Study on Pulmonary Delivery of Salmon Calcitonin in Rats: Effects of Protease Inhibitors and Absorption Enhancers

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Effects of protease inhibitors and absorption enhancers on the absorption of salmon calcitonin (sCT) were evaluated after intratracheal coadministration to rats using the plasma Ca level as an index. Remarkable absorption enhancement could be attained with unsaturated fatty acids such as oleic acid and poloxamethylene oleyl ether (absorption enhancers) and with chymostatin, bacitracin, potato carboxypeptidase inhibitor and phosphoramidon (protease inhibitors). sCT degrading enzymes had four times higher activity per total protein in membrane fraction of lung homogenates than the activity in cytosol fraction. These enzymes are thought to be serine proteases and metalloenzymes from the in vitro action profile of protease inhibitors. A good correlation between the in vitro activity of protease inhibitors and the in vitro enhancing effect of sCT activity suggested that membrane enzymes are responsible for the inactivation of sCT. Metabolic degradation and low permeability of sCT may be possible barriers to the absorption of sCT.

KEY WORDS: salmon calcitonin; rat alveolar absorption; protease inhibitor; absorption enhancer.

INTRODUCTION

The pulmonary route has attracted attention in addition to nasal, dermal and rectal routes for its potential for noninvasive systemic administration of peptide and protein drugs (1,2). The present study was undertaken to evaluate the effect of protease inhibitors and absorption enhancers on the absorption of salmon calcitonin (sCT) in rats after intratracheal coadministration. The possible absorption barrier and enzymatic barrier of the alveolar epithelial mucosa are also discussed.

MATERIALS AND METHODS

Reagents

Synthetic sCT and protease inhibitors [chymostatin, potato carboxypeptidase inhibitor (pCPH), phosphoramidon, antipain, leupeptin, bestatin, foroxymithin, amastatin, pepstatin, Tos-Lys-chloromethylketone (TLCK), Tos-Phe-chloromethylketone (TPCK), 3,4-dichloroisocumarin (3,4-DCI), trans-epoxysuccinyl-leucylamido(4-guanido)butane (E-64)] were purchased from Sigma Chemical Co. Other protease inhibitors used were D-Tyr-Pro-Arg-chloromethylketone (CK) purchased from Bachem Co., (p-amidinophenyl)methanesulfonyl fluoride (p-APMSF), soy bean trypsin inhibitor (STI), disopropyl fluorophosphate (DFP), bacitracin and benzamidine from Wako Pure Chemical, and aprotinin from Behringwerke AG. Fatty acids and surfactants were purchased from Nihon Oil & Fats, which had been synthesized from highly pure oleic acid (purity: ≥95%).

As dilution buffers for reagents, phosphate buffered saline (PBS) and 0.1M Tris-HCl buffer (pH 7.5) were used for animal and in vitro studies, respectively. sCT was dissolved in purified water to prepare a high conc. solution, which was diluted with the dilution buffers containing the below mentioned absorption enhancers/protease inhibitors. Pepsin and chymostatin were dissolved in 0.1N HCl; TPCK and 3,4-DCI, in dimethyl sulfoxide (DMSO); and other protease inhibitors, in purified water all at high concentrations. These high conc. solutions were diluted with the dilution buffer. The final concentration of DMSO in the drug solution was ≤1%. As absorption enhancers, surfactants and fatty acids were also dissolved or suspended in the dilution buffer, followed by 2-min sonication. Exceptionally, palmitic acid was melted at 60°C before the addition of an adequate amount of the buffer. Palmitic acid suspension was obtained by 2-min sonication at 37°C. All fatty acid/surfactant solutions or suspensions were prepared just before use.

Animal Studies

Male Sprague-Dawley rats, 7–9 weeks of age and weighing 200–300 g, were fasted for 18–20 hr prior to the experiment. Intratracheal (i.t.) administration was done according to the method of Enna and Schanker (3). The rats were anesthetized with urethane and the trachea was exposed. A polyethylene tube was inserted through a tracheal incision. The drug solution was instilled through the polyethylene tube with a 100-μl microsyringe (100 μl/300 g body weight). The animals were fixed in a supine position on a plate kept at 37°C. In the intramuscular (i.m.) administration group, the same volume of the drug solution was injected into the thigh muscle. The control animals received PBS solution without sCT.

Determination of sCT Biological Activity

Blood (100 μl) was collected from the tail vein using a heparinized capillary tube at designated intervals for 5 hr after the administration and centrifuged at 1000 xg to separate plasma. Plasma Ca levels were measured with Calcium Test Wako C (Wako Pure Chemical). The Ca reduction curve was drawn with reduction rates of plasma Ca levels at 15 min, 30 min, 1 hr, 2 hr, 3 hr, 4 hr and 5 hr postadministration against the plasma Ca level just before the administration. The area between the plasma Ca level versus time curves of the sCT administered groups and that of the control (PBS) group for 0–5 hr was calculated by the trapezoidal method. The obtained value (Area of Ca Reduction: ACR) was used as an index of sCT biological activity. Each dose group con-
sisted of 4 to 6 rats. Student’s t-test was used for the statistical analysis.

Degradation of sCT in Lung Homogenate

The lungs of 6 rats were pooled, washed with cool saline solution, and homogenized with a 4-fold volume of 0.1M Tris-HCl (pH 7.4) containing 0.2M sucrose per gram of the tissue weight with ice cooling. The homogenate was centrifuged at 4°C, 4500 xg for 30 min. The supernatant was further centrifuged at 50,000 xg for 30 min, and the resultant supernatant was used as the cytosol fraction. The precipitate was washed with the same buffer twice by centrifugation in a similar manner, and the resultant precipitate was used as the membrane fraction.

The substrate solution was prepared with 0.1M Tris-HCl buffer (pH 7.4) to a final sCT concentration of 50 µM. To 100 µl of the substrate solution, 25 µl of purified water or an enzyme inhibitor/absorption enhancer solution/suspension and 25 µl of enzyme solution were added in this order. The mixture was allowed to react at 37°C for 30 min.

The reaction was stopped with 10 µl of 10% trifluoroacetic acid. After centrifugation at 7000 xg for 3 min to remove proteins, the residual sCT concentration in the supernatant was determined by HPLC to calculate the enzyme activity. HPLC conditions were: column, Vydac Protein & Peptide 0.4 x 15 cm; measurement wavelength, at 215 nm; mobile phase, 32% acetonitrile solution containing 0.1% trifluoroacetic acid; and flow rate, 1.7 ml/min. Under these conditions, an sCT peak appeared at a retention time of about 7 min. The protein content of the enzyme solution was determined with a Protein-Assay Kit (Bio-Rad).

RESULTS

Intramuscular Administration

Fig. 1 shows the reduction rate of plasma Ca levels after i.m. injection of sCT. At 0.2 and 0.4 mg/kg, a maximum reduction rate of 10–15% was observed at 1 hr postadministration, and then the plasma Ca level increased gradually to reach a normal level at 5 hr. At 1, 2, 4, and 8 µg/kg, the Ca level decreased until 5 hr postadministration in a similar fashion for each dose showing the saturation of Ca reduction effect. The control group showed no change in the plasma Ca level.

Intratracheal Administration

Fig. 2 shows the reduction rate of plasma Ca levels after i.t. administration of sCT. Plasma Ca levels decreased dose-dependently. At 4 and 8 µg/kg, a similar reduction pattern to that after i.m. injection of 1 µg/kg or higher was observed. No change of the plasma Ca level was seen at 0.2 µg/kg and in the control group.

Comparison of sCT Activity Between Intramuscular and Intratracheal Administration

In Fig. 3, the ACR is plotted against each dose level. In the i.m. injection group, a linear relationship was observed between ACR and the logarithm of doses at 0.2, 0.4, 1 and 2 µg/kg. In the i.t. administration group, a linear relationship was obtained at 0.4, 1, 2 and 4 µg/kg. The relative i.t. bio-

Fig. 1. Efficacy of salmon calcitonin after intramuscular administration in rats. Doses were given at 0.2 µg/kg (△), 0.4 µg/kg (○), 1 µg/kg (●), 2 µg/kg (□), 4 µg/kg (×) and 8 µg/kg (▲). Each point represents the mean ± S.E. of plasma Ca reduction (%) (n = 6).

Fig. 2. Efficacy of salmon calcitonin after intratracheal administration in rats. Doses were given at 0.2 µg/kg (△), 0.4 µg/kg (○), 1 µg/kg (●), 2 µg/kg (□), 4 µg/kg (×) and 8 µg/kg (▲). Each point represents the mean ± S.E. (n = 6).

availability (BA) against i.m. administration was calculated to be 30%.

Enhancing Effect of Fatty Acid and Surfactant on sCT Absorption in Intratracheal Administration

Free unsaturated fatty acids of oleic acid, palmitoleic acid and linoleic acid exhibited very strong enhancing effect (Fig. 4). Sodium olate was also effective to the same degree as oleic acid. POE oleyl ether showed very excellent enhancing activity. In contrast, sorbitan trioleate, POE sorbitan monooleate and POE sorbitan trioleate exhibited moderate enhancing effect, and the effect of glycerol trioleate, ethyl olate, oleyl alcohol, palmitic acid and stearic acid was poor.

Enhancing Effect of Protease Inhibitor on sCT Absorption in Intratracheal Administration

As shown in Fig. 5, bacitracin, chymostatin, pCPI and phosphoramidon exhibited remarkable enhancing effect. Antipain, leupeptin, DFP and TLCK promoted Ca reduction to