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Improvement of intestinal absorption of human calcitonin by chemical modification with fatty acids: synergistic effects of acylation and absorption enhancers

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Abstract

To improve the intestinal absorption of human calcitonin (hCT), novel lipophilic derivatives of hCT were synthesized by chemical modification with short-chain length fatty acids such as acetic acid and caproic acid, and the intestinal absorption and stability of these derivatives were examined in rats. The intestinal absorption properties of hCT and its acyl derivatives, acetylated hCT (Ac-hCT) and caproylated hCT (Cap-hCT), were estimated by measuring its hypocalcemic effects. Their intestinal absorption and stability in the intestinal mucosal homogenates were improved by increasing the carbon number of the fatty acid. Furthermore, we investigated the effects of co-administered absorption enhancers, sodium glycocholate (Na-GC), *N*-lauryl-β-D-maltoside (LM), sodium deoxycholate (Na-DC) and sodium salicylate (Na-Sal), on the intestinal absorption of hCT and its acyl derivatives. Their intestinal absorption was slightly enhanced by the addition of various absorption enhancers except for Na-Sal. However, the extent of the promoting effect was a little higher for a native hCT rather than for acyl-hCT derivatives. These results suggested that it may be possible to achieve the further absorption enhancement of hCT by using a combination of acylation and absorption enhancers.

Keywords: Acylation; Acetylated human calcitonin; Caproylated human calcitonin; Intestinal absorption; Chemical modification; Peptide delivery

1. Introduction

A wide variety of biologically active peptides and proteins are expected as candidates as therapeutically applicable drugs. However, these peptide and protein drugs are still limited to administration by injection because, when orally administered, they are degraded by the proteolytic enzymes in the small intestinal luminal proteases such as trypsin, chymotrypsin and carboxypeptidase and brush border enzymes such as various aminopeptidases (Ginsburg and Schachman, 1960; Schilling and Mitra, 1991). In addition, they

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are impermeable across the intestinal mucosa due to their high hydrophilicity and high molecular weights (Lee and Yamamoto, 1990). Therefore, alternative administration routes have been investigated to avoid pain on injection and improve patient compliance. In our series of investigations, we improved the intestinal permeability of peptides and proteins by the application of absorption enhancers such as mixed micelles (Muranishi, 1990) and *N*-lauryl-β-maltoside (LM) (Murakami et al., 1992) and protease inhibitors (Yamamoto et al., 1994a) such as bacitracin, soybean trypsin inhibitor and aprotinin. Moreover, to improve the bioavailability of the peptide drugs such as insulin and calcitonin, we examined their absorption properties from the non-oral routes such as the pulmonary (Morita et al., 1994; Yamamoto et al., 1994b) and rectal (Yamamoto et al., 1992) routes.

Chemical modification of peptide and protein drugs is a potentially useful approach because this method can alter the various physicochemical properties of peptides and proteins, such as an increase in their molecular weights (Fujita et al., 1990, 1992) and lipophilicity (Hashimoto et al., 1989; Bundgaard and Møss, 1990; Møss et al., 1990; Tenma et al., 1993) and alter their in vivo pharmacokinetic behavior (Fujita et al., 1994; Takakura et al., 1994). Furthermore, it has been reported that chemically modified peptides can increase resistance against enzymatic degradation (Laster and Walsh, 1968; Haga et al., 1990). In order to improve the intestinal absorption of peptide drugs, we have synthesized novel lipophilic derivatives of various peptides such as thyrotropin-releasing hormone (TRH) (Yamada et al., 1992), tetragastrin (Tenma et al., 1993; Yodoya et al., 1994), and insulin (Hashimoto et al., 1989; Hashizume et al., 1992; Asada et al., 1994, 1995) by chemical modification with various fatty acids. Furthermore, we found that these acyl-peptides were more permeable across the intestine than the native peptides while retaining their biological activities (Muranishi et al., 1992). In addition, such acylated peptides were more stable than the parent peptides in plasma and the intestinal mucosae (Yamada et al., 1992; Asada et al., 1994; Yodoya et al., 1994).

Human calcitonin (hCT) which consists of 32 amino acid residues is one of the calcium-regulating peptide hormones. Calcitonin is clinically used for the treatment of increasing bone resorption in metabolic bone disorders such as postmenopausal osteoporosis (McDermott and Kidd, 1987) and Paget's disease (Muff et al., 1990), in particular for osteoporosis in older women. Therefore, there is a great clinical demand for calcitonin. In practice, calcitonin has only been administered from subcutaneous and intramuscular routes. Recently, non-oral routes, such as nasal (Hirai et al., 1981a), pulmonary (Morita et al., 1994) and vaginal (Yamamoto et al., 1992) routes, have been investigated.

In the present study, we synthesized chemically modified hCT with acetic and caproic acids and examined their intestinal absorption characteristics by an in situ loop method. In addition, in order to further improve the intestinal absorption of hCT, we also investigated the synergistic effects of acylation and absorption enhancers on its intestinal absorption.

2. Materials and methods

2.1. Chemicals

hCT and polyoxygenated (60 M) hydrogenated caster oil (HCO-60®) were kindly supplied by SUNTORY Ltd. (Osaka, Japan) and Nikko Chemical Co. (Tokyo, Japan). Acetic anhydride, caproic anhydride, and sodium salicylate (Na-Sal) were purchased from Nacalai Tesque Co. (Kyoto, Japan). Sodium deoxycholate (Na-DC), sodium glycocholate (Na-GC) and *N*-lauryl-β-D-maltoside (LM) were obtained from Sigma Chemical Company (St. Louis, MO, USA). All other chemicals and solvents were of analytical grade, available without further purification.

2.2. Synthesis of acyl-hCT derivatives

New lipophilic derivatives of hCT were synthesized by chemical attachment to ε-amino group of lysine which was the eighteenth amino group from N-terminal one with various fatty acids. In brief,

hCT (20 mg: 0.0056 mmol) was dissolved in 1 ml of *N,N*-dimethylformamide. This solution was stirred with acetic anhydride (1.13 mg: 0.011 mmol) or caproic anhydride (2.4 mg: 0.011 mmol) at room temperature for 10 min, respectively. The afforded solid materials were recrystallized from dry ether.

2.3. Determination of lipophilicity of hCT and its acyl derivatives

hCT and its acyl derivatives were analyzed by a reversed-phase HPLC (Shimadzu LC-10A system) using an ODS column (Cosmosil AR-300: 4.6 × 150 mm, Nacalai Tesque, Kyoto, Japan). The column was eluted with a linear gradient (10–80%, 40 min) in 0.1% trifluoroacetic acid at a flow rate of 1.0 ml/min. Lipophilic index values for acyl-hCT derivatives were calculated using the following equation (Asada et al., 1994; Yodoya et al., 1994):

$$\text{Lipophilic index} = Log(t_r - t_{zero})/t_{zero}$$

where t_r is the retention time of the acyl-hCT derivatives and t_{zero} is that of the solvent.

2.4. Stability of acyl-hCT derivatives in homogenates of the small and large intestinal mucosae

The rat intestinal mucosal homogenates were prepared by the modified method of Yamamoto et al. (1990), as reported previously. The protein concentrations in the small and large intestinal homogenates were determined by the method of Lowry et al. (1951) using bovine serum albumin as the standard.

The stability of acyl-hCT derivatives in the intestinal homogenate was examined by incubating 100 μl of homogenates and 100 μl of phosphate buffered saline (PBS), which had been preincubated at 37°C for 15 min, with 300 μl of 0.057 mM of acyl-hCT derivatives. At an appropriate time, 50 μl of sample were withdrawn from the incubation mixture, to which was added 100 μl of acetonitrile to terminate the reaction. The reaction mixture was immediately centrifuged for 10 min to remove precipitated proteins. One hundred microliters of the supernatant were injected into HPLC.

2.5. Preparation of test solutions

Dosing solutions with acyl-hCT derivatives were prepared in PBS containing 0.5% HCO-60 to yield a final concentration of 20 μg/ml and 50 μg/ml for the small and large intestinal administration, respectively. In certain experiments, the dosing solutions were added with absorption enhancers such as Na-GC, LM, Na-DC or Na-Sal at a final concentration of 10 mM.

2.6. Animal experiments

Male Wistar rats (180–200 g) were fasted for about 18 h before the experiment (but given water ad libitum) and anesthetized with an intraperitoneal injection of sodium pentobarbital during the experiment. Absorption experiments were performed by an in situ closed loop method (Hashida et al., 1984). An intestinal loop was prepared by cannulation with 3 cm silicon tubing (i.d. 3 mm; o.d. 5 mm) at the small and large intestine. The drug solution, warmed at 37°C, was introduced into the intestinal loop, which was closed by clipping with forceps at the cannulated position of each tubing. The administered volumes were 5 ml and 2 ml for the small and large intestines, respectively (about 500 μg/kg). Blood samples were collected from the jugular vein to obtain plasma. In some experiments, acyl-hCT derivative solution was administered intravenously at a dose of 10 μg/kg to determine the pharmacological availability in each experiment.

The intestinal absorption of an acyl-hCT derivative was estimated by measuring its hypocalcemic effect. The blood sample was separated by centrifugation at 10000 rev./min for 5 min, and the plasma (50 μl) was used for a calcium assay. The plasma calcium level was determined by an *o*-cresolphthalein complexone method using a Calcium-C test Wako (Wako Pure Chemical Industries Ltd., Osaka, Japan). A decrease in the plasma calcium concentration (A %) was calculated by the modified method of Hirai et al. (1981a), as reported previously. The pharma-

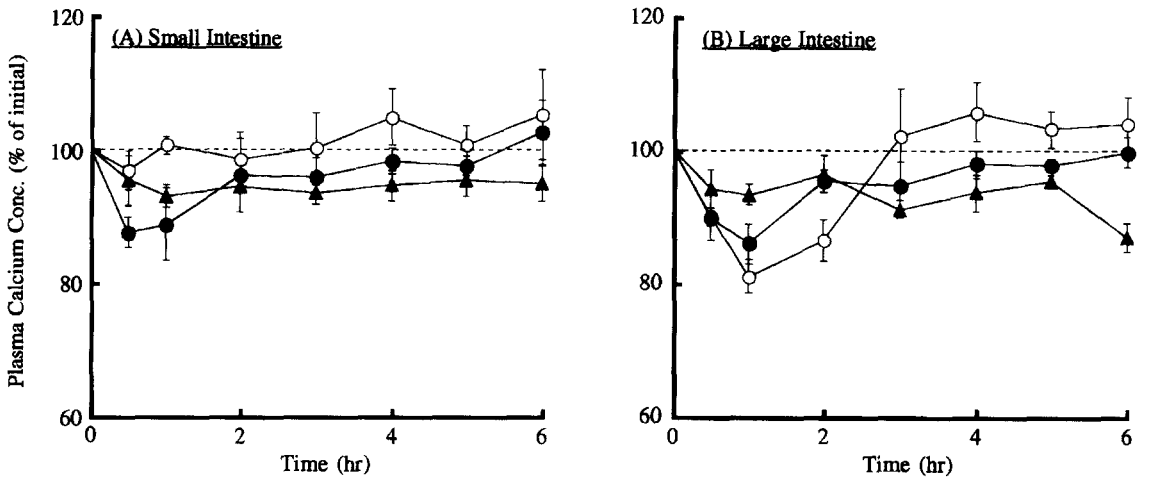

Fig. 1. Concentration-time profiles of calcium in plasma after the small and large intestinal administration of hCT and its acyl derivatives in rats. Each value represents the mean ± S.E. of three to six experiments. ○, hCT; ●, Ac-hCT; ▲, Cap-hCT.

cological availability (PA %) after the intestinal administration of acyl-hCT derivatives was calculated as follows:

$$PA\ \% = (A\ \%_{intestine}/A\ \%_{i.v.}) \times (Dose_{i.v.}/Dose_{intestine}) \times 100$$

where A $\%_{intestine}$ and A $\%_{i.v.}$ are percent of decrease in the plasma calcium concentration after the intestinal and intravenous administration, respectively.

2.7. Statistical analyses

Results were expressed as the means ± S.E. and statistical significance was assessed with Student's *t*-test.

3. Results

3.1. Chemistry and pharmacological activities of acyl-hCT

The synthetic derivatives of hCT were purified by recrystallization using dry ether. These purified compounds showed single peaks at different positions than native hCT on HPLC (data not shown), indicating that the acyl-derivatives were homogenous compounds. The lipophilic indices calculated by HPLC retention times were 0.847, 0.877 and 0.976 for hCT, Ac-hCT and Cap-hCT, respectively. The lipophilic index value increased with increasing carbon number of fatty acids attached to hCT, indicating that the acylation of hCT enhanced its lipophilicity.

The pharmacological activities of acyl-hCT derivatives were estimated by measuring their hypocalcemic effects after intravenous injection to rats. The pharmacological activities of Ac-hCT and Cap-hCT were approximately 70 and 40%, respectively. This finding indicates that the pharmacological activity of hCT was reduced with increasing the carbon numbers of fatty acids introduced to hCT.

3.2. Absorption characteristics of acyl-hCT derivatives from the small and large intestine

Fig. 1 shows the concentration-time profiles of calcium in plasma after the small and large intestinal administration of acyl-hCT derivatives in rats. Significant and continuous hypocalcemic effects were observed after small intestinal administration of Ac-hCT and Cap-hCT (Fig. 1A). However, when native hCT was administered to the small intestine, no significant decrease in plasma

calcium concentration was observed (Fig. 1A). On the other hand, in all compounds, there were significant hypocalcemic responses following large intestinal administration (Fig. 1B). Following native hCT administration, plasma calcium level was markedly and rapidly decreased and recovered to basal level up to 3 h (Fig. 1B). On the contrary, the hypocalcemic effects of acyl-derivatives after large intestinal administration were lower than that of hCT; however, their effects were continuous. Table 1 summarizes A % and PA % of acyl-hCT derivatives after intestinal administration to rats. In both regions, PA % of hCT was significantly increased by chemical modification with fatty acids.

3.3. Stability of acyl-hCT derivatives in the small and large intestinal mucosal homogenates

The degradation of acyl-hCT derivatives in the intestinal homogenates followed first-order kinetics (data not shown). Table 2 shows the half-lives for the hydrolysis of hCT and its acyl derivatives in the small and large intestinal mucosal homogenate. The half-lives were significantly prolonged by chemical modification with fatty acids in both small and large intestines, indicating that the acyl derivatives were more stable than the native hCT. The rank of order of stability among these compounds was Cap-hCT > Ac-hCT > native hCT. Furthermore, the acyl-hCT derivatives were much more stable in the large intestine than in the small intestines.

Table 1
Effects of acyl modification on the intestinal absorption of hCT in rats

		A %	PA %
Small intestine	hCT	2.36 ± 0.77	0.29 ± 0.10
	Ac-hCT	5.82 ± 1.19	3.65 ± 0.75**
	Cap-hCT	5.58 ± 1.96	4.26 ± 1.49*
Large intestine	hCT	6.43 ± 1.91	0.79 ± 0.24
	Ac-hCT	5.44 ± 0.70	3.41 ± 0.44***
	Cap-hCT	6.37 ± 1.18	4.86 ± 0.90**

PA %: Pharmacological availability % = $(A\%_{intestine})/(A\%_{i.v.}) \times (Dose_{i.v.})/(Dose_{intestine}) \times 100$.
Each value represents the mean ± S.E. (n = 3–6).
*P < 0.05; **P < 0.01; ***P < 0.001, compared with hCT.

Table 2
Half-lives for the hydrolysis of acyl-hCT derivatives in the small and large intestinal mucosal homogenates

	Half-life (min)	
	Small intestine	Large intestine
hCT	3.98 ± 0.08	22.2 ± 0.29
Ac-hCT	13.5 ± 0.13***	78.7 ± 1.27***
Cap-hCT	43.6 ± 0.42***	118 ± 2.33***

Each value represents the mean ± S.E. (n = 3).
***P < 0.001, compared with hCT.

3.4. Effect of various absorption enhancers on the intestinal absorption of acyl-hCT derivatives

We next examined the hypocalcemic effects of the acyl-hCT derivatives in the presence of absorption enhancers. Figs. 2 and 3 show concentration-time profiles of calcium in plasma after the small and large intestinal administration of the acyl-hCT derivatives in the presence of LM and Na-GC, respectively. In the absence of these additives, a slight hypocalcemic effect was obtained following intestinal administration (Fig. 1). On the other hand, in the presence of the absorption enhancers, a significant decrease in plasma calcium levels was obtained and their effects were more predominant in the large intestine than in the small intestine.

Table 3 and Table 4 summarize the effect of these absorption enhancers on the intestinal absorption of hCT and its acyl derivatives from the small and large intestine, respectively. When the absorption enhancers were co-administered with the acyl-hCT derivatives into the small and large intestine, their PA % values were increased except for Na-Sal. Therefore, it is suggested that the addition of various absorption enhancers further enhanced the intestinal absorption of the acyl-hCT derivatives. The rank order of the promoting effectiveness of these enhancers was as follows: Na-DC ≈ Na-GC > LM > Na-Sal (= no additive).

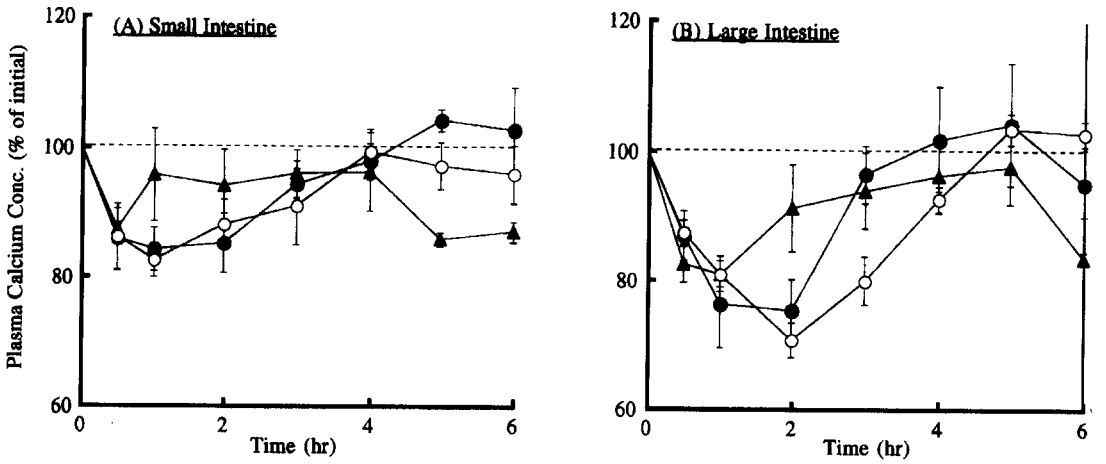

Fig. 2. Concentration-time profiles of calcium in plasma after the small and large intestinal co-administration with acyl-hCT and 10 mM LM in rats. Each value represents the mean ± S.E. of three to six experiments. ○, hCT; ●, Ac-hCT; ▲, Cap-hCT.

3.5. Effects of absorption enhancers on the relationship between PA % of acyl-hCT derivatives and their lipophilicity

Fig. 4 shows the correlation between PA % values of acyl-hCT derivatives in the presence of various absorption enhancers and their lipophilicity. In the presence of absorption enhancers, the PA % values of acyl-hCT derivatives were increased with their lipophilicity, compared with in the absence of absorption enhancer. However, the PA % values of Cap-hCT were almost similar to those of Ac-hCT. These tendencies were observed in both the small and large intestines. The promoting effects [PA % (enhancer)/PA % (no additive)] of these absorption enhancers were less pronounced for the absorption of acyl-hCT derivatives than for native hCT. Overall, the promoting effects of these absorption enhancers were more predominant in the large intestine than in the small intestine.

4. Discussion

In the present study, we synthesized chemically modified hCT with acetic acid (Ac-hCT) and caproic acid (Cap-hCT) whose biological activities were approximately 70% and 40% of native hCT, respectively, as assessed by the hypocalcemic effects after intravenous injection to rats. The reason for the reduced biological activity of hCT by acylation is not clearly understood. However, we presumed that the amphiphilic α-helix structure, which is essential for preserving the calcitonin activity (Inoue et al., 1991), might be broken and/or the affinity of hCT to the receptor, which is concerned with C-terminus (Kozono et al., 1992), might be decreased by chemical modification.

In the intestinal absorption experiment, the increase in A % was observed by acylation of hCT in the small intestine but not in the large intestine. However, in the present study, it was difficult to assess the intestinal absorption characteristics of acyl-hCT derivatives exactly using A % value because their A % values did not reflect the reduction of the intestinal absorption of these derivatives. Therefore, we used the PA %, which normalized the reduction of biological activity of hCT by acylation with A % for the intravenous administration of acyl-hCT. The acylation of hCT remarkably increased its PA % value in both the small and large intestinal administration.

To clarify the contribution to the stability of hCT by acylation, we next examined the stability of acyl-hCT derivatives in the intestinal homogenate. Peptides and proteins are generally

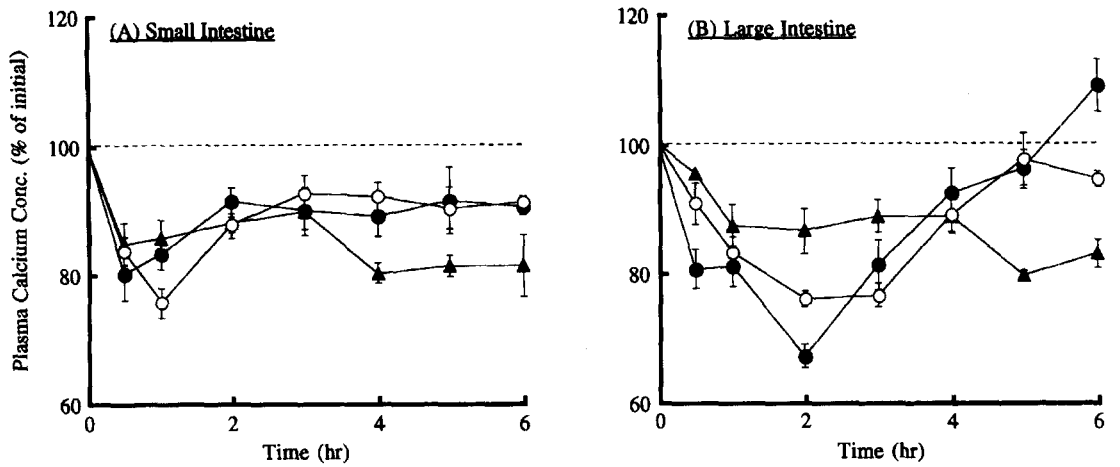

Fig. 3. Concentration-time profiles of calcium in plasma after the small and large intestinal co-administration with acyl-hCT and 10 mM Na-GC in rats. Each value represents the mean ± S.E. of three to six experiments. ○, hCT; ●, Ac-hCT; ▲, Cap-hCT.

known to be degraded in various intestinal fractions such as mucosa, brush-border membrane surface and cytosol in which proteases and other metabolic enzymes are ubiquitous (Kobayashi et al., 1994). In the stability experiment, the acyl-hCT derivatives were more stable than native hCT in the intestinal homogenate (Table 2). These findings suggested that the chemical modification of hCT with fatty acids might protect the degradation of hCT against proteolytic enzymes such as trypsin, chymotrypsin and aminopeptidases (Lee and Yamamoto, 1990). Similar effects were observed in our previous reports of thyrotropin-releasing hormone (Yamada et al., 1992), tetragastrin (Yodoya et al., 1994) and insulin (Asada et al., 1994).

In general, peptides are absorbed more from the small intestine than the large intestine because the small intestine has higher proteolytic enzyme activities as compared with the large intestine. Indeed, relatively high PA % value of hCT was observed in the large intestinal administration. On the other hand, no significant difference of PA % was observed between Ac-hCT and Cap-hCT in the small and large intestinal administration. Thus, it is suggested the chemical modification of hCT with fatty acids might improve its intestinal absorption by inhibiting the proteolytic degradation of hCT by various proteases rather than by increasing its lipophilicity.

The above finding suggested that chemical modification of hCT with fatty acids can increase its lipophilicity and improve its absorption up to about 3.5% and 4.5% for Ac-hCT and Cap-hCT, respectively, from the small and large intestine (Tables 3 and 4). However, its bioavailability was not necessarily enough. Thus, we tried to further improve the intestinal absorption of hCT with combining the acylation and various absorption enhancers. Three types of absorption enhancers, such as LM, Na-GC and Na-DC, enhanced the intestinal absorption of acyl-hCT derivatives approximately 2-fold as compared with the acylation alone. However, the enhancing ratio [PA % (enhancer)/PA % (no additive)] for hCT was slightly larger than for acyl-hCT. Kimura et al. (1971) reported that sodium cholate, one of the absorption enhancers, considerably enhanced the intestinal absorption of low lipophilic drugs. In our previous report, linoleic acid-HCO-60 mixed micelles enhanced the large intestinal absorption of native insulin, but not chemically modified insulin, with palmitic acid, a highly lipophilic derivative (Hashizume et al., 1992). Thus, the present finding was in agreement with these previous reports.

Among absorption enhancers, bile salts such as Na-DC and Na-GC significantly enhanced the intestinal absorption of acyl-hCT derivatives (Ta-

Table 3
Effects of various absorption enhancers on the small intestinal absorption of acyl-hCT derivatives in rats

	Absorption enhancers (10 mM)	A %	PA %	*P*	
				vs. hCT	vs. no additive
hCT	No additive	2.36 ± 0.77	0.29 ± 0.10	–	
	LM	9.04 ± 2.50	1.11 ± 0.31	–	< 0.05
	Na-DC	15.6 ± 2.97	1.91 ± 0.37	–	< 0.01
	Na-GC	11.5 ± 1.62	1.41 ± 0.20	–	< 0.01
	Na-Sal	3.54 ± 1.17	0.44 ± 0.14	–	n.s.
Ac-hCT	No additive	5.82 ± 1.19	3.65 ± 0.75	< 0.01	
	LM	7.47 ± 0.97	4.81 ± 0.61	< 0.01	n.s.
	Na-DC	11.4 ± 2.16	7.17 ± 1.35	< 0.01	n.s.
	Na-GC	11.0 ± 0.81	6.92 ± 0.51	< 0.001	< 0.01
	Na-Sal	3.56 ± 0.78	2.23 ± 0.49	< 0.05	n.s.
Cap-hCT	No additive	5.58 ± 1.96	4.26 ± 1.49	< 0.05	
	LM	8.40 ± 0.86	6.41 ± 0.66	< 0.001	n.s.
	Na-DC	10.2 ± 1.98	7.75 ± 1.51	< 0.05	n.s.
	Na-GC	14.9 ± 1.30	11.3 ± 0.99	< 0.001	< 0.01
	Na-Sal	4.63 ± 0.91	3.53 ± 0.69	< 0.01	n.s.

PA%: Pharmacological availability % = $(A\%_{small})/(A\%_{i.v.}) \times (Dose_{i.v.})/Dose_{small} \times 100$.
Each value represents the mean ± S.E. (n = 3–6).
n.s., not significant.

Table 4
Effect of various absorption enhancers on the large intestinal absorption of acyl-hCT derivatives in rats

	Absorption enhancers (10 mM)	A %	PA %	*P*	
				vs. hCT	vs. no additive
hCT	No additive	6.43 ± 1.91	0.79 ± 0.24	–	–
	LM	13.0 ± 1.67	1.60 ± 0.21	–	< 0.05
	Na-DC	25.9 ± 0.95	3.18 ± 0.12	–	< 0.001
	Na-GC	13.7 ± 1.32	1.69 ± 0.16		< 0.05
	Na-Sal	3.54 ± 0.64	0.44 ± 0.08		n.s.
Ac-hCT	No additive	5.44 ± 0.70	3.41 ± 0.44		< 0.001
	LM	11.4 ± 1.01	7.17 ± 0.64	< 0.001	< 0.01
	Na-DC	12.8 ± 0.52	8.03 ± 0.32	< 0.001	< 0.001
	Na-GC	14.6 ± 1.28	9.17 ± 0.80	< 0.001	< 0.001
	Na-Sal	5.39 ± 2.06	3.38 ± 1.29	n.s.	n.s.
Cap-hCT	No additive	6.37 ± 1.18	4.86 ± 0.90		< 0.01
	LM	9.51 ± 0.61	7.20 ± 0.47	< 0.001	n.s.
	Na-DC	14.3 ± 0.54	10.9 ± 0.42	< 0.001	< 0.001
	Na-GC	12.8 ± 1.61	9.79 ± 1.23	< 0.01	< 0.01
	Na-Sal	6.94 ± 1.06	5.29 ± 0.81	< 0.01	n.s.

PA %: Pharmacologicalavailability % = $(A\%_{large})/(A\%_{i.v.}) \times (Dose_{i.v.})/(Dose_{large}) \times 100$.
Each value represents the mean ± S.E. (n = 3–6).
n.s., not significant

bles 3 and 4). It is reported that bile acids have not only absorption enhancing activities (Gordon et al., 1985), but also stabilizing activities for peptides (Helenius and Simons, 1975). In particular, Na-GC was reported to inhibit leucine aminopeptidase (Hirai et al., 1981b).

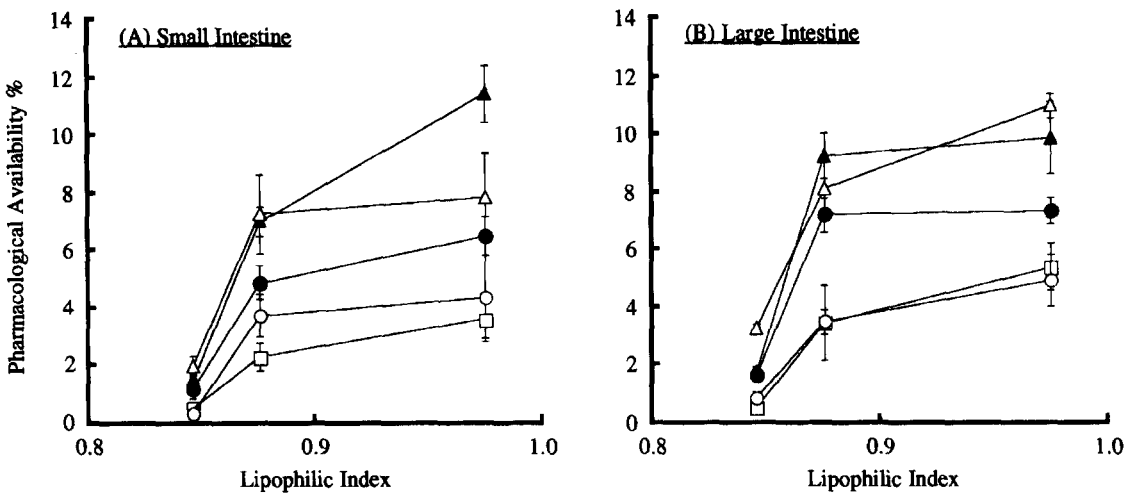

Fig. 4. Correlation between pharmacological availability of acyl-hCT derivatives after the small and large intestinal administration in the presence of 10 mM absorption enhancers and their lipophilicity. Each value represents the mean ± S.E. of three to six experiments. ○, no additive; ●, LM 10 mM; △, Na-DC 10 mM; ▲, Na-GC 10 mM; □, Na-Sal 10 mM.

Therefore, it is suggested that its inhibitory action against degradative enzymes contributes to improving the intestinal absorption of native hCT. LM, an alkylsaccharide, has recently been found to lower surface tension and to have absorption enhancing activity in the gastrointestinal tract (Murakami et al., 1992). In this study, the intestinal absorption of acyl-hCT derivatives was significantly improved by the co-administration of LM. We found that LM exhibited the absorption enhancing effect even at considerably low concentration and did not cause local irritation in the gastrointestinal tract (Murakami et al., 1992). Therefore, it is demonstrated that LM is a suitable absorption enhancer for enhancing the intestinal absorption peptide drugs. Na-Sal was reported to increase the transcellular and paracellular transport of drugs by calcium chelation at a relatively high concentration (Nishihata et al., 1983, 1986). In this study, however, no effect was observed for absorption enhancement of acyl-hCT at the tested dose (10 mM). This low concentration may be one of the main reasons by which Na-Sal did not affect the absorption enhancement of acyl-hCT derivatives.

In conclusion, we indicated that the intestinal absorption of hCT was enhanced by chemical modification with fatty acids and it was further promoted by the combination of acylation and absorption enhancers. In our preliminary study, we are studying colon-specific peptide delivery using azopolymers and chitosan capsules for improving the peptide absorption. Combining the above techniques, it will be expected to achieve further high bioavailability of peptide drugs including hCT after oral administration. The above results will be described in future reports.

References

Asada, H., Douen, T., Mizokoshi, Y., Fujita, T., Murakami, M.. Yamamoto, A. and Muranishi, S., Stability of acyl derivatives of insulin in the small intestine: relative impor-tance of insulin association characteristics in aqueous solution. *Pharm. Res.*, 11 (1994) 1115–1120.

Asada, H., Douen, T., Waki, M., Adachi, S., Fujita, T., Yamamoto, A. and Muranishi, S., Absorption characteristics of chemically modified insulin derivatives with various fatty acids. *J. Pharm. Sci.*, 85 (1995) 682–687.

Bundgaard, H. and Møss, J., Prodrug of peptide. 6. Bioreversible derivatives of thyrotropin-releasing hormone (TRH) with increased lipophilicity and resistance to cleavage by the TRH-specific serum enzyme. *Pharm. Res.*, 7 (1990) 885–892.

Fujita, T., Yasuda, Y., Takakura, Y., Hashida, M. and Sezaki, H., Alteration of biopharmaceutical properties of drugs by their conjugation with water-soluble macromolecules: Uricase-dextran conjugates. *J. Control. Release*, 11 (1990) 149–156.

Fujita, T., Nishikawa, M., Tamaki, C., Takakura, Y., Hashida, M. and Sezaki, H., Targeted delivery of human recombinant superoxide dismutase by chemical modification with mono- and polysaccharide derivatives. *J. Pharmacol. Exp. Ther.*, 263 (1992) 971–978.

Fujita, T., Nishikawa, M., Ohtsubo, Y., Ohno, J., Takakura, Y., Sezaki, H. and Hashida, M., Control of in vivo fate of albumin utilizing combined chemical modification. *J. Drug Targeting*, 2 (1994) 157–165.

Ginsburg, A. and Schachman, H.K., Studies on the enzymatic breakdown of proteins. I. Action of chymotrypsin on insulin. *J. Biol. Chem.*, 235 (1960) 108–114.

Gordon, G.S., Moses, A.C., Silver, R.D., Flier, J.S. and Carey, M.C., Nasal absorption of insulin: Enhancement by hydrophobic bile salts. *Proc. Natl. Acad. Sci. USA*, 82 (1985) 7419–7423.

Haga, M., Saito, K., Ikegaki, K., Sato, F. and Yokoo, N., Hypoglycemic effect of intestinally administered monosaccharide-modified insulin derivatives in rats. *Chem. Pharm. Bull.*, 38 (1990) 1983–1986.

Hashida, N., Murakami, M., Yoshikawa, H., Takada, K. and Muranishi, S., Intestinal absorption of carboxyfluorescein entrapped in liposomes in comparison with its lipid-surfactant mixed micelles. *J. Pharmacobio–Dyn.*, 7 (1984) 193–195.

Hashimoto, M., Takada, K., Kiso, Y. and Muranishi, S., Synthesis of palmitoyl derivatives of insulin and their biological activities. *Pharm. Res.*, 6 (1989) 171–176.

Hashizume, M., Douen, T., Murakami, M., Yamamoto, A., Takada, K. and Muranishi, S., Improvement of large intestinal absorption of insulin by chemical modification with palmitic acid in rats. *J. Pharm. Pharmacol.*, 44 (1992) 555–559.

Helenius, A. and Simons, K., Solubilization of membranes by detergents. *Biochim. Biophys. Acta*, 415 (1975) 29–79.

Hirai, S., Yashiki, T. and Mima, H., Effect of surfactants on the nasal absorption of insulin in rats. *Int. J. Pharm.*, 9 (1981a) 165–172.

Hirai, S., Yashiki, T. and Mima, H., Mechanism for the enhancement of the nasal absorption of insulin by surfactants. *Int. J. Pharm.*, 9 (1981b) 173–184.

Inoue, A., Shikano, M., Komatsu, Y., Obata, J., Ochiai, J., Nishide, H., Ito, N., Nakao, H., Kondo, K., Tunemoto, D., Hemmi, H. and Numao, N., Structure/activity relationship of eel calcitonin. *Eur. J. Biochem.*, 201 (1991) 607–614.

Kimura, T., Inui, K. and Sezaki, H., Effect of bile salts on the gastrointestinal absorption of drugs III. Effect of sodium cholate on the intestinal absorption of sulfa drugs. *Yakuzaigaku*, 31 (1971) 167–174.

Kobayashi, S., Kondo, S. and Juni, K., Study on pulmonary delivery of salmon calcitonin in rats: Effects of protease inhibitors and absorption enhancers. *Pharm. Res.*, 11 (1994)1239–1243.

Kozono, T., Hirata, M., Endo, K., Satoh, K., Takanashi, H., Miyauchi, T., Fukushima, N., Kumagai, E., Abe, S., Matsuda, E., Ohba, M., Hoshi, T., Suda, T. and Murayama, E., A chimeric analog of human and salmon calcitonin eliminates antigenicity and reduces gastrointestinal disturbances. *Endocrinology*, 131 (1992) 2885–2890.

Laster, L. and Walsh, J.H., Enzymatic degradation of C-terminal tetrapeptide amide of tertagastrin by mammalian tissue extracts. *Fed. Proc.*, 27 (1968) 1328–1330.

Lee, V.H.L. and Yamamoto, A., Penetration and enzymatic barriers to peptide and protein absorption. *Adv. Drug Delivery Rev.*, 4 (1990) 171–207.

Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J., Protein measurement with folin phenol reagent. *J. Biol. Chem.*, 193 (1951) 265–275.

McDermott, M.T. and Kidd, G.S., The role of calcitonin in the development and treatment of osteoporosis. *Endocrinol. Rev.*, 8 (1987) 377–390.

Morita, T., Yamamoto, A., Takakura, Y., Hashida, M. and Sezaki, H., Improvement of pulmonary absorption of (Asu1,7)-eel calcitonin by various protease inhibitors in rats. *Pharm. Res.*, 11 (1994) 909–913.

Møss, J., Buur, A. and Bundgaard, H., Prodrug of peptide. 8. In vitro study of intestinal metabolism and penetration of thyrotropin-releasing hormone (TRH) and its prodrug. *Int. J. Pharm.*, 66 (1990) 183–191.

Muff, R., Dambacher M.A., Perrenoud, A., Simon, C. and Fischer, J.A., Efficacy of intranasal human calcitonin in patients with Paget's disease refractory to salmon calcitonin. *Am. J. Med.*, 89 (1990) 181–184.

Murakami, M., Kusanoi, Y., Takada, K. and Muranishi, S., Assessment of enhancing ability of medium chain alkylsaccharides as new absorption enhancers in rat rectum. *Int. J. Pharm.*, 79 (1992) 159–169.

Muranishi, S., Absorption enhancers. *Crit. Rev. Ther. Drug Carrier Syst.*, 4 (1990) 171–207.

Muranishi, S., Murakami, M., Hashizume, M., Yamada, K., Tajima, S. and Kiso, Y., Trials of lipid modification of peptide hormones for intestinal delivery. *J. Controlled Release*, 19 (1992) 179–188.

Nishihata, T., Rytting, J.H., Kamada, A., Higuchi, T., Routh, M. and Caldwell, L., Enhancement of rectal absorption of insulin using salicylates in dogs. *J. Pharm. Pharmacol.*, 35 (1983) 148–151.

Nishihata, T., Ngheim, B.T., Yoshitomi, H., Lee, C.S., Dillsaver, M., Higuchi, T., Choh, R., Suzuka, T., Furuya, A. and Kamada, A., Changes in intestinal mucosal permeability caused by nonprotein thiol loss in rats. *Pharm. Res.*, 3 (1986) 345–351.

Schilling, R.J. and Mitra, A.K., Degradation of insulin by trypsin and alpha-chymotrypsin. *Pharm. Res.*, 8 (1991) 721–727.

Takakura, Y., Fujita, T., Furitsu, H., Nishikawa, M., Sezaki, H. and Hashida, M., Pharmacokinetics of succinylated

proteins and dextran sulfate in mice: implications for hepatic targeting of protein drugs by direct succinylation via scavenger receptors. *Int. J. Pharm.*, 105 (1994) 19–29.

Tenma, T., Yodoya, E., Tashima, S., Fujita, T., Murakami, M., Yamamoto, A. and Muranishi, S., Development of new lipophilic derivatives of tetragastrin: physicochemical characteristics and intestinal absorption of acyl-tetragastrin derivatives in rats. *Pharm. Res.*, 10 (1993) 1488–1492.

Yamada, K., Murakami, M., Yamamoto, A., Takada, K. and Muranishi, S., Improvement of intestinal absorption of thyrotropin releasing-hormone by chemical modification with lauric acid. *J. Pharm. Pharmacol.*, 44 (1992) 717–721.

Yamamoto, A., Hayakawa, E. and Lee, V.H.L., Insulin and proinsulin proteolysis in mucosal homogenate of the albino rabbit: Implication in peptide delivery from nonoral routes. *Life Sci.*, 47 (1990) 2465–2474.

Yamamoto, A., Hayakawa, E., Kato, Y., Nishiura, A. and Lee, V.H.L., A mechanistic study on enhancement of rectal permeability to insulin in the albino rabbit. *J. Pharmacol. Exp. Ther.*, 263 (1992) 25–31.

Yamamoto, A., Taniguchi, T., Rikyuu, K., Tsuji, T., Fujita, T., Murakami, M. and Muranishi, S., Effects of various protease inhibitors on the intestinal absorption and degradation of insulin in rats. *Pharm. Res.*, 11 (1994a) 1496–1500.

Yamamoto, A., Umemori, S. and Muranishi, S., Absorption enhancement of intrapulmonary administered insulin by various absorption enhancers and protease inhibitors. *J. Pharm. Pharmacol.*, 46 (1994b) 14–18

Yodoya, E., Uemura, K., Tashima, S., Fujita, T., Murakami, M., Yamamoto, A. and Muranishi, S., Enhancement permeability of tetragastrin across the intestinal membrane and its reduced degradation by acylation with various fatty acids in rats. *J. Pharmacol. Exp. Ther.*, 271 (1994) 1509–1513.