 27 000-*g* pellet, rich in brush-border membrane, 100 000-*g* pellet, and 100 000-*g* supernatant, rich in cytosol, were determined and compared for both the small intestine and colon. In colonic fractions, the cytosol had highest activity (g protein)$^{-1}$ degrading three out of four peptides tested, while in small intestinal fractions, the 27 000-*g* pellet had the highest activity (g protein)$^{-1}$, degrading four out of five peptides tested. In both small intestine and colon, the cytosol had a higher percentage of total proteolytic activity degrading each of the above polypeptides and the highest insulin-degrading activity (g protein)$^{-1}$. The results suggest that at pH 7·5, proteolytic activities (g protein)$^{-1}$ in the fraction of subcellular organelles are much lower than those in cytosol and that cytosolic proteolytic activities degrading polypeptides and analogues are significant.

Recently, an analogue of vasopressin has been marketed even though it has an oral bioavailability of only 1%. Both poor membrane permeability and extensive lumenal degradation impede oral absorption of peptides and peptide analogues. Ileal mucosal homogenates and colonic epithelium metabolize peptide hormones rapidly (Atchison et al 1989; Yamamoto et al 1990). Clearly, intestinal epithelium is also a barrier which metabolically limits bioavailability of large peptide drugs.

There are two putative pathways, paracellular and transcellular transport, in intestinal absorption of large peptides, and the former, selective for cations as well as excluding molecules with hydrodynamic radii greater than 4–8 Å at the resting state (Pappenheimer & Reiss 1987), will probably prevent penetration of polypeptides. The paracellular mechanism cannot explain the large differences in the intestinal transport rates of cyclic peptides of oxytocin, vasopressin, and their analogues with similar mol. wt (1000 Da) but varying hydrophobicity (Vilhardt & Lundin 1986); cyclosporin (mol. wt 1203) has a better than 25% absorption in-vivo (Lee et al 1991), which is unlikely to be due to the paracellular pathway. Further, SMS 201-995, an octapeptide, was found to be absorbed transcellularly (Fricker et al 1991). Clearly, the transcellular pathway is much more important in in-vivo absorption. Endocytosis and passive diffusion are all transcellular processes. Even though absorption mechanisms of proteins in adult intestine are less well characterized, endocytotic uptake and a subsequent degradation in lysosomes have been observed (Marcon-Genty et al 1989). Presumably, if taken up by enterocytes via passive diffusion, large peptides will be exposed to brush-border membrane, cytosol or membrane peptidases on intracellular organelles, and may also be translocated to lysosomes via specific mechanisms.

Lysosomes involved in eliminating intracellular abnormal and unnecessary proteins via specific transport or signals of specific amino acid sequences are established in isolated mammalian cells and have also been observed in-vivo (Wing et al 1991; Dice 1992). However, considering the amino acid sequences of bioactive peptides, specific transport is unlikely, due to lack of signalling sequences in their structures, while random intracellular encounter is most probably the main factor when intestinal absorption is low (Dice 1992). Cellular organelles participating in intracellular production and transport of proteins may have various types of proteases on their membranes. Random encounter will subject polypeptides to cytosolic peptidases and membrane peptidases of organelles, which usually have activity at pH above 7.

Colonic brush-border has little proteolytic activity while small intestinal brush-border has an array of exo- and endopeptidases (Adibi & Kim 1981; Bai et al 1992). According to studies on protein digestion, the cytoplasm of small intestinal enterocytes have only di- and tripeptidases (Adibi & Kim 1981). However, various reports have suggested the existence of cytosolic proteolytic enzymes capable of degrading large peptides, such as post-proline cleaving enzyme and a multicatalytic high-molecular-weight protease complex in the intestine (Zolfaghari et al 1987; Arrigo et al 1988; Tsuji & Kurachi 1989; Lundin et al 1989). To design successful delivery schemes for bioactive peptides and analogues, it is essential to determine whether the cytoplasm and intracellular organelles contain proteolytic activities at cytosolic pH. In-vivo bioavailability is determined by absorption and metabolism at the cellular level. Using in-vitro subcellular fractionation, this study aimed to compare distribution of enzymes which degrade large peptides in three subcellular fractions, intracellular organelles, brush-border membrane, and cytosol, in the small intestine and colon of rat.

Materials and Methods

Materials

Neurotensin, acetylneurotensin(8-13), GRF(1-29)NH_2, insulin B-chain, and pentobarbitone were obtained from Sigma Chemical Co. (St Louis, MO, USA). ^{125}I-(A14)-Human recombinant insulin was obtained from Amersham Corporation (Arlington Heights, IL, USA). GRF(1-29)NH_2, and (desNH_2Tyr^1,D-Ala^2,Ala^{15})-GRF(1-29)NH_2 were gifts from Dr Arthur M. Felix (Peptide Research Department, Hoffmann-La Roche Nutley, NJ). 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.

Animals

Male Sprague-Dawley rats, 250–300 g, were used.

Preparation of subcellular fractions

The rat was killed by an overdose of pentobarbitone and its abdomen was opened by a mid-line incision. The small intestine and colon were removed and cut longitudinally to expose the mucosal surface (epithelium), from which the mucosa was scraped off.

One gram mucosal tissue was suspended in 10 mL 50 mM Tris/HCl buffer (pH 7·5) and 125 mM NaCl. Individual mucosal tissue was then homogenized by a glass/Teflon Potter homogenizer in an ice bath with 10 strokes at the speed of 1140 rev min^{-1}. $CaCl_2$ (1 M) was added to intestinal homogenates to achieve a final concentration of 10 mM. Homogenate was subjected to a series of differential centrifugations at 4°C: 10 000 *g* (20 min), 27 000 *g* (30 min), and 100 000 *g* (1 h) (Kessler et al 1978; Bai 1993). The protein concentration of each subcellular fraction was determined using the Bradford method (Bradford 1976). Enzyme markers for Golgi membrane (α-D-mannosidase), mitochondria (succinate dehydrogenase), and cytosol (lactate dehydrogenase) were assayed to confirm preparation of subcellular fractions (Miura et al 1983; Moktari et al 1986; Bai 1993; Reim et al 1993).

Proteolysis by subcellular fractions

Proteolysis of all the peptides and analogues except insulin was performed as published previously (Bai 1993). The incubation mixture (300 μL) consisted of 50 mM pH 7·5 Tris/HCl buffer, NaCl (150 mM), a substrate, and subcellular fraction protein (0·1–2 mg). In the incubation mixture, the final substrate concentration was 0·02 mM. The concentration of each substrate was the same for all the subcellular fractions studied. This would ensure that results of distribution of subcellular degradative activities would be truly delineated since hydrolysis rate (g protein)$^{-1}$ depends on the substrate concentration. For all colonic subcellular fractions, incubation was performed at 37°C; for the small intestinal subcellular fractions, neurotensin, acetylneurotensin (8-13), and GRF(1-29)NH_2 were incubated at room temperature (21°C) to reduce rapid degradation, and others were incubated at 37°C. Samples were taken periodically and the reaction was stopped using 0·1 M HCl.

For insulin, degradation was studied using the trichloroacetic acid (TCA) method (Duckworth et al 1972). The incubation mixture consisted of 50 mM Tris/HCl buffer (pH 7·5 at 37°C), 1% bovine serum albumin (w/v), 125 mM NaCl, 30 pM ^{125}I-(A14)-insulin, and proteins of the subcellular fraction (100–600 μg mL^{-1}). Periodically, 200 μL samples were taken and mixed with 200 μL 10% TCA. The mixture was centrifuged at 3000 *g*. The supernatant containing insulin fragments was counted in a γ-counter. The amount of insulin degraded was then estimated from standard curves.

In the control group, proteins in buffer were denatured by TCA or inactivated by HCl and then mixed with substrates. No hydrolysis of any peptides or analogues was observed in the control groups for any of the subcellular fractions.

Assay methods

Analysis of peptides was by HPLC using an SIL autoinjector, an LC-600 pump, an SPD-6A UV spectrophotometric detector and a CR 601 recorder (Shimadzu Corporation, Kyoto, Japan). All analyses were with a C_{18} phase on 5 μm particles packed in 4·6 mm × 15 cm columns from either Beckman Instrument (Berkley, CA, USA) or Vydac (Hesperia, CA, USA). Other HPLC conditions are summarized in Table 1. ^{125}I-(A14)-Human recombinant insulin was assayed using a γ-counter.

Data analysis

Initial hydrolysis rate was obtained from the first 10–20% proteolysis. After normalization for protein concentration, average hydrolysis rate was obtained by averaging results from three experimental groups. Proteolytic activity was expressed as the amount of substrate hydrolysed (g protein)$^{-1}$ min^{-1}.

Results and Discussion

The results of the study are summarized in Tables 2–5. Homogenates and subcellular fractions are the simplest in-vitro tools to examine overall proteolytic activities at the

Table 1. Assay of peptides by HPLC analysis using reverse-phase C_{18} columns.

Peptide	Mobile phase	Flow rate (mL min^{-1})
Neurotensin	Acetonitrile : 0·05% TFA (25 : 75)	1·5
Acetylneurotensin (8-13)	Acetonitrile : 0.05% TFA (25 : 75)	1·0
Insulin B-chain	Acetonitrile : 0.05% TFA (32 : 68)	1·0
GRF(1-29)NH_2	Acetonitrile : 0.05% TFA (35 : 65)	1·5
(desNH_2Tyr^1,D-Ala^2,Ala^{15})-GRF(1-29)NH_2	Acetonitrile : 0.05% TFA (35 : 65)	1·5

TFA = trifluoroacetic acid.

Table 2. Distribution (%) of various enzyme markers in subcellular fractions.

Enzyme	10 000-*g* pellet	27 000-*g* pellet	100 000-*g* pellet	100 000-*g* supernatant
Colon				
Lactate dehydrogenase	13	0·3	0·3	86·3
α-D-Mannosidase	93	6·5	0	0
Small intestine				
Lactate dehydrogenase	17	1·3	0·5	81
Succinate dehydrogenase	82	5·6	2·3	10
α-D-Mannosidase	91	7	2·3	0

Each result was the average of three experiments.

Table 3. Small intestinal subcellular distribution of proteolytic activities degrading peptides and analogues.

Compounds	10 000-*g* pellet	27 000-*g* pellet	100 000-*g* pellet	100 000-*g* supernatant
($DesNH_2Tyr^1$,D-Ala^2,Ala^{15})-GRF(1-29)NH_2	0·10 (0·03)	0·20 (0·05)	0·37 (0·01)	0·30 (0·10)
Insulin B-chain	1·40 (0·10)	9·30 (0·83)	9·63 (0·43)	2·34 (0·16)
Insulin	0·32 (0·03)	0·65 (0·10)	0·73 (0·17)	2·10 (0·30)
Acetylneurotensin (8-13)	0·67 (0·01)	2·23 (0·03)	1·82 (0·06)	0·68 (0·05)
Neurotensin	0·76 (0·02)	4.38 (0·31)	3·29 (0·07)	0·85 (0·02)
GRF(1-29)NH_2	2·64 (0·37)	8·33 (0·34)	4·63 (0·58)	1·76 (0·50)

Results are mean (s.d.) values of three determinations and are expressed as substrate hydrolysed (g protein)$^{-1}$ min^{-1}.

Table 4. Colonic subcellular distribution of proteolytic activities degrading peptides and analogues.

Compounds	10 000-*g* pellet	27 000-*g* pellet	100 000-*g* pellet	100 000-*g* supernatant
($DesNH_2Tyr^1$,D-Ala^2,Ala^{15})-GRF(1-29)NH_2	0·50 (0·01)	0·28 (0·05)	0·05 (0·00)	1·10 (0·20)
Acetylneurotensin (8-13)	0·22 (0·03)	0·30 (0·09)	1·90 (0·16)	0·14 (0·03)
Neurotensin	1·60 (0·1)	0·55 (0·02)	1·71 (0·15)	3·80 (0·20)
Insulin	0·32 (0·02)	0·40 (0·03)	1·01 (0·21)	1·43 (0·13)

Results are mean (s.d.) values of three determinations and are expressed as substrate hydrolysed (g protein)$^{-1}$ min^{-1}.

Table 5. Distribution (%) of degradative activities degrading peptides and analogues.

Enzyme	10 000-*g* pellet	27 000-*g* pellet	100 000-*g* pellet	100 000-*g* supernatant
Small intestine				
Acetylneurotensin (8-13)	47	9·3	3·9	39
Neurotensin	42	14	6	38
GRF(1-29)NH_2	57	10	3	30
($DesNH_2Tyr^1$,D-Ala^2,Ala^{15})-GRF(1-29)NH_2	27	3	4	66
Insulin	13	2·8	2·7	82
Insulin B-chain	34	13	7	46
Colon				
Acetylneurotensin (8-13)	41	4	17	38
Neurotensin	22	0·6	1·2	76·3
($DesNH_2Tyr^1$,D-Ala^2,Ala^{15})-GRF(1-29)NH_2	24	1	0	75
Insulin	12	1	2	84

Results are the mean values of three determinations.

administration sites and the distribution of intracellular potential metabolic activities against large peptides, respectively (Kessler et al 1978; Chowdhary et al 1985; Zolfaghari et al 1987; Yamamoto et al 1990; Kominami et al 1991). Isolation and separation of subcellular compartments via centrifugational forces is determined by density and hydrodynamic diameter of organelles and subcellular compartments; this has been well established for more than three decades. For example, cytoplasm or cytosol is usually obtained from the 100 000-*g* supernatant; brush-border membranes are usually obtained at 27 000 *g*; lysosomes are usually obtained with mitochondria in the 10 000-*g* pellet (Kominami et al 1991; Bai et al 1992). Agreeing with Kessler's observation, this study showed that 10 000-*g* pellets were rich in mitochondria, Golgi apparatus; 27 000-*g* pellets were rich in brush-border membrane;

Table 6. Tissue proteolytic enzymes degrading peptides and analogues.

Peptides and analogues	Proteolytic enzymes reported to degrade individual peptide	References
GRF(1-29)NH_2	Dipeptidyl peptidase IV Trypsin-like activity	Frohman et al (1989)
($DesNH_2Tyr^1$,D-Ala^2,Ala^{15})-GRF(1-29)NH_2	Unknown	
Insulin	Insulin-degrading enzyme	Duckworth (1988)
Insulin B-chain	Endopeptidase-2, endopeptidase-24.11 Insulin-degrading enzyme	Kenny & Ingram (1987) Duckworth (1988)
Acetylneurotensin (8-13)	Unknown	
Neurotensin	Endopeptidase-24.11 Angiotensin converting enzyme Endopeptidase-2	Turner (1987) Kenny & Ingram (1987)

100 000-*g* supernatant was rich in cytosol. According to Kessler's results, nuclei were also included with mitochondria at 3000 *g* (Kessler et al 1978); therefore 10 000-*g* pellet would also contain nuclei. Results of enzyme marker measurements (Table 2) indicated that there was little cross-contamination in the three subcellular fractions. As the study aimed only to characterize proteolytic activities in the brush-border membrane, cytosol, and on the external organelle membranes, pH 7·5 was used. Lysosomal proteases have high activities at acidic pH (Kirschke & Barrett 1987); results at pH 7·5 will not reflect the significance of lysosomal proteolytic activities in the 10 000-*g* pellet.

Neurotensin and acetylneurotensin (8-13) are short oligopeptides subject to various membrane and soluble peptidases (Table 6). Since those enzymes also exist in brush-border membrane, neurotensin and its analogue are likely to be degraded by the fraction rich in brush-border membrane (Kenny & Ingram 1987; Turner 1987; Bai 1993; Bai & Amidon 1992). The results confirmed this and showed that in small intestinal subcellular fractions, neurotensin and its analogue were degraded most rapidly by the fractions which were rich in brush-border membrane and plasma membrane fragments (microsomes) (100 000-*g* pellet). Colonic 27 000-*g* pellet, rich in brush-border membrane, had weak activities on neurotensin and acetylneurotensin (8-13). The former was degraded most rapidly by colonic cytosol, and the latter by the microsomal fraction. It is unknown why microsomes had such high activity against acetylneurotensin (8-13). Post-proline cleaving enzyme was suggested to exist in enterocyte cytosol (Lundin et al 1989). Although neurotensin and acetylneurotensin (8-13) contain proline, the involvement of post-proline cleaving enzyme in degrading neurotensin and acetylneurotensin (8-13) needs to be verified.

In the small intestinal preparation, GRF(1-29)NH_2 was degraded most rapidly by brush-border membrane, while ($desNH_2Tyr^1$,D-Ala^2,Ala^{15})-GRF(1-29)NH_2 was most rapidly degraded by the microsomal fraction and cytosol. Although ($desNH_2Tyr^1$,D-Ala^2,Ala^{15})-GRF(1-29)NH_2 was more stable than GRF(1-29)NH_2, its degradation by enterocyte proteolytic activities was substantial. In colonic subcellular fractions, the cytosol degraded ($desNH_2Tyr^1$,D-Ala^2,Ala^{15})-GRF(1-29)NH_2 most rapidly. Since GRF(1-29)NH_2 and its analogue do not have proline residues, the compounds are not subject to attack by the post-proline cleaving enzyme. It is known that GRF(1-29)NH_2 is a substrate for dipeptidyl peptidase IV (Frohman et al 1989), a brush-border membrane peptidase. Dipeptidyl peptidase IV may be a contributor to GRF(1-29)NH_2 degradation by the small intestinal 27 000-*g* pellet.

In both small intestinal and colonic preparations, the cytosol was the major subcellular localization of insulin-degrading activity. Insulin is degraded by enzymes in hepatocytes, adipocytes, and other enzymes (Duckworth 1988). It remains to be verified whether insulin-degrading enzyme exists in intestinal enterocytes, and whether it was responsible for the results observed in this study. Several membrane endopeptidases attack insulin B-chain (Kenny & Ingram 1987), and it is not surprising to observe rapid degradation of insulin B-chain by subcellular fractions of brush-border membrane and microsomes.

In the small intestine, the cytosol had higher activities than the 10 000-*g* pellet (subcellular organelles) against all the peptides except acetylneurotensin (8-13) and GRF(1-29)NH_2; in the colon, the cytosol had the highest activities against neurotensin, insulin, and GRF(1-29)NH_2 analogue. Further, except for degradation of acetylneurotensin (8-13), neurotensin, and GRF(1-29)NH_2 by small intestinal fractions and degradation of acetylneurotensin (8-13) by colonic fractions, the cytosol had the highest percentage of proteolytic activities against large peptides for both the small intestine and colon. Apparently, the cytosol, in addition to di- and tripeptidases, has other enzymes capable of degrading insulin, insulin B-chain, GRF(1-29)NH_2, and ($desNH_2Tyr^1$,D-Ala^2,Ala^{15})-GRF(1-29)NH_2.

In summary, the results suggest that, in addition to small intestinal brush-border membrane which is significant in limiting intact absorption of peptides and analogues, small intestinal and colonic cytosolic proteolytic activities are also significant. To achieve oral efficacy of peptides and peptidiomimetics, it is essential to characterize the cytosolic enzymes of intestinal enterocytes.

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