JPP Journal of Pharmacy and Pharmacology

JPP 2003, 55: 1523–1529

Received April 9, 2003
Accepted July 16, 2003
DOI 10.1211/0022357022052
ISSN 0022-3573

Potential utility of various protease inhibitors for improving the intestinal absorption of insulin in rats

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Abstract

The aim of the investigation was to study the effects of protease inhibitors on the absorption of insulin in-situ from closed small and large intestinal loops in rats and to investigate the mechanism of various protease inhibitors in different intestinal loops. The intestinal absorption of insulin was evaluated by its hypoglycaemic effect and serum insulin level in the presence or absence of luminal contents. No marked hypoglycaemic effect was observed after administration of insulin alone in either region in the presence or absence of luminal contents. A significant hypoglycaemic effect of insulin was obtained in the large intestinal loop in the presence or absence of luminal contents when insulin was co-administered with bacitracin (20, 30 mM), sodium glycocholate (20, 40 mM), bestatin (29 mM), leupeptin (21 mM) and cystatin (0.8 mM). In contrast, there was no hypoglycaemic effect in the small intestinal loop in the presence of luminal contents following small intestinal co-administration of insulin with these protease inhibitors. The effectiveness of protease inhibitors was susceptible to their categories, concentrations and activity of proteolytic enzymes in different regions. The degree of improving insulin absorption in intestine was in the order of leupeptin > sodium glycocholate > bacitracin > bestatin > cystatin. At the same time, the percutaneous enhancement effect was observed in the presence of either sodium glycocholate or bacitracin. These results suggest that protease inhibitors could increase the insulin efficacy more effectively in the large intestine than in the small intestine.

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Acknowledgement and funding: The authors would like to thank Mrs Yu-Fen Jiang and Ms Xin-Rei Liu for their skilful technical assistance. This study was supported by grants from the Natural Science Foundation of Hubei Province, China (2002AB115).

Introduction

The increasing number of peptide and protein drugs that are being identified using biotechnology and recombinant DNA techniques will widely serve clinical therapy because of the authentic curative effect with less side effects. Advances made in the understanding of the role of the multitude of these regulatory agents in physiology and pathology, coupled with their availability in sufficient quantities in a much purer form at reasonable costs with reduced immunogenicity and antigenicity, have expanded their field of application, and now they represent an increasingly important group of drugs. However, despite these advantages, relatively little progress has been made in reaching the target of safe and effective oral formulations for peptides and proteins.

The difficulties associated with developing effective oral formulations for peptides and proteins have been elucidated in a number of excellent review articles (Fasano 1998; Lee 2002). The main barriers to success are normally ascribed to: poor intrinsic permeability of peptides and proteins across biological membranes due to their hydrophilic nature and large molecular size (Lee et al 1991; Yamamoto 2001; Bernkop-Schnurch & Clausen 2002); susceptibility to enzymatic attack by intestinal proteases and peptidases (Vyas et al 1997; Zhaopeng et al 1998); and chemical instability, including tendency to aggregate or nonspecifically adsorb to a variety of physical and biological surfaces (Borchardt 1999). Strategies, including dosage form modifications and alteration of administration route (Jung et al 2000; Bernkop-Schnurch & Walker 2001; Sakuma et al 2001; Tozaki et al 2001), alteration of physiological factors by absorption enhancers and enzyme inhibitors (Yamamoto et al 1994; Fukuda et al 1995) and modifications of native proteins by chemical synthesis of prodrugs and analogues (Yamamoto 1998; Gudmundsson et al 1999; Adessi & Soto 2002), have

been or are being employed to improve the bioavailability of peptides and proteins. It was proved that these methods could enhance permeability absorption and bioavailability of peptides and proteins from the parenteral route. Of these approaches, though some studies suggest that co-administration of insulin with different enzyme inhibitors has shown potential for increasing the absorption of bioactive insulin from the intestine (Bernkop-Schnurch 1998; Uchiyama et al 1999), it remains to be determined what is the mechanism of some protease inhibitors in different intestinal regions. It is uncertain whether the promoting effects of these protease inhibitors are site-dependent, and whether the quantities and kinds of protease inhibitors reflect the intestinal absorption of peptides and proteins.

In this study, insulin was chosen as a model peptide and the regional differences in the effects of various protease inhibitors on the absorption of insulin from the small and large intestine of rats were investigated. The biological efficacy of insulin following intestinal loops was evaluated by comparison with the intravenous route. The objectives were two-fold. First, it was desirable to compare the hypoglycaemic effect in different intestinal regions in the presence or absence of luminal contents when insulin was co-administered with different kinds and concentrations of protease inhibitors. The second objective, then, was to determine the mechanism of protease inhibitors and site-dependent characteristics of various protease inhibitors.

Materials and Methods

Materials and animals

Insulin ($28\,IU\,mg^{-1}$) was kindly supplied by Xuzhou Biochemical Co. (Jiangsu, China). Bacitracin (BAC), sodium glycocholate (SGC), cystatin of molecular weight range 12 700 daltons (CTT) (Anastasi et al 1983; Henskens et al 1994), sodium pentobarbital and insulin enzyme immunoassay kit were purchased from Sigma Chemical Co. (St Louis, MO). Leupeptin (LPT), bestatin (BTT) and Accu-Chek Advantage were obtained from Roche Diagnostics Co. (Indianapolis, IN). Phosphate-buffered saline (PBS: 137 mM NaCl, 2.6 mM KCl, 6.4 mM $Na_2HPO_4 \cdot 12\,H_2O$ and 1.4 mM KH_2PO_4; pH 7.4; 37 °C) and all other chemicals were of reagent grade.

The Wistar rats were housed under controlled environmental conditions (general food and water were freely available) and treated according to the Guiding Principles for the Administration and Use of Laboratory Animals authorized by the Ministry of Health of China. The institutional animal experimentation ethics committee approved the study protocol.

Animal experiments

Male Wistar rats, 240–300 g, were anaesthetized by an intraperitoneal injection of $50\,mg\,kg^{-1}$ sodium pentobarbital. Rats were restrained in a supine position on a board which was kept at a surface temperature of 37 °C for about 16 h before the experiments, with water freely available. A small midline incision was made in the abdomen, and a small or large intestinal loop was prepared by cannulation with polyethylene L-tube ligated at the proximal and distal ends of the small or large intestine, respectively. In experiments designed to remove luminal contents, a washing step (Doluisio et al 1969; Lennernas 1997) was performed on either intestinal loop after the same operation as in the presence of luminal contents was finished. The loop was washed gently with 20 mL of warmed PBS until the exudates became limpid. The remaining PBS in the loop was then washed out with air. The left jugular vein was cannulated for collection of blood samples. The rats were fixed on the warmed board for 1 h before various test solutions were injected into the loops.

Insulin was dissolved in PBS to a final concentration of $60\,IU\,kg^{-1}$ body weight. Protease inhibitors in varied concentrations were added to dosing solutions. Approximately 5 min before insulin dosing, a 0.5- mL blood sample was taken from the cannulated jugular vein. Subsequent blood samples were taken at 15, 30, 60, 120, 180 and 240 min after dosing.

To calculate the efficacy of insulin intestinal administration relative to intravenous insulin, solutions were administered intravenously via the jugular vein. In this case, the same operation as in the intestinal administration experiments was performed on another group of rats. The insulin intravenous doses were $0.5\,IU\,kg^{-1}$ body weight. A 0.5- mL blood sample was collected from the cannulated jugular vein on the opposite side to the injection 5 min before and at 5, 10, 20, 30, 60, 120, 180 and 240 min after dosing. Serum was separated by centrifugation at 5000 $rev\,min^{-1}$ for 10 min and kept frozen until analysis.

Analytical methods

The serum glucose concentrations were determined by Accu-Chek Advantage (measurement range: 0–6 $g\,L^{-1}$; accuracy: $\pm(5\% + 2)$; relative standard deviation (RSD): $\leq 8\%$). The decrement in serum glucose level (D) was calculated by a method similar to that described by Hirai et al (1981) from equation 1:

$$D = \left(1 - \frac{AUC_{G1\ 0\rightarrow 240\,min}}{100\% \times 240\,min}\right) \times 100\% \quad (1)$$

In this work, the area above the 100% line was ruled out for calculating the area under the serum glucose concentration–time curve ($AUC_{G1\ 0-240\,min}$). The D value was 2.44% when insulin solution ($0.5\,IU\,kg^{-1}$) was intravenously administered by jugular injection. The pharmacological availability (PA) was calculated from equation 2:

$$PA = \frac{D_{i.p.} \times Dose_{i.v.}}{D_{i.v.} \times Dose_{i.p.}} \times 100\% \quad (2)$$

The serum insulin concentrations were determined by enzyme immunoassay (EIA) using insulin EIA kit. The

area under the serum insulin concentration–time curve (AUC_{In}) was calculated using the linear trapezoidal rule to the last measured serum insulin concentration.

Statistical analysis

Data are expressed as the mean ± s.d. of 4 rats. For group comparisons, an analysis of variance with a one-way layout was applied, followed by Student's unpaired *t*-test. A difference between means was considered to be significant when the *P* value was less than 0.05.

Results

Effects of various protease inhibitors on the intestinal absorption of insulin

In the presence of luminal contents

Figure 1 shows the changes in serum glucose levels following administration of insulin with or without various protease inhibitors in the presence of luminal contents. No hypoglycaemic effect was observed following small intestinal administration of insulin with or without protease inhibitors. However, the profiles of serum glucose levels were decreased significantly after large intestinal co-administration of insulin with protease inhibitors.

In the absence of luminal contents

Figure 2 shows the changes in serum glucose levels following administration of insulin with or without various protease inhibitors in the absence of luminal contents. No hypoglycaemic effect was observed when insulin was administered to the small or large intestine alone. Serum glucose levels were decreased more or less when insulin was co-administered with various protease inhibitors in the small or large intestinal loops as compared with control group. However, no significant difference ($P > 0.05$) was observed following large intestinal administration of insulin with protease inhibitors in the presence or absence of luminal contents.

Effects of various protease inhibitors on D and PA of insulin in the intestine

Table 1 summarizes the decrements in serum glucose levels (D) and pharmacological availability (PA) of insulin following small or large intestinal administration with various protease inhibitors. In the presence of luminal contents, a marked decrease ($P < 0.01$) in serum glucose levels was observed after large intestinal co-administration of insulin with BAC (30 mM), BTT (29 mM), LPT (21 mM) and SGC (20, 40 mM) as compared with insulin alone, whereas a slight hypoglycaemic effect ($P < 0.05$) was observed with BAC (20 mM) and CTT (0.8 mM). In the absence of luminal contents, the hypoglycaemic effect was also observed after small intestinal co-administration of insulin with BAC (30 mM), BTT (29 mM), LPT (21 mM) and SGC (20, 40 mM). Overall, these protease inhibitors were more effective in enhancing insulin absorption from the large intestine than from the small intestine, and LPT, SGC and BAC showed the greatest enhancing effects.

Effects of different levels of BAC and SGC dose on the intestinal absorption of insulin

In the small and large intestine, the peak serum insulin levels were elevated in a BAC dose-related fashion. The AUC_{In} was linearly related to the BAC dose. In the absence of small and large luminal contents, the regression lines with BAC (10, 20, 30 mM) were $AUC_{In} = 0.533 + 0.054\ Dose_{BAC}$ ($r = 0.999$) and $AUC_{In} = 0.867 + 0.092\ Dose_{BAC}$ ($r = 0.999$), respectively. In the presence of large luminal contents, the regression line with BAC (10, 20, 30 mM) was $AUC_{In} = 0.667 + 0.087\ Dose_{BAC}$ ($r = 0.999$). A dose-dependent effect of SGC (5, 20, 40 mM) on the intestinal absorption of insulin in the absence or presence of luminal contents was not observed (data not shown).

Discussion

A significant difference in hypoglycaemic effect ($P < 0.05$) was observed following small intestinal administration of protease inhibitors in the presence or absence of luminal contents. The luminal contents contain large quantities of proteolytic enzymes. These enzymes such as trypsin, chymotrypsin and other pancreatic proteases exist mainly in microvilli brush border of small intestinal epithelial membranes, intracellular cytoplasm and small intestinal secretory liquid. They are also adsorbed within the glycocalyx, which is located external to the microvilli, and play a crucial role in terminal digestion (Tozaki et al 1998). In the washing step, the luminal surface enzymes and parts of enzymes adsorbed within the glycocalyx were washed. The absence of these enzymes was the most likely cause of good hypoglycaemic effect. Further, it was observed that the brush border membrane did possess endopeptidase activity (Bai & Amidon 1992). Therefore, we suggested that insulin was degraded not only in the internal and external mucosa of small intestine, but also in small intestinal secretory liquid. In addition, no significant difference in hypoglycaemia was observed following large intestinal administration of insulin with protease inhibitors in the presence or absence of luminal contents. It is suggested this may be attributed to the intrinsic low activity of enzymes that exist in both secretory layer and mucosal layer of the large intestine.

There was no evidence of absorption of insulin from the small intestine when insulin was co-administered with protease inhibitors in the presence of luminal contents. In the absence of luminal contents, these protease inhibitors were more effective in improving insulin absorption from the large intestine than from the small intestine. We suggested that regional differences exist (i.e. protease inhibitors may thus have site-dependent effects on intestinal absorption of insulin). This result may be explained by

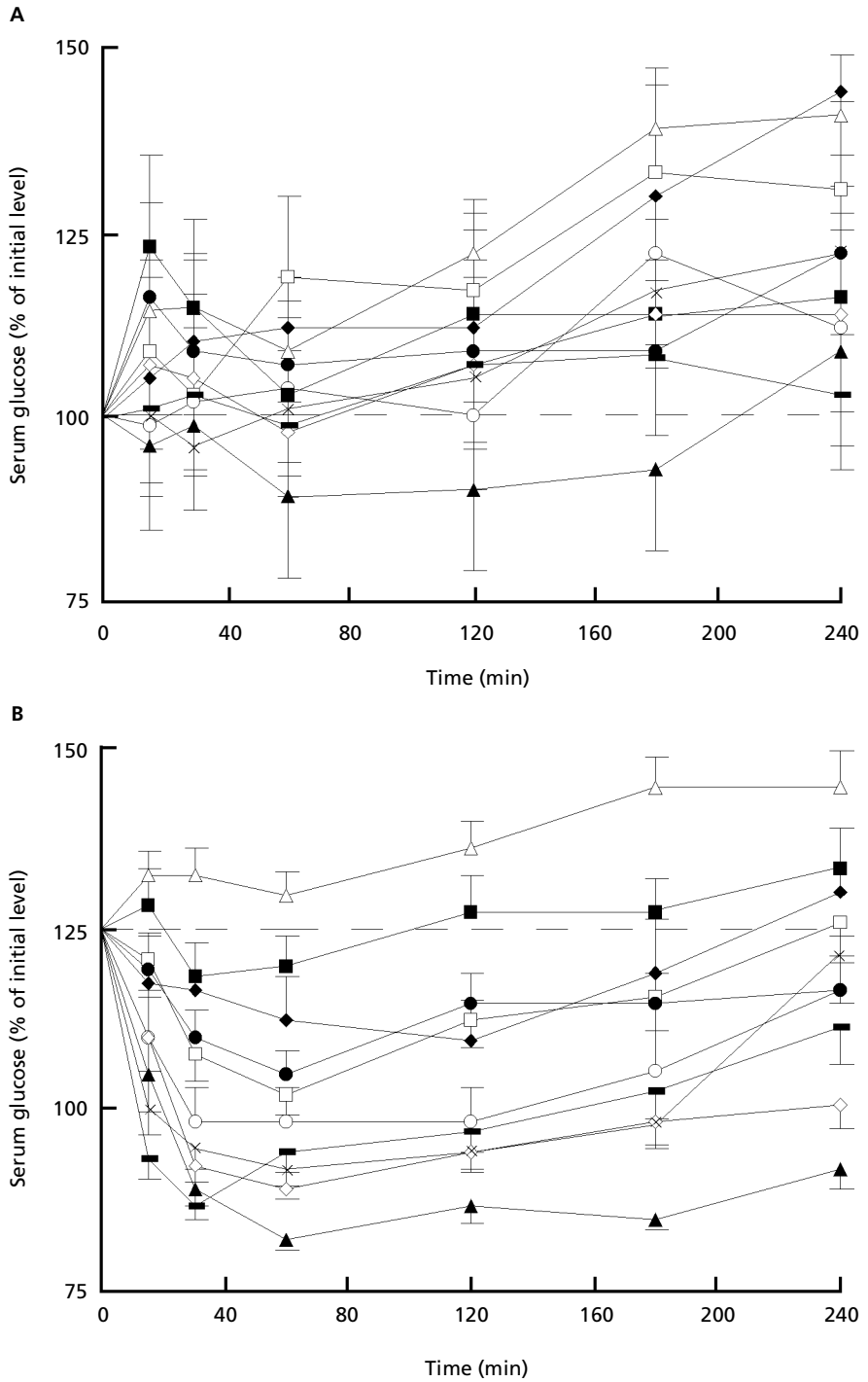

Figure 1 Serum glucose concentration–time curves after the small (A) and large (B) intestinal administration of insulin with various protease inhibitors in the presence of luminal contents. Control (insulin only) (△); 10 mM BAC (◆); 20 mM BAC (□); 30 mM BAC (×); 29 mM BTT (○); 0.8 mM CTT (●); 21 mM LPT (▲); 5 mM SGC (■); 20 mM SGC (◇); 40 mM SGC (▬). Data represent the mean ± s.d. of four experiments.

the differences in physiology and morphology of large and small intestine (Fujita & Muranishi 1998). The numbers and activity of the proteolytic enzymes responsible for the degradation of insulin in the small intestine are greater than those in the large intestine. In addition, the small intestine has longer villi and more loops, which result in a greater surface area for absorption and clearance per unit length of intestine (Bai & Chang 1995). All these morphological differences seem to be a major barrier for the absorption of peptides and proteins from the small intestine.

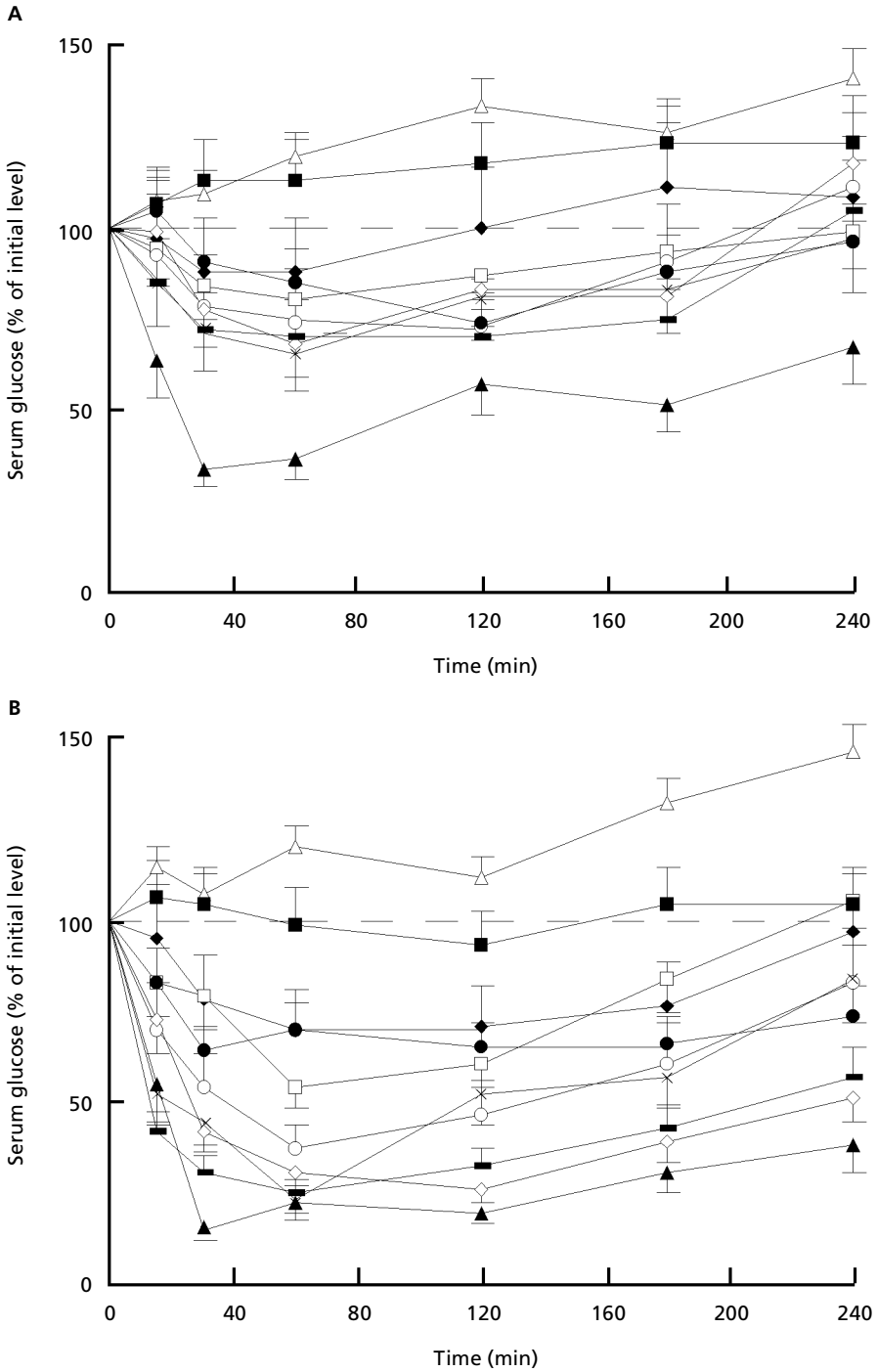

Figure 2 Serum glucose concentration–time curves after the small (A) and large (B) intestinal administration of insulin with various protease inhibitors in the absence of luminal contents. Control (insulin only) (△); 10 mM BAC (◆); 20 mM BAC (□); 30 mM BAC (×); 29 mM BTT (○); 0.8 mM CTT (●); 21 mM LPT (▲); 5 mM SGC (■); 20 mM SGC (◇); 40 mM SGC (▬). Data represent the mean ± s.d. of four experiments.

In the large intestine, a hypoglycaemic effect was observed following intestinal administration of insulin with SGC, but no dose-dependent relationship was obtained. Some reports (Scott-Moncrieff et al 1994; Dangi et al 1998) supported the mechanism involved in the hypoglycaemic effect achieved by the administration of insulin with the bile salt being as follows: reduction of the degradation of insulin; enhancement of the intrinsic permeability of insulin; decrease in the aggregation of insulin. In our study, we demonstrated that SGC under critical micelle concentration (CMC) had protease inhibitory action, but no permeation enhancing action and chemical stability action. SGC that achieved, or went beyond, CMC had all above three actions. Further, no

Table 1 Effects of various protease inhibitors on decrements in serum glucose levels (D) and pharmacological availability (PA) of insulin in the small and large intestine.

Conditions	Protease inhibitors	Dose (mM)	Small intestine		Large intestine	
			D (%)	PA (%)	D (%)	PA (%)
In the presence of luminal contents						
	Control		0.0	0.0	0.0	0.0
	BAC	10	0.0	0.0	1.7 ± 0.4	0.7
		20	0.0	0.0	2.8 ± 0.5*	1.0
		30	0.2 ± 0.2	0.0	3.8 ± 1.2**	1.4
	BTT	29	0.2 ± 0.1	0.0	3.3 ± 0.4**	1.2
	CTT	0.8	0.0	0.0	2.5 ± 0.5*	0.9
	LPT	21	1.2 ± 0.2	0.4	6.0 ± 1.4**	2.2
	SGC	5	0.0	0.0	0.6 ± 0.2	0.2
		20	0.2 ± 0.1	0.0	3.9 ± 0.8**	1.4
		40	0.3 ± 0.1	0.1	4.2 ± 1.1**	1.5
In the absence of luminal contents						
	Control		0.0	0.0	0.0	0.0
	BAC	10	1.2 ± 0.5	0.4	2.0 ± 0.3	0.7
		20	1.8 ± 0.2	0.7	3.0 ± 0.3**	1.1
		30	2.5 ± 0.2*	0.9	4.2 ± 1.5**	1.6
	BTT	29	2.2 ± 0.1*	0.8	3.8 ± 0.2**	1.4
	CTT	0.8	1.8 ± 0.4	0.7	2.7 ± 0.4*	1.0
	LPT	21	4.3 ± 1.0**	1.6	6.3 ± 1.6**	2.3
	SGC	5	0.1 ± 0.0	0.0	0.6 ± 0.3	0.2
		20	2.6 ± 0.4*	1.0	4.1 ± 0.9**	1.5
		40	2.8 ± 0.6*	1.0	4.5 ± 1.3**	1.7

Data represent the mean ± s.d. of 4 experiments. *$P < 0.05$, **$P < 0.01$, compared with the control.

significant difference in hypoglycaemic effect was found with SGC concentration beyond CMC. Thus, we suggested that, at concentrations above the CMC (= 2–5 mM), SGC interacts with epithelial membranes to form a reverse mixed micelle which can take up substances. The small micelle, which acted as a channel to increase permeation by the test solution, was inserted in the intervillous space and then diffused to the surface of intestinal cells. The substance that the reverse mixed micelle took up could become in contact with epithelial membranes and diffuse into the epithelial membranes.

Bacitracin, a cyclic polypeptide antibiotic obtained from *Bacillus licheniformis*, is known to be a leucine aminopeptidase inhibitor. It has been used to increase the absorption of peptides and proteins from various absorptive mucosae (Gotoh et al 1996; Quan et al 1999). This previous study correlated well with the present observation that the absorption of insulin from large intestine was improved by co-administration with BAC by inhibiting its degradation in the large intestine mucosa. Furthermore, the finding that serum insulin levels were linearly related to the BAC dose demonstrated that BAC had not only the proteolytic inhibitory action but also the absorption-enhancing capability, and the hypoglycaemic effect of BAC reflected both of these actions.

The reason for the enhancement of hypoglycaemic effect of insulin on co-administration with protease inhibitors correlated well with the types and quantities of protease inhibitors (Woodley 1994; Bernkop-Schnurch 1998; Yamamoto 2001). It was assumed that there exist aminopeptidase-like and trypsin-like proteases, which may play an important role in the degradation of insulin in intestine. LPT had the greatest hypoglycaemic action because it could inhibit both aminopeptidase-like and trypsin-like proteases. Both BAC and SGC had not only the proteolytic inhibitory action but also the permeation-enhancing capability (Yamamoto et al 1990; Tozaki et al 1997). It has been reported that BTT could enhance the permeability of thyrotropin-releasing hormone and insulin across rabbit trachea (Morimoto et al 2000). Therefore, our result suggested that BTT might have inhibitory effect on the activity of aminopeptidases and leucine aminopeptidases and thus increase the absorption of insulin from large intestine. CTT may inhibit the activity of trypsin-like proteases, and also prevent the active radical double sulfur bond of insulin from breaking down while retaining chemical stability action.

Conclusions

This study demonstrated that the effects of protease inhibitors on the absorption of insulin from the intestine were site-dependent. Protease inhibitors were more effective in enhancing the absorption of insulin from the large intestine than from the small intestine. Additional studies of pharmacological toxicity and irritation of these protease inhibitors are required for their clinical application because of the complicated mechanism of protease inhibitors.

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