Improvement of the Pulmonary Absorption of (Asu[1,7])-Eel Calcitonin by Various Protease Inhibitors in Rats

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The effects of protease inhibitors, Na-glycocholate, bacitracin, bestatin, nafamostat mesilate and soybean trypsin inhibitor (STI) on the pulmonary absorption of (Asu[1,7])-eel calcitonin (ECT, molecular weight 3363) were investigated in rats. The pulmonary absorption of ECT was estimated by measuring its hypocalcemic effect. When ECT alone was administered into the lung, the pharmacological availability of ECT was 2.7%. Co-administration with STI or bestatin did not change the pharmacological effect of ECT. However, Na-glycocholate, bacitracin and nafamostat mesilate caused a significant hypocalcemic effect following the pulmonary absorption of ECT and a maximal effect was noted in the presence of 20 mM bacitracin, approaching the effect after intravenous administration of ECT. Bacitracin and Na-glycocholate reduced the degradation of ^{111}In-ECT in rat lung homogenate. Therefore, protease inhibitors effectively improved the pulmonary absorption of ECT.

KEY WORDS: pulmonary absorption; protease inhibitor; calcitonin; Na-glycocholate; bacitracin; pharmacological availability.

INTRODUCTION

Peptides and proteins, such as insulin and calcitonin, are usually given only by injection because, when taken orally, they are degraded by the proteolytic enzymes in the gastrointestinal tract or impermeable to the intestinal mucosa because of their hydrophilicity and large molecular size (1). Therefore, non-oral routes, such as nasal (2), buccal (3), rectal (4), ocular (5), vaginal (6) and pulmonary (7–10), are being investigated as alternative routes for systemic delivery of peptides and proteins. The pulmonary route is promising for delivering peptide and protein drugs, since many drugs that are poorly absorbed from the other mucosal sites, are well absorbed from the lung because of the large absorptive surface area and the short distance of the air-blood exchange pathway. In addition, drugs avoid first pass metabolism in the liver after pulmonary administration.

Peptide drugs, such as insulin (8), LH-RH (9), bovine IgG, bovine serum albumin and 1-deamino-cysteine-8-D-arginine vasopressin (10), are absorbed from the lung, although they are poorly absorbed from the gastrointestinal tract. Recently, Okumura et al. (11) reported that the pulmonary absorption of insulin was enhanced in the presence of various adjuvants. In addition, we found a significant and continuous hypoglycemic effect after insulin administration with various absorption enhancers and protease inhibitors (12).

In this study, (Asu[1,7])-eel calcitonin (ECT), a calcitonin analogue, was chosen as a model peptide and the pulmonary absorption of ECT was examined in rats. Further, we investigated the effect of various protease inhibitors on the pulmonary absorption of ECT in rats.

MATERIALS AND METHODS

Chemicals

ECT was kindly supplied from Asahi Chemical Industry Co., Ltd. (Shizuoka, Japan). Bacitracin, STI, Na-glycocholate and bestatin were purchased from Sigma Chemical Co., (St. Louis, MO, U.S.A.). Nafamostat mesilate was kindly supplied from Torii Pharmaceuticals Co., Ltd. (Tokyo, Japan). All other reagents used were of analytical grade.

Preparation of Drug Solutions

Dosing solutions containing ECT were prepared in Krebs-Ringer bicarbonate buffer (pH 7.4) to yield a final concentration of 0.1 – 50 μg/100 μl. In the case of nafamostat mesilate, the drug solution was prepared in HEPES buffer (pH 7.4) to yield the same concentration as in the case of other protease inhibitors, since nafamostat mesilate was hardly dissolved in the Krebs-Ringer bicarbonate buffer. For an in vitro study, ECT was labeled with ^{111}In using a bifunctional chelating agent, diethylenetriaminepentaacetic acid anhydride (Dojindo Labs, Kumamoto, Japan) according to the method of Hnatowich et al (13).

Pulmonary Absorption of ECT

To investigate the pulmonary absorption of ECT, male Wistar rats, weighing 230 to 250 g, were anesthetized by means of an intraperitoneal injection of sodium pentobarbital (30 mg/kg) and prepared surgically according to the method of Enna and Schanker (14). Briefly, the trachea was exposed through a ventral midline incision in the neck, and a 2.5 cm length of polyethylene tubing (i.d. 1.2 mm, o.d. 2 mm) was inserted through an incision between the fourth and fifth tracheal rings caudal to the thyroid cartilage to a depth of 0.6 cm. Fifty μl of the drug solution was administered intratracheally through a tube inserted to the trachea by a 250 μl glass syringe. After administration of the drug solution, 150 μl of blood sample was periodically collected from the cannula inserted into the carotid artery for up to 5 hrs. In another experiment, various amounts of ECT were injected intravenously into rats, and the blood sample was similarly collected.

For the determination of the remaining percentage of ^{111}In-labeled ECT (^{111}In-ECT) in the lung, the rats were sacrificed with an intravenous overdose injection of Na-pentobarbital solution and the lung was excised. One or two hours after the administration of ^{111}In-ECT, the lung, to-

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gether with the heart, esophagus, trachea and attached cannula, were removed from the body. After removing the blood by circulating a Krebs-Ringer bicarbonate buffer solution (pH 7.4) from the pulmonary artery, the lung and trachea were isolated, weighed, and prepared for determining the remaining radioactivity in the pulmonary tissue. Then, the lung was homogenized in a Krebs-Ringer bicarbonate buffer using a glass homogenizer, and centrifuged at 3000 rpm for 10 min. The supernatant was then applied to Sephadex G-25 column (Pharmacia, LKB, Sweden) for fractionation.

Stability of ECT in the Rat Lung Homogenate

The lung was isolated from the rat after removing blood, and weighed, the 10% homogenate was prepared using a glass homogenizer under ice cold condition. The homogenate was, then, centrifuged at 3000 rpm for 10 min at 4°C, and the supernatant was used for the stability experiment. ^{111}In-ECT was added to the supernatant of the homogenate in the absence or presence of various protease inhibitors and incubated for 2 hrs at 37°C. After filtering the incubation mixture by a Millex filter (0.45 μm, Millipore Co. Ltd., Japan), the filtrate sample was applied to Sephadex G-25 column (Pharmacia, LKB, Sweden) for a gel filtration. The percentage of intact ^{111}In-ECT was calculated by extrapolating the slope of the first peak of the gel filtration and the percentage of degradation products was obtained by subtracting the percentage of intact drug from the total amount.

Analytical Methods

The absorption of ECT was estimated by measuring its hypocalcemic effect. Shortly, the blood sample was separated by centrifugation at 5000 rpm for 5 min, and the plasma (50 μl) was collected and 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 Chemicals Industries LTD., Osaka, Japan). The ^{111}In radioactivity was determined in a gamma scintillation counter (ARC-500, Aloka, Japan).

RESULTS

Effect of Dose on the Pulmonary Absorption of ECT

Figure 1 shows the effect of ECT dose on the concentration-time profiles of calcium in plasma after intrapulmonary administration of ECT in rats. ECT was administered at various doses from 0.1 to 50 μg/rat. The plasma calcium levels decreased, as the dose of ECT increased. The relationship between dose and pharmacological effects (the decrease in plasma calcium levels) of ECT after intravenous and intrapulmonary administration was plotted in Fig. 2. The pharmacological effects were evaluated by the maximal depletion of plasma calcium level. We found sigmoidal dose-response curves following both intravenous and intrapulmonary administration of ECT, although the pharmacological response in the latter case was much less than that in the former case.

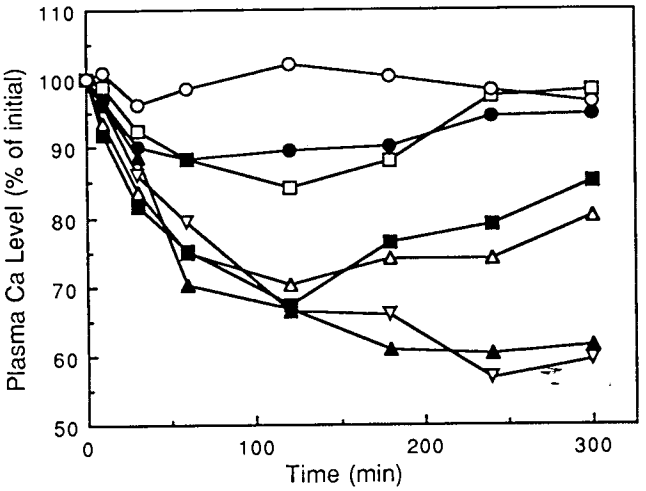

Fig. 1. Dose dependency of changes in plasma calcium levels after pulmonary administration of (Asu^{1,7})-eel calcitonin in rats. Key: (○) 0 μg/rat, (●) 0.1 μg/rat, (□) 0.5 μg/rat, (■) 1 μg/rat, (△) 2 μg/rat, (▲) 20 μg/rat, (▽) 50 μg/rat. Each point represents the mean of 3 or 4 rats.

The experimental data were fitted to the following Hill's equation

$$E = E_{max} * Dose^n/(ED_{50}^n + Dose^n) \quad (1)$$

where E and n represents the maximal depletion of calcium level (ΔCa%) and the shape factor of the sigmoidal dose-response curve. E_{max} and ED_{50} indicate the maximal effect and 50% effective dose, respectively. The values of these parameters were calculated by fitting these data to the equation (1) and were summarized in Table I. ED_{50} values for the intravenous and intrapulmonary administration of ECT were 0.015 and 0.56 μg/rat, respectively. The pharmacological availability of ECT following intrapulmonary administration was 2.7%, as compared with the case of intravenous administration.

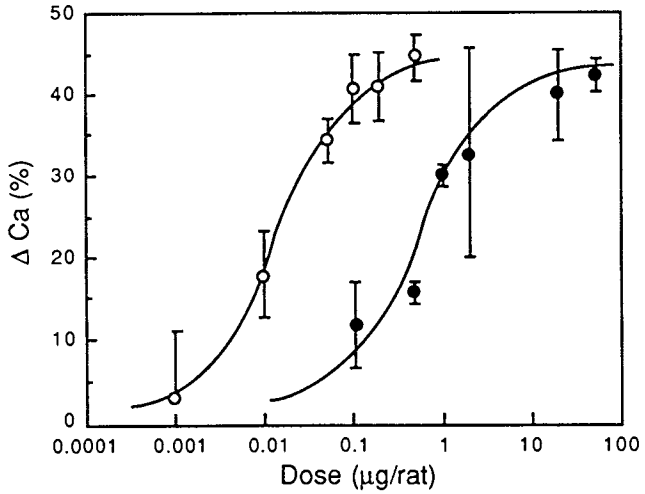

Fig. 2. Relationship between dose and pharmacological effect of (Asu^{1,7})eel-calcitonin after intravenous and pulmonary administration in rats. Key: (○) intravenous administration, (●) pulmonary administration. Each point represents the mean ± S.D. of 3 or 4 rats.

Table I. Pharmacodynamic Parameters Following Intravenous or Intrapulmonary Administration of [Asu[1,7]]-Eel Calcitonin

Parameters[a]	Administration route	
	intravenous route	intrapulmonary route
E_{max} (%)	46	44
ED_{50} (μg/rat)	0.015	0.56
n	1.0	0.75

a. Each parameter was calculated from the Hill's equation (1).

Effect of Various Protease Inhibitors on the Pulmonary Absorption of ECT

Table II summarizes minimum calcium levels, time to minimum calcium levels and pharmacological availability of ECT after intrapulmonary administration of ECT in the presence of various protease inhibitors. When Na-glycocholate, bacitracin and nafamostat mesilate were coadministered with ECT from the lung, the pharmacological availability of ECT reached to 8.3–100% of its intravenous administration. Especially, the coadministration of 20 mM bacitracin reached the same level as its intravenous administration. However, no significant effect was noted in the pharmacological availability of ECT in the presence of STI or bestatin.

The Stability of ECT in the Rat Lung Homogenate

Figure 3 shows the degradation of ^{111}In-ECT after incubation in the supernatant of the rat lung homogenate. ^{111}In-ECT was incubated for 0.5 to 5 hrs in the supernatant of the rat lung homogenate (pH 7.4) at 37°C. As the incubation time increased, a fraction of intact ^{111}In-ECT (first peak) decreased, whereas a low molecular weight fraction (second peak), degradation products of ECT, inversely increased after the incubation. At low temperature (4°C), we found a small amount of degradation products of ECT (data not shown). From these findings, it was suggested that ECT may be degraded by proteolytic enzymes in the rat lung homogenate. The degradation rate constant of ^{111}In-ECT was 0.55 hr^{-1}.

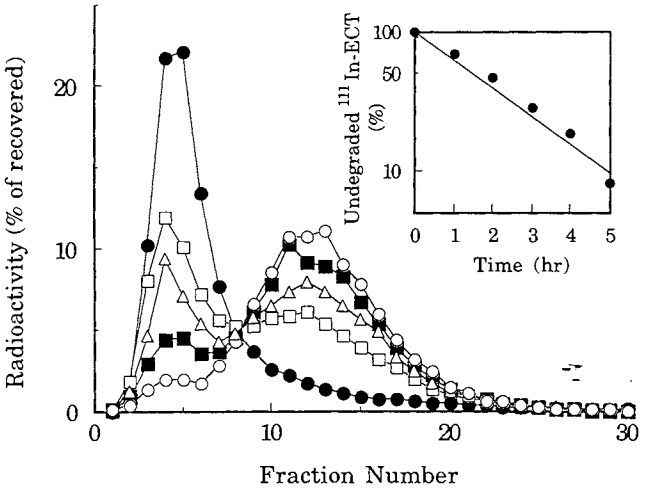

Fig. 3. The degradation of ^{111}In-(Asu[1,7])-eel calcitonin after incubation in the supernatant of the rat lung homogenate at 37°C (pH 7.4). Key: (●) in pH 7.4 Krebs-Ringer bicarbonate buffer solution (0 hr), (□) 0.5 hr, (△) 1 hr, (■) 2 hr, (○) 5 hr.

Table III summarizes the effect of these protease inhibitors on the degradation of ^{111}In-ECT in the supernatant of the rat lung homogenate at 37°C (pH 7.4). A remarkable increase in the undegraded ^{111}In-ECT was observed in the presence of 2 mM of Na-glycocholate and 0.2 or 2 mM of bacitracin. The inhibitory percentages of ECT degradation were 28% for 2 mM of Na-glycocholate, 30% or 78% for 0.2 or 2 mM of bacitracin, respectively. However, 0.2 mM of Na-glycocholate and bestatin (0.02 mM or 0.2 mM) did not inhibit the degradation of ^{111}In-ECT in the rat lung homogenate at 37°C (pH 7.4).

Table II. Effects of Protease Inhibitors on Decrease in Plasma Calcium Levels after Pulmonary Administration of (Asu[1,7])—Eel Calcitonin (0.1 μg/rat)

Additives	Concentration (mM)	n	Minimum[a] Ca Levels (% of initial)	T minimum[b] (min)	Pharmacological[a,c] Availability (%)	p value[d]
None		3	88 ± 5	60	5.4 ± 2.4	
Glycocholate	2	4	82 ± 7	60	9.3 ± 3.7	NS[e]
	20	4	76 ± 6	120	17 ± 4	$p < 0.01$
Bacitracin	2	3	76 ± 4	120	17 ± 3	$p < 0.01$
	20	4	58 ± 2	240	100	$p < 0.001$
Nafamostat	2	4	84 ± 3	60	8.3 ± 1.5	NS[e]
	20	4	74 ± 6	120	21 ± 5	$p < 0.01$
Bestatin	0.2	4	86 ± 10	30	6.8 ± 4.9	NS[e]
	2	4	91 ± 3	60	3.6 ± 1.2	NS[e]
STI	0.2	4	83 ± 9	60	8.9 ± 4.9	NS[e]
	2	4	86 ± 4	30	6.6 ± 2.0	NS[e]

a. Mean ± S.D.
b. Time showing minimum plasma Ca levels.
c. These values were calculated from the dose and pharmacological effect relationship at a dose of 1 μg/rat.
d. Statistical analyses were carried out for bioavailability using Student's T-test.
e. Not significant.

Table III. Inhibitory Effects of Protease Inhibitors on Degradation of ^{111}In-ECT in the Supernatant of Rat Lung Homogenate at 37°C (pH7.4)

Additives	Concentration (mM)	Undegraded ^{111}In-ECT (%)	Inhibitory Effect (%)
None	—	41	—
Glycocholate	0.2	43	4.0
	2	57	28
Bacitracin	0.2	59	30
	2	87	78
Bestatin	0.02	41	1.0
	0.2	39	−2.3

The Degradation and Absorption of ^{111}In-ECT in the Pulmonary Absorption Studies

Figure 4 represents the disappearance of ^{111}In-ECT from the lung and its degradation after pulmonary administration in the absence or presence of various protease inhibitors in rats. In the absence of protease inhibitors, the radioactivity rapidly disappeared from the lung, and only 62% of total radioactivity was remaining after 2 hrs. However, most of the radioactivity remaining in the lung was in a degraded fraction and the percentage of intact ^{111}In-ECT was less than 1% after 2 hr. This indicates that almost all ECT was degraded in the lung before reaching the systemic circulation and little intact ECT was absorbed from the lung. In the presence of 20 mM of Na-glycocholate or bacitracin, the disappearance percentage of ^{111}In-ECT from the lung increased. There was a significant increase in the percentages of undegraded ^{111}In-ECT in the lung and the percentages of undegraded drug were higher than its degraded percentages when coadministered with 20 mM of Na-glycocholate or bacitracin. This result suggests that more than 50% of ^{111}In-ECT which disappeared from the lung was absorbed in an intact form in the presence of these protease inhibitors, especially 20 mM of bacitracin. However, STI did not increase the undegraded percentage of ^{111}In-ECT remaining in the lung, suggesting that ECT was metabolized in the lung and converted to the degradation products even in the presence of STI.

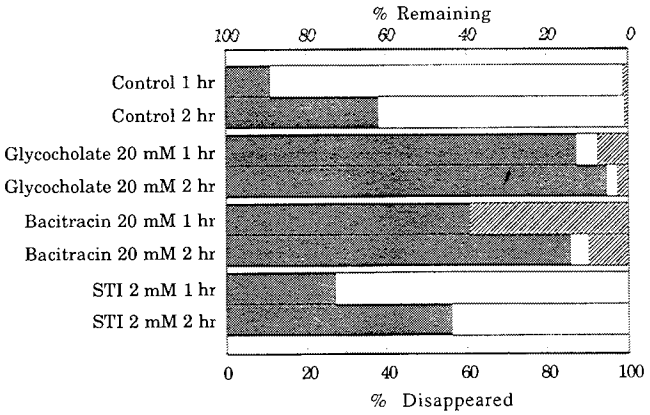

Fig. 4. Disappearance and degradation of ^{111}In-(Asu^{1,7})-eel calcitonin (ECT) after pulmonary administration in the presence of various protease inhibitors in rats. Key: (▩) ^{111}In-ECT disappeared from the lung (□) degradation products of ^{111}In-ECT remaining in the lung, (▨) undegraded ^{111}In-ECT remaining in the lung.

DISCUSSION

Fluorescein isothiocyanate labeled dextrans (FDs) with various molecular weights and insulin were previously shown to be absorbed from the lung, although they are poorly absorbed from the gastrointestinal tract (12,15,16). These results suggest that the lung is suitable for delivering macromolecular compounds to the systemic circulation.

In the present study, a small amount of ECT was absorbed from the lung even in the absence of protease inhibitor, and the pharmacological availability of ECT from the lung was 2.7%. Compared with the pulmonary route, Morimoto et al reported that the biavailability of ECT from the rectum was 0.8% (17). In addition, Ogiso et al. reported that ECT did not penetrate across the skin in the absence of additives such as Na-taurocholate and n-octyl-β-D-thioglucoside (OTC), while the bioavailability in their presence was about 4.6% (18). Therefore, without various additives, the bioavailability of ECT from the lung is higher than in the rectum and the skin.

We previously reported that the pulmonary absorption of FDs depends on their molecular weights, and that FD with an approximate molecular weight of 4000 (FD-4) was absorbed by 10% over 2 hrs (15). Further, Ohtani et al. also found that the % absorption of FD-4 from the lung reached 55% over 6.5 hrs (16). In comparison, the pulmonary absorption of ECT was lower, although the molecular weight of ECT is similar to that of FD-4. This result may be attributed to the enzymatic hydrolysis of ECT in rat lung, which was inhibited by protease inhibitors, while FD-4 is generally known to be stable.

Our present result indicated that the coadministration of Na-glycocholate, bacitracin and nafamostat mesilate with ECT was effective for improving its pulmonary absorption (Table II). Insulin absorption from the lung was also improved by the coadministration of Na-glycocholate (11,12). On the other hand, it was reported that bile salts inhibited aminopeptidase activity (2), and Na-glycocholate was effective for reducing the proteolysis of insulin and proinsulin in the various absorptive mucosae (19). Similarly, the degradation products of ^{111}In-ECT were reduced when coadministered with 20 mM Na-glycocholate. Therefore, Na-glycocholate not only increased the pulmonary absorption of ECT by its absorption-enhancing action but also inhibited the activity of proteolytic enzymes, thereby reducing the degradation of ECT in the lung.

Bacitracin has been used to inhibit degradation of various peptides and proteins and increase their absorption from various absorptive mucosae (20,21), such as the pulmonary absorption of insulin in rats (12). Similarly, bacitracin increased the nasal absorption of gonadorelin and buserelin after nasal administration of these peptides in rats (21). Unexpectedly, the pulmonary absorption of undegraded ^{111}In-ECT was increased in the presence of bacitracin as well as Na-glycocholate (Fig. 4). This finding suggests that bacitracin may also have an absorption enhancing activity, although the mechanisms for the enhancing action of bacitracin is unclear.

Nafamostat mesilate strongly inhibits a variety of proteases such as trypsin, plasmin and kallikrein (22,23). Therefore, our present result suggests that nafamostat mesilate inhibits proteolytic enzymes that degrades ECT and may increase the pulmonary absorption of ECT.

The reason why STI and bestatin did not affect pulmonary ECT absorption is unclear. Their concentrations may have been too low to inhibit ECT degradation, since trypsin activity was reduced by nafamostat and STI and the aminopeptidase activity was inhibited in the presence of Na-glycocholate, bacitracin and bestatin.

We have not examined the type of proteolytic enzymes responsible for the hydrolysis of ECT in the lung. Aminopeptidase-like and trypsin-like proteases may play a role in the degradation of ECT in lung homogenate, because both bacitracin, a potent aminopeptidase inhibitor, and nafamostat mesilate, a trypsin inhibitor decreased ECT proteolysis, thereby increasing the pulmonary absorption of ECT.

In conclusion, a small amount of ECT administered intratracheally was absorbed from the lung, although ECT was mostly degraded in the lung tissues. Further, some protease inhibitors such as Na-glycocholate, bacitracin and nafamostat mesilate were useful for improving the ECT absorption from the lung by inhibiting its degradation in the lung.

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