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Effects of Different Absorption Enhancers on the Permeation of Ebiratide, an ACTH Analogue, across Intestinal Membranes

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Abstract

The permeation of ebiratide (H-Met(O_2)-Glu-His-Phe-D-Lys-Phe-NH(CH_2)$_8$$NH_2$), a novel ACTH analogue, across the intestinal mucosae has been examined by use of isolated intestinal membranes from rats in a modified Ussing chamber.

Regional differences were observed in the permeation of ebiratide across intestinal membranes; the order of membrane permeability was jejunum > ileum > duodenum > colon. Overall, the permeation of ebiratide was relatively poor. The effects of various absorption enhancers were examined to increase the intestinal permeability to ebiratide. Sodium glycocholate and sodium caprate had no significant enhancing effect on the permeability of the jejunal membrane, but significantly enhanced the permeation of ebiratide through the colonic membrane. On the other hand, *N*-dodecyl-β-D-maltopyramoside (LM) significantly enhanced the permeation of ebiratide through both jejunal and colonic membranes. In general, the absorption-enhancing effects of these agents were more predominant in the colon than in the jejunum. Membrane damage by the absorption enhancers was evaluated by measuring the amount of protein released from the intestinal membrane. It was found that all the absorption enhancers slightly increased the amount of protein released, but that the amounts of protein released in the presence of these enhancers were much less than in the presence of ethylenediaminetetraacetic acid (EDTA), used as a positive control.

These findings suggest that the absorption enhancers, especially LM might be useful adjuvants for improving the intestinal absorption of peptide and protein drugs, including ebiratide.

The intestinal absorption of peptide and protein drugs after oral administration is usually poor. This has been attributed to their extensive hydrolysis by the proteolytic enzymes in the gastrointestinal tract or their poor membrane permeation characteristics, or both (Lee & Yamamoto 1990). Therefore, various approaches such as alternative routes of administration (Banga & Chien 1988), absorption enhancers (Muranishi 1990), protease inhibitors (Saffran et al 1988), chemical modification (Yodoya et al 1994; Asada et al 1995; Tanaka et al 1996) and different dosage forms (Patel & Ryman 1976) have been examined in attempts to overcome problems with the delivery of these peptides and proteins via the gastrointestinal tract.

The absorption enhancers used include surfactants, bile salts, chelating agents and fatty acids (Lee & Yamamoto 1990). Nishihata et al (1983) found that sodium salicylate and 5-methoxysalicylate both increased the rectal absorption of insulin in dogs. In addition, Yoshioka et al (1982) found that co-administration of sodium 5-methoxysalicylate greatly enhanced the rectal bioavailability of pentagastrin and gastrin. Furthermore, we observed by use of an in-vitro Ussing chamber method that the rectal transport of insulin and enkephalin was remarkably improved in the presence of various bile salts and polyoxyethylene-9-lauryl ether (Yamamoto et al 1992).

Ebiratide (H-Met(O_2)-Glu-His-Phe-D-Lys-Phe-NH(CH_2)$_8$ NH_2) is a newly synthesized ACTH derivative, developed for the treatment of Alzheimer-like dementia, which acts directly on the central nervous system. It has been reported to have potent neurotrophic effects in rats and mice after peripheral administration (Hock et al 1988), and its biological stability is superior to that of the native $ACTH_{4-10}$ fragment and that of the $ACTH_{4-9}$ analogue (H-Met(O_2)-Glu-His-Phe-D-Lys-Phe-NH_2; Shimura et al 1991). In a previous paper we showed that ebiratide was extremely stable in rat small intestinal fluid but was degraded in a variety of mucosal homogenates (Okagawa et al 1994). We also found that the degradation of ebiratide was markedly inhibited by aminopeptidase inhibitors such as sodium glycocholate, puromycin, bestatin and bacitracin (Okagawa et al 1994). However, the intestinal absorption of ebiratide and the effect of absorption enhancers on its absorption were not fully examined, although the mechanisms of ebiratide transport through the blood–brain barrier has been investigated (Terasaki et al 1992).

In this study, therefore, we first examined the intestinal transport of ebiratide across the various regions of the intestine using the in-vitro modified Ussing chamber method. We also used the method to investigate the effects of various absorption enhancers on the intestinal transport of ebiratide and on mucosal toxicity.

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Materials and Methods

Materials

Ebiratide was kindly supplied by Hoechst AG (Frankfurt, Germany). Sodium glycocholate was purchased from Sigma (St Louis, MO). *N*-Dodecyl-β-D-maltopyranoside (LM) was supplied by Nippon Seika (Osaka, Japan). Sodium caprate was purchased from Tokyo Kasei Industries (Tokyo, Japan).

Ethylenediaminetetraacetic acid (EDTA) was obtained from Nacalai Tesque (Kyoto, Japan). All other chemicals were obtained from commercial sources and were of analytical grade and used without further purification.

Intestinal transport of ebiratide

The transport of ebiratide across small and large intestinal membranes was evaluated by the method of Buur et al (1990) with slight modification. In brief, male Wistar rats (Japan SLC, Shizuoka, Japan), 250–300 g, were fasted for approximately 24 h before the experiments; water was freely available. The rats were anaesthetized with sodium pentobarbital (32 mg kg^{-1} i.p.) and were killed by exsanguination from the inferior vena cava. The intestine was excised and rinsed in saline. Avoiding Peyer's patches, experimental segments were obtained and the underlying muscularis was removed before mounting in a modified Ussing chamber. Glutathione bicarbonate Ringer's solution (2·5 mL) pre-adjusted to pH 7·4 and containing various absorption enhancers was added to the reservoir bathing the mucosal side. Each compartment of the chamber was mixed by bubbling with 95% O_2/5% CO_2, and the temperature within the chamber was maintained at 37°C by means of a circulating water bath. At predetermined times for up to 3 h, 100-μL samples were taken from the serosal side and immediately replaced with an equal volume of transport buffer. The apparent permeability coefficient, P_{app} (cm s^{-1}), was calculated from the formula, $P_{app} = (dX_R/dT) \times (1/A) \times (1/C_0)$, where X_R is the amount of ebiratide (mol) on the receptor side, A is the diffusion area (cm^2) and C_0 is the initial concentration of ebiratide (mol mL^{-1}) on the donor side. The viability of intestinal membrane during the test period was monitored by measuring the transport of trypan blue dye. There was no transport of dye during the incubation.

Release of protein from the intestinal mucosa by absorption enhancers

The release of proteins from the donor side of the intestinal membrane was used as an index to evaluate the membrane damage caused by the absorption enhancers. Buffer solution (2 mL) with or without absorption enhancer (20 mM) was administered to the donor side of intestinal membrane in a manner similar to that used in the penetration experiments. Tissues were left for 4 h after the administration of the solution and at the end of this experiment the fluid in the donor side was removed and assessed, by the method of Lowry et al (1951) using bovine serum albumin as the standard, for the release of protein from the intestinal membrane.

Assay of ebiratide

Ebiratide was assayed by reversed-phase HPLC on a 300×5 mm Vydac 214TP54 C_4 column. The mobile phase was 1% phosphoric acid adjusted to pH 3·0 with triethylamine and acetonitrile (8:1), the flow rate was 1·0 mL min^{-1}; detection was performed at 220 nm (Okagawa et al 1994).

Statistical analysis

Results are expressed as the mean ± s.e. and statistical significance was assessed by use of Student's *t*-test.

Results

Regional differences in permeation of ebiratide across the intestinal membrane

Fig. 1 shows the regional differences between the permeation of ebiratide across the mucosal membranes isolated from different regions of the rat intestine. The order of membrane permeability to ebiratide (1·0 mM) was: jejunum > ileum > duodenum > colon. As seen in Table 1, the P_{app} value for ebiratide across the jejunal membrane was 2·3 times that across the colonic membrane. Overall, however, we observed that permeation characteristics of ebiratide across the intestine were poor and the amount of ebiratide remaining at the donor site was more than 95% of the administered dose in every region (data not shown).

The permeation of ebiratide across the intestinal membrane in the presence of various absorption enhancers

To increase the permeation of ebiratide across the intestinal membrane, ebiratide was co-administered with various absorption enhancers (20 mM) to the donor site. Fig. 2 shows the effects of various absorption enhancers on the permeation of ebiratide (1·0 mM). The P_{app} values of ebiratide across the jejunal and colonic membranes are shown in Table 2. Regional differences were observed in their enhancing effects. Sodium glycocholate and sodium caprate had no significant enhancing effect on the permeation of ebiratide through the jejunal membrane but the permeability of the colonic membrane was

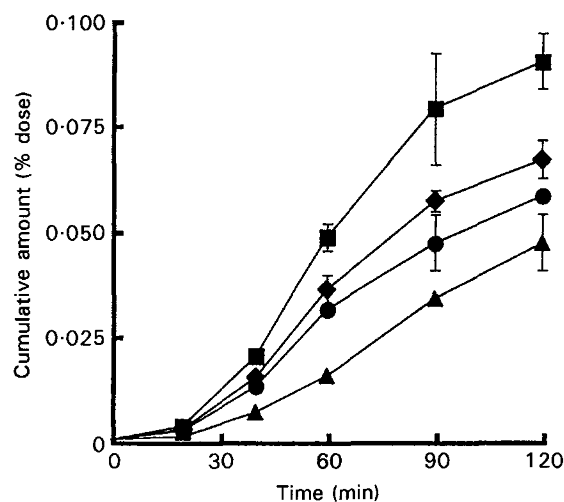

FIG. 1. Regional differences in the permeation of ebiratide (1·0 mM) across rat intestinal mucosae: duodenum (●), jejunum (■), ileum (◆), colon (▲). Data are means ± s.e. of results from three experiments

Table 1. Apparent permeability coefficients of ebiratide (1·0 mM) across rat intestinal mucosae.

Tissue	Apparent permeability coefficient (×10^{-6} cm s^{-1})	Ratio
Duodenum	0·984±0·04	0·64
Jejunum	1·531±0·14	1·00
Ileum	1·137±0·05	0·74
Colon	0·612±0·03	0·40

Data are means ±s.e. of results from three experiments.

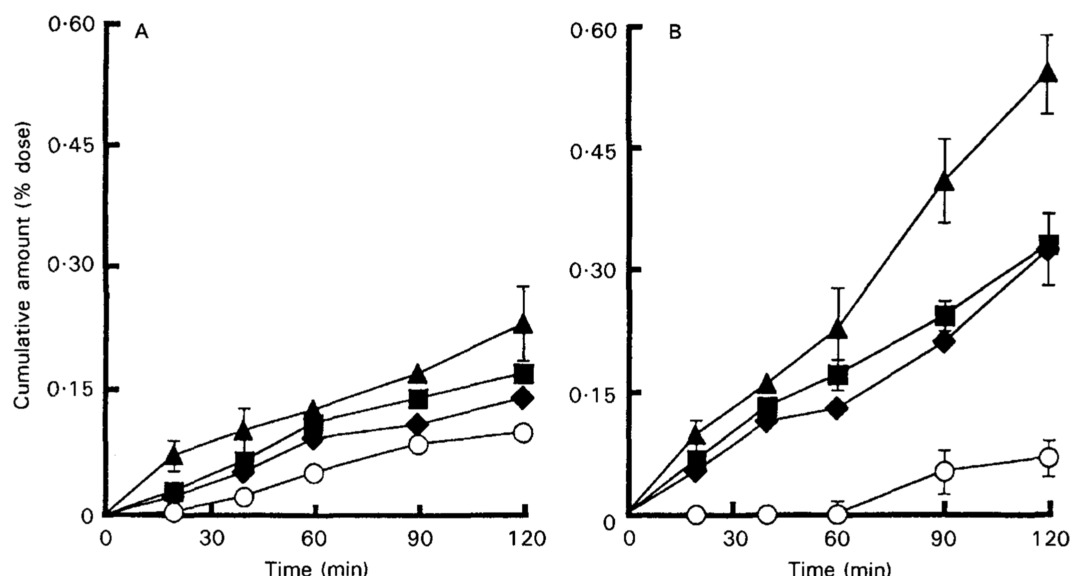

FIG. 2. Effects of various absorption enhancers on the permeation of ebiratide (1·0 mM) across the (A) jejunal and (B) colonic mucosae: control (○); sodium glycocholate, 20 mM (◆); sodium caprate, 20 mM (■); LM, 20 mM (▲). Data are means ± s.e. of results from three experiments.

significantly enhanced. On the other hand, LM significantly enhanced the permeation of ebiratide through both jejunal and colonic membranes. In general, however, the enhancement effect for the colonic membrane was higher than that for the jejunal membrane.

Release of protein from the intestinal mucosa by absorption enhancers

Membrane damage by these absorption enhancers was evaluated by measuring the release of protein from the intestinal membrane. The values obtained for the jejunal and colonic membranes are shown in Table 3. All the enhancers slightly increased the release of protein from the intestinal membrane. In the presence of sodium glycocholate, the amount of protein release in the jejunum was less than that in the colon. The opposite tendency was observed in the presence of sodium caprate and LM. In general, however, the amount of protein released in the presence of these enhancers was much less than that in the presence of EDTA as a positive control.

Discussion

This study has demonstrated regional differences in the permeation of ebiratide across the various intestinal membranes. The permeation of ebiratide across the jejunal membrane was much higher than across the colonic membrane. We have previously observed that the apparent permeability coefficient for insulin across the jejunal membrane was approximately 3-fold higher than the value for the colonic membrane (Asada et al 1995). Similarly, the absorption of thyrotropin-releasing hormone (TRH) from the small intestine was significantly higher than that from the large intestine (Tanaka et al 1996). Therefore, these previous findings were consistent with our current result. The reason for the regional difference in the permeability to ebiratide is not clearly understood. One possibility is that ebiratide is transported, like TRH, by a carrier-mediated process. However, in our pilot studies the percentage of ebiratide transported was almost 0·1%, irrespective of the initial concentration of ebiratide (0·05–1·0 mM). Moreover, because the transport of ebiratide was not inhibited by the addition of 2,4-dinitrophenol (DNP), it might not be plausible that ebiratide was transported across the intestinal membrane by a carrier-mediated process. Presumably, morphological differences in the thickness of the mucous layer, the unstirred water layer and membrane components between the small and the large intestine might influence the percentage permeation of ebiratide across the intestinal membrane.

During the transport studies, we examined the formation of ebiratide metabolites in both donor and receiver sites of the Ussing chamber. However, we found no evidence of formation of ebiratide metabolites and over 95% of the administered dose of ebiratide remained on the donor side after transport studies,

Table 2. Effect of absorption enhancers on the absorption of ebiratide (1·0 mM) across the rat jejunal and colonic mucosae.

	Jejunum Apparent permeability coefficient ($\times 10^{-6}$ cm s^{-1})	Ratio	Colon Apparent permeability coefficient ($\times 10^{-6}$ cm s^{-1})	Ratio
Control	1·53 ± 0·14	1·00	0·61 ± 0·03	1·00
Sodium glycocholate (20 mM)	1·93 ± 0·20	1·26	2·39 ± 0·40*	3·91
Sodium caproate (20 mM)	2·30 ± 0·55	1·50	2·35 ± 0·14†	3·84
N-Dodecyl-β-D-maltopyramoside (20 mM)	2·86 ± 0·23†	1·87	4·30 ± 0·25†	7·02

Data are means ± s.e. of results from three experiments. * $P < 0·05$, † $P > 0·01$ compared with control.

Table 3. Effects of various absorption enhancers on the release of protein from the jejunal and colonic mucosae.

	Jejunum		Colon	
	Protein (mg)	Ratio	Protein (mg)	Ratio
Control	0·12 ± 0·01	1·00	0·09 ± 0·02	1·00
NaGC	0·23 ± 0·06	1·92	0·03 ± 0·01*	3·33
NaCap	0·62 ± 0·05*	5·17	0·32 ± 0·01*	3·56
LM	0·77 ± 0·02*	6·42	0·46 ± 0·05*	5·11
EDTA	1·21 ± 0·11*	10·1	0·93 ± 0·08*	10·3

Results are expressed as the mean ± s.e. of three experiments. $*P < 0{\cdot}01$, compared with the control.

suggesting that ebiratide was extremely stable during the transport studies. This finding is in good agreement with the previous finding that ebiratide was comparatively stable in the intestinal fluid of rats, although it was degraded in homogenates of various intestinal mucosae (Okagawa et al 1994).

As mentioned above, because the transport of ebiratide across the intestinal membrane was very poor, absorption enhancers were required to improve the compound's low permeation across the intestinal membrane. We found that some absorption enhancers effectively enhanced the permeation of ebiratide across intestinal membranes, especially the colonic membrane.

Co-administration with sodium glycocholate resulted in less effective enhancement of the permeation of ebiratide in the jejunal membrane than in the colonic membrane. It has been reported that bile salts enhance the permeability of various drugs (Gordon et al 1985; Hersey & Jackson 1987) and that bile salts including sodium glycocholate are known to have inhibitory action on protease activities such as leucine aminopeptidase and other aminopeptidases (Hirai et al 1981). However, the enhancement effect of sodium glycocholate on the absorption of ebiratide might not be because of its protease inhibitory action, because ebiratide itself was very stable even in the absence of any additive in the gastrointestinal tract, as mentioned above.

We found that sodium caprate significantly enhanced the permeation of ebiratide in the colon, but not in the jejunum. Tomita et al (1988) reported that in rats the jejunal absorption of cefmetazole, a poorly absorbable antibiotic, was significantly enhanced by sodium caprate, but to a lesser extent than colonic absorption. Furthermore, Morishita et al (1993) reported that the enhancement effect of sodium caprate for insulin absorption was more predominant in the jejunum than in the colon. Thus, our current finding concurs with previous findings.

Our current study demonstrated that LM effectively enhanced the permeation of ebiratide through both jejunal and colonic mucosae. The P_{app} value of ebiratide co-administered with LM in the colonic mucosae was about seven times higher than in the control, whereas its jejunal P_{app} value in the presence of LM was about twice as high as in the control. This finding is consistent with our previous finding that LM enhanced the absorption of 6-carboxyfluorescein (CF) to a greater extent in the lower regions of the intestine (Murakami et al 1992). These positive effects of LM in improving the intestinal absorption of drugs is also supported by the observation that the pulmonary absorption of fluorescein isothiocyanate-labelled dextrans (FDs) and insulin were markedly enhanced by the co-administration of LM (Ohtani et al 1991; Yamamoto et al 1994).

When these absorption enhancers are used in practice it is essential they do not affect the membrane integrity of the epithelium. Many methods have been used to evaluate the membrane toxicity of absorption enhancers (Lee et al 1991). Morphological observation and haemolysis have been widely used to assess local toxicity of absorption enhancers. However, sometimes the results obtained by haemolysis cannot be extrapolated directly to the mucosal cells exposed to the enhancers. Moreover, neither of these methods can be used for quantitative assessment of local toxicity to the mucosal membrane. On the other hand, biological markers such as protein and phospholipid release are suitable for quantitative analysis (Uchiyama et al 1996; Yamamoto et al 1996). Because of this, we examined membrane toxicity by measuring protein release from the intestinal mucosae. We found that all the enhancers slightly increased the amount of protein released from the intestinal membrane, but that the amount of protein in the presence of these enhancers was much less than that in the presence of EDTA as a positive control. In particular, the amount of protein released by the colon after addition of sodium caprate and LM was less than in the jejunum, although the absorption-enhancing effects of these compounds were more predominant in the colon than in the jejunum. Therefore, sodium caprate and LM, which have low membrane toxicity in the large intestine, were suitable adjuvants for improving the absorption of ebiratide from the large intestine.

In conclusion, our results demonstrate that the overall permeation of ebiratide across the intestine was poor, but was effectively enhanced by some absorption enhancers, especially in the colon. The permeability of the colonic membrane to ebiratide was remarkably improved by co-administration of LM in particular. Thus, these absorption enhancers were suitable for improving the intestinal absorption of ebiratide, because they are of low membrane toxicity. However, we have not determined the effect of LM dose on the intestinal absorption of ebiratide. The relationship between the dose of LM and its enhancing effect need to be examined in the future.

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