J. Pharm. Pharmacol. 1995, 47: 698–701
Received December 6, 1994
Accepted February 9, 1995

The Involvement of Dipeptidyl Peptidase IV in Brush-border Degradation of GRF(1-29)NH_2 by Intestinal Mucosal Cells

JANE P. F. BAI AND LI-LING CHANG

College of Pharmacy, University of Minnesota, Minneapolis, MN 55455, USA

Abstract

GRF(1-29)NH_2 is degraded mainly by dipeptidyl peptidase IV (DPP IV) in plasma, resulting in inactivated GRF(3-29)NH_2. To understand whether improving stability of GRF(1-29)NH_2 in the plasma will result in enhanced stability in intestinal mucosal cells, stability of GRF(1-29)NH_2 and [desNH$_2$Tyr^1,D-Ala^2,Ala^{15}]-GRF(1-29)NH_2 in rat intestine brush-border membrane and homogenate was examined.

[desNH$_2$Tyr^1,D-Ala^2,Ala^{15}]-GRF(1-29)NH_2, resistant to plasma DPP IV, was much more stable than GRF(1-29)NH_2 in enterocytes. Gradient HPLC analysis, mass balance analysis and studies of inhibitor effects revealed that GRF(3-29)NH_2 was the major metabolite of GRF(1-29)NH_2 due to the action of DPP IV during incubation with brush-border membranes.

It is concluded that the design of peptide analogues to resist plasma enzymes dramatically increases stability in intestinal epithelium.

GRF(1-29)NH_2 is the shortest analogue of GRF (human growth hormone releasing factor) with full biological activity (Rivier et al 1982; Grossman et al 1984). GRF is also present in the gut and regulates the release of somatostatin into the portal blood (Arimura et al 1984). By stimulating the release of growth hormone, GRF(1-29)NH_2 has potential clinical uses for treating children deficient in growth hormone (Frohman & Jansson 1986). Several studies have shown that the major metabolite of GRF(1-29)NH_2 in plasma is GRF(3-29)NH_2 resulting from attack by dipeptidyl peptidase IV (DPP IV) (Frohman et al 1989a, b; Su et al 1991). Elimination of the *N*-terminal α-amino group greatly enhances the stability of GRF(1-29)NH_2 in plasma (Felix 1991). Furthermore, retaining the α-helix conformation of GRF(1-29)NH_2 increases its biological potency (Felix 1991). [desNH$_2$Tyr^1,D-Ala^2,Ala^{15}]-GRF(1-29)NH_2 was thus designed and has been shown to have enhanced biological activity and plasma stability, that is, resistant to plasma DPP IV (Su et al 1991).

Insulin and insulin-like growth factor I, which are much larger than GRF(1-29)NH_2, are absorbed across the intestinal epithelium (Quadros et al 1994). GRF(1-29)NH_2 also has a secondary structure. Hence, the theory that linear small peptides require a large amount of desolvation energy in order to be absorbed into the membrane may not be applicable to GRF(1-29)NH_2 (Karis et al 1991). To evaluate the intestinal absorption of GRF(1-29)NH_2, it is necessary to understand its stability in the gut wall.

No previous study has investigated intestinal metabolism and stability of GRF(1-29)NH_2. There are at least two intestinal brush-border membrane endopeptidases, and several exopeptidases are present in plasma (Adibi & Kim 1981; Ishida et al 1989). We have investigated whether GRF(1-29)NH_2 is mainly degraded by DPP IV in a mucosal cell brush-border membrane preparation and homogenate, as in plasma. If so, it is expected that [desNH$_2$Tyr^1,D-Ala^2,Ala^{15}]-GRF(1-29)NH_2, which is designed to resist plasma DPP IV (Frohman et al 1989b), should be much more stable.

Correspondence: J. P. F. Bai, 9-104 HSUF, College of Pharmacy, University of Minnesota, Minneapolis, MN 55455, USA.

Materials and Methods

Materials

GRF(1-29)NH_2, diisopropyl fluorophosphate (dip-F), diprotin A, and pentobarbitone were obtained from Sigma Chemical Co. (St. Louis, MO). GRF(1-29)NH_2, GRF(3-29)NH_2, and [desNH$_2$Tyr^1,D-Ala^2,Ala^{15}]-GRF(1-29)NH_2 were gifts from Dr. Arthur M. Felix (Peptide Research Department, Hoffmann-La Roche). 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.

Animals

Male Sprague-Dawley rats, 250–300 g, were fed with regular laboratory rodent chow. Rats were killed with an overdose of pentobarbitone.

Preparation of brush-border membranes

The first 8 cm of rat small intestine was used as the duodenum, the next 35 cm as the jejunum, the last 20 cm 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 just distal to the ileum. 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 from homogenates as reported previously (Kessler et al 1978). Briefly, $CaCl_2$ (1 M) was added to the homogenates to achieve a final concentration

Table 1. Effects of pH on proteolysis of GRF(1-29)NH_2 and [desNH$_2$Tyr^1,D-Ala^2,Ala^{15}]-GRF(1-29)NH_2 by rat intestine mucosal homogenate.

Intestinal segment	GRF(1-29)NH_2[a]		[desNH$_2$Tyr^1,D-Ala^2,Ala^{15}]-GRF(1-29)NH_2[b]
	pH 7·5	pH 4·5	pH 7·5
Duodenum	2·1 ± 0·7	0·8 ± 0·0[c]	
Jejunum	0·5 ± 0·1	0·9 ± 0·1	0·2 ± 0·1 ($P < 0·05$)[d]
Jejuno-ileal junction	0·9 ± 0·3	0·8 ± 0·1	
Ileum	1·0 ± 0·1	0·8 ± 0·1	0·3 ± 0·0[c] ($P < 0·005$)[d]
Caecum	0·4 ± 0·2	0·6 ± 0·0[c]	

Mean ± s.d. (n = 3). Proteolysis rate: μmol min^{-1} (g protein)$^{-1}$. [a]At room tempeature (22°C); [b]At 37°C. [c]Standard deviations are rounded off to 0 when only one digit after the decimal point is considered. [d]Compared with degradation of GRF(1-29)NH_2 at pH 7·5.

of 10 mM and then the mixtures were centrifuged at 3000 *g* for 15 min. Supernatants were collected and centrifuged at 27 000 *g* for 30 min, the pellets were suspended in a 150 mM NaCl/10 mM Tris/HCl (pH 7·5) solution and homogenized with a glass/Teflon Potter homogenizer. Centrifugations at 3000 and 27 000 *g* were repeated to obtain brush-border membranes. Protein concentrations were determined using the Bradford method and γ-globulin as the protein standard (Bradford 1976). Activity of alkaline phosphatase was determined to assess the purity of brush-border membranes which were 12 times purified (Miura et al 1983).

Proteolysis

The incubation mixture of 300 μL consisted of 50 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 0·1–0·2 mg homogenate proteins. For the studies at pH 4·5, 50 mM acetate buffer was used. Proteolysis of GRF(1-29)NH_2 and [desNH$_2$Tyr^1,D-Ala^2,Ala^{15}]-GRF(1-29)NH_2 was performed at room temperature (22°C) and 37°C, respectively. Aliquots were taken periodically and the reaction was stopped using 0·1 M HCl. For each study, there were 3 experimental and 3 control groups. In the control group, proteins were denatured by boiling for 10 min before being added to the incubation mixture. For the inhibitor studies, dip-F and diprotin A were added to the incubation mixture to achieve a concentration of 30 and 0·73 mM, respectively (Umezawa et al 1984; Kirschke & Barrett 1987).

Assay methods

HPLC systems consisted of a SIL autoinjector, LC-600 pump, SPD-6A UV spectrophotometric detector, and a CR 601 recorder (Shimadzu Corporation, Kyoto, Japan), and a C_{18} (Vydac 201 TP C18) column (5 μ, 4·6 mm × 25 cm, Vydac, Hesperia, CA). GRF(1-29)NH_2 and [desNH$_2$-Tyr^1,D-Ala^2,Ala^{15}]-GRF(1-29)NH_2 were assayed by a gradient system of mobile phases of A (0·05% TFA) and B (acetonitrile: 0·05% TFA = 95 : 5). The gradient started with 30% B and reached 45% B in 60 min (Frohman et al 1989a). The two compounds were eluted at 7 min at a flow rate of 1·5 mL min^{-1}.

Data analysis

An initial hydrolysis rate was obtained from the first 10 to 20% of proteolysis. After normalization for the protein concentration, the average hydrolysis rate was obtained by averaging results from 3 experimental groups. Proteolytic activity was expressed as the amount of substrate hydrolysed (g protein)$^{-1}$ min^{-1}. All data reported are mean ± s.d.

Results and Discussion

Degradation of GRF(1-29)NH_2 at 37°C was too rapid to attain an appropriate evaluation of the hydrolysis rate. Therefore, GRF(1-29)NH_2 was studied at room temperature (22°C). GRF(1-29)NH_2 and [desNH$_2$Tyr^1,D-Ala^2,Ala^{15}]-GRF(1-29)NH_2 are chemically stable in the experimental conditions, with 103 and 99% left after 2 h incubation in the buffer and boiled tissue preparations at room temperature and 37°C, respectively.

Degradation by homogenate

At room temperature, degradation of GRF(1-29)NH_2 in homogenates showed the order of duodenum > ileum ~ jejuno-ileal junction > jejunum > caecum at pH 7·5, and the order of jejunum ~ duodenum ~ jejuno-ileal junction ~ ileum > caecum at pH 4·5 (Table 1). There are brush-border, cytosolic, and lysosomal peptidases in mucosal-cell homogenate. In general brush-border and cytosolic enzymes have high activities at pH 7·5 while lysosomal enzymes have high activities at acidic pH (Kirschke & Barrett 1987; Bai & Amidon 1992). Therefore, degradation in the homogenate at different pHs is due to different sources of peptidases. At pH 7·5, [desNH$_2$Tyr^1,D-Ala^2,Ala^{15}]-GRF(1-29)NH_2 was much more stable at 37°C than GRF(1-29)NH_2 at room temperature, and it had similar degradative rates in both jejunal and ileal homogenates.

GRF(1-29)NH_2 degradation by duodenal homogenate at pH 7·5 was inhibited 41% by diprotin A (a specific inhibitor of DPP IV) and 92·3% by dip-F (serine inhibitor, Table 2). Its degradation by jejunal homogenates was also inhibited 64% by diprotin A (Table 2). The IC50 of diprotin A against DPP IV was 1·1 μg mL^{-1} (Umezawa et al 1984). Dip-F at 1 mM completely inhibited DPP IV activity (Puschel et al 1982). The inhibition was < 50% in the duodenum and < 70% in the jejunum by diprotin A, but the almost

Table 2. Effects of inhibitors on GRF(1-29)NH_2 proteolysis by rat intestine homogenate.

	Proteolysis rate (μmol min^{-1} (g protein)$^{-1}$)	
	Duodenum	Jejunum
Control	2·4 ± 0·7	0·42 ± 0·14
Diprotin A	1·4 ± 0·0 ($P < 0·025$)[a]	0·15 ± 0·07 ($P < 0·05$)[a]
Dip-F	0·18 ± 0·07 ($P < 0·005$)[a]	

Mean ± s.d. (n = 3). Studies were performed at pH 7·5, and room temperature (22°C). Diprotin A; 0·73 mM; dip-F (diisopropyl fluorophosphate): 30 mM. [a]Compared with individual controls.

Table 3. Effects of pH on proteolysis of GRF(1-29)NH_2 and [desNH_2Tyr^1,D-Ala^2,Ala^{15}]-GRF(1-29)NH_2 by rat intestine brush border membranes.

Intestinal segment	GRF(1-29)NH_2[a]		[desNH_2Tyr^1,D-Ala^2,Ala^{15}]-GRF(1-29)NH_2[b]
	pH 7·5	pH 4·5	pH 7·5
Duodenum	3·8 ± 0·3	0·4 ± 0·1	
Jejunum	11 ± 1	0·5 ± 0·7	1 ± 0·1
Jejunoileal junction	6 ± 2	0·9 ± 0·1	
Ileum	6·0 ± 0·3	0·2 ± 0·1	1·2 ± 0·1
Caecum	4·2 ± 0·6	0·3 ± 0·1	

Mean ± s.d. (n = 3). Proteolysis rate: μmol min^{-1} (g protein)$^{-1}$. [a]At room temperature (22°C); [b]At 37°C.

complete inhibition in the duodenum by dip-F suggests that other unknown serine peptidases contribute to the degradation.

Degradation by brush-border membrane

Degradation of GRF(1-29)NH_2 by brush-border membrane was much faster than degradation by homogenate at pH 7·5, but was slower at pH 4·5, revealing that brush-border enzymes contribute to a large portion of homogenate proteolytic activities at pH 7·5 (Table 3). Degradation of GRF(1-29)NH_2 at room temperature showed the order of jejunum > ileum ~ jejuno-ileal junction > caecum ~ duodenum at pH 7·5, and the order of jejuno-ileal junction > jejunum ~ duodenum > caecum ~ ileum at pH 4·5. The regional differences of brush-border stability of GRF(1-29)NH_2 agree with the observation that DPP IV has high activity in the mid and distal small intestine (Bai 1994).

At pH 7·5, [desNH_2Tyr^1,D-Ala^2,Ala^{15}]-GRF(1-29)NH_2 was much more stable at 37°C then GRF(1-29)NH_2 at room temperature. Its rate of degradation was slightly higher in the ileum than in the jejunum ($P > 0·05$) (Table 3). Clearly, [desNH_2Tyr^1,D-Ala^2,Ala^{15}]-GRF(1-29)NH_2 is much more stable than its parent peptide when incubated with both homogenate and brush-border membranes.

The HPLC analysis of degradation by brush-border membranes revealed that a peak at 39·5 min corresponding to GRF(3-29)NH_2 was the major metabolite at pH 7·5. This peak first increased, and then decreased with time when the peak of GRF(1-29)NH_2 was decreasing (Fig. 1). The metabolite peaks produced are summarized in Table 4. Minor unidentified secondary metabolites that eluted at 5·9 and 7·8 min appeared after 5 min incubation; another peak appeared after 12 min incubation and was apparently a tertiary metabolite that eluted at 4·9 min. In the presence of diprotin A or dip-F, only a trace amount of GRF(3-29)NH_2 was detected after 20 min incubation, major unidentified metabolites eluted at 3·2, 4·8, 6·3, 7·3 min were detected when both inhibitors were used. In the presence of dip-F, an additional metabolite was detected at 15 min. Comparison of unidentified metabolites revealed by HPLC analysis indicates some similarity in the metabolite peaks produced when dip-F or diprotin A was used. At pH 4·5, DPP IV had no activity against its standard substrate, Gly-Pro-nitroanilide (data not shown) whereas GRF(1-29)NH_2 was still hydrolysed. At pH 4·5, no GRF(3-29)NH_2 was detected, and HPLC analysis suggests that degradation was due to unidentified enzymes producing a major primary unidentified metabolite that eluted at 37 min, and minor primary metabolites that eluted at 4·3, 4·8 and 7·4 min.

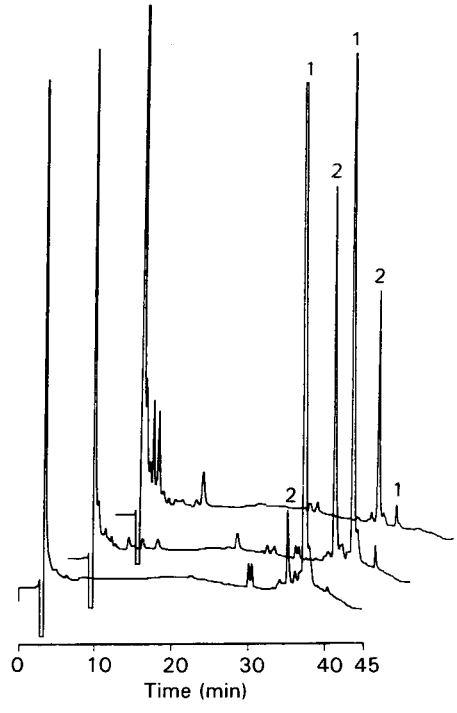

FIG. 1. Chromatograms of GRF(1-29)NH_2 after GRF(1-29)NH_2 was incubated with ileal brush-border membranes at pH 7·5 and room temperature (22°C). The gradient system consisted of mobile phases of A (0·05% TFA) and B (ACN: 0·05% TFA = 95 : 5%). The gradient started with 30% B and reached 45% B in 60 min. Peak 1 was GRF(1-29)NH_2 whose peak height decreased with time while peak 2 was GRF(3-29)NH_2 whose peak height increased and then decreased with time.

Table 4. Metabolites of GRF(1-29)NH_2 produced by incubation with brush-border membranes in different incubation conditions.

Conditions	Metabolites
pH 7·5	Major primary metabolite: GRF(3-29)NH_2. Minor secondary or tertiary metabolites: peaks eluted at 4·9, 5·9, 7·8 min.
pH 4·5	Major primary metabolite: the peak eluted at 37 min. Minor primary metabolites: peaks eluted at 4·3, 4·8, 7·4 min.
Dip-F (30 mM)	Major primary metabolites: peaks eluted at 3·2, 4·8, 6·3, 7·3 min.
Diprotin A (0·73 mM)	Major primary metabolites: peaks eluted at 3·2, 4·8, 6·3, 7·3, 15 min.

Dip-F: diisopropyl fluorophosphate.

Table 5. Mass balance analysis.

Incubation time	GRF(1-29)NH_2	GRF(3-29)NH_2	Total	Missing
10 s	91·3 ± 3·7%	2 ± 0·2%	92·7 ± 3·1%	7%
45 s	72·3 ± 1·9%	5·3 ± 0·5%	77·6 ± 1·9%	22%
1·5 min	51·7 ± 1·9%	8·1 ± 0·4%	59·8 ± 1·5%	40%
5 min	6 ± 1·5%	9·3 ± 1·2%	15·3 ± 1·5%	85%
11·5 min	N.D.	4·8 ± 0·2%	4·8 ± 0·2%	95%

GRF(1-29)NH_2 was incubated with ileal brush-border membranes at pH 7·5 and room temperature (22°C). Mean ± s.d. N.D.: no detection (detection limit of 1 μM).

Table 6. Effects of inhibitors on GRF(1-29)NH_2 proteolysis during incubation with rat jejunal brush-border membranes.

	Proteolysis rate (μmol min^{-1} (g protein)$^{-1}$)
Control	3·12 ± 0·90
Diprotin A	1·50 ± 0·14 ($P < 0·02$)[a]
Dip-F	0·15 ± 0·16 ($P < 0·01$)[a]

Mean ± s.d. (n = 3). Studies were performed at pH 7·5, and room temperature (22°C). Diprotin A; 0·73 mM; dip-F (diisopropyl fluorophosphate): 30 mM. [a]Compared with the control.

From the mass balance (Table 5), GRF(3-29)NH_2 was the major likely initial metabolite produced from degradation of GRF(1-29)NH_2 by ileal brush-border membranes. The missing amount was probably due to further rapid degradation of GRF(3-29)NH_2. In another single experiment using jejunal brush-border membranes, the mass balance analysis also revealed that GRF(3-29)NH_2 was the major initial metabolite (data not shown). Degradation of GRF(1-29)NH_2 by duodenal brush-border membranes at pH 7·5 was partly inhibited by diprotin A and almost completely by dip-F (Table 6). Since dip-F is a serine protease inhibitor, almost complete inhibition by dip-F suggest that there is other serine protease other than DPP IV capable of degrading GRF(1-29)NH_2 or that diprotin A is not as strong an inhibitor of DPP IV as dip-F. It is not known whether another serine brush-border protease can degrade GRF(1-29)NH_2. The effects of inhibitors and the stability of [desNH$_2$Tyr^1,D-Ala^2,Ala^{15}]-GRF(1-29)NH_2 show that DPP IV is the major brush-border membrane enzyme cleaving GRF(1-29)NH_2.

The results clearly indicate that [desNH$_2$Tyr^1,D-Ala^2,Ala^{15}]-GRF(1-29)NH_2 is much more stable during incubation with brush-border membranes and homogenate. DPP IV is the major brush-border enzyme degrading GRF(1-29)NH_2 to GRF(3-29)NH_2. Other brush-border enzymes can degrade GRF(1-29)NH_2 to a lesser extent.

Acknowledgements

This work was supported by Grant-In-Aids Program, Graduate School, University of Minnesota.

References

Adibi, S. A., Kim, Y. S. (1981) Peptide absorption and hydrolysis, In: Johnson, L. R. (ed.) Physiology of the Gastrointestinal Tract, 1st edn. Raven Press, New York, pp 1073–1094

Arimura, A., Culier, M. D., Matsumoto, K., Kanda, M., Itoh, Z., Murphy, W., Shively, J. E., Palkovits, M. (1984) Growth hormone releasing factors in the brain and the gut: chemistry, actions, and localization. Peptides 5: 41–44

Bai, J. P. F. (1994) Distribution of brush-border membrane peptidase along the rat intestine. Pharm. Res. 11: 897–900

Bai, J. P. F., Amidon, G. L. (1992) Structural specificity of mucosal cell transport and metabolism of amino acids and peptide drugs: implication for oral peptide drug delivery. Pharm. Res. 9: 969–978

Bradford, M. M. (1976) 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

Felix, A. M. (1991) Growth hormone-releasing factor. Pharm. Technol. May: 28–32

Frohman, L. A., Jansson, J. O. (1986) Growth hormone-releasing hormone. Endocr. Rev. 7: 223–253

Frohman, L. A., Downs, T. R., Williams, T. C., Heimer, E. P., Pan, Y. C. E., Felix, A. M. (1989a) Rapid enzymatic degradation of growth hormone-releasing hormone by plasma in vitro and in vivo to a biologically inactive product cleaved at the NH_2 terminus. J. Clin. Invest. 78: 906–913

Frohman, L. A., Downs, T. R., Heimer, E. P., Felix, A. M. (1989b) Dipeptidylpeptidase IV and trypsin-like enzymatic degradation of human growth hormone-releasing hormone in plasma. J. Clin. Invest. 83: 1533–1540

Grossman, A., Savage, M. O., Lytras, N., Preece, M. A., Diaz-Sueiras, J., Coy, D. H., Rees, L. H., Besser, G. M. (1984) Responses to analogues of growth hormone-releasing hormone in normal subjects, and in growth-hormone deficient children and young adults. Clin. Endocrinol. 21: 321–330

Ishida, H., Scicli, G., Carretero, O. A. (1989) Contributions of various rat plasma peptidases to kinin hydrolysis. J. Pharmacol. Exp. Ther. 251: 817–820

Karis, M. S., Rush, B. D., Wilkinson, K. F., Vidmar, T. J., Burton, P. D., Ruwart, M. J. (1991) Desolvation energy: a major determinant of absorption, but not clearance, of peptides in rats. Pharm. Res. 12: 1477–1491

Kessler, M., Acuto, O., Storelli, C., Murer, H., Muller, M., Semenza, G. (1978) A modified procedure for the rapid preparation of efficiently transporting vesicles from small intestinal brush border membranes. Biochim. Biophys. Acta 506: 136–154

Kirschke, H., Barrett, A. J. (1987) Chemistry of lysosomal proteases. In: Glaumann, H., Ballerd, F. J. (eds) Lysosomes: Their Role in Protein Breakdown, Academic Press, London, pp 193–238

Miura, S., Song, I. S., Morita, A., Erickson, R. H., Kim, Y. S. (1983) Distribution and biosynthesis of aminopeptidase N and dipeptidyl aminopeptidase IV in rat small intestine. Biochim. Biophys. Acta 761: 66–75

Puschel, T., Mentlein, R., Heymann, E. (1982) Isolation and characterization of dipeptidyl peptidase IV from human placenta. Eur. J. Biochem. 126: 359–365

Quadros, E., Landzert, N. M., LeRoy, S., Gasparini, F., Worosila, G. (1994) Colonic absorption of insulin-like growth factor I in vitro. Pharm. Res. 11: 226–230

Rivier, J., Spiess, J., Thorner, M., Vale, W. (1982) Characterization of a growth hormone-releasing factor from a human pancreatic islet tumour. Nature 300: 276–278

Su, C. M., Heimer, E. P., Felix, A. M., Pan, Y. C. E., Mowles, T. F. (1991) In vitro stability of growth hormone releasing factor (GRF) analogs in porcine plasma. Horm. Metab. Res. 23: 15–21

Umezawa, H., Aoyagi, T., Ogawa, K., Naganawa, H., Hamada, M., Takeuchi, T. (1984) Diprotins A and B, inhibitors of dipeptidyl aminopeptidase IV, produced by bacteria. J. Antibiotics 37: 422–425