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Distribution and Metabolism of [Asu^{1,7}]-Eel Calcitonin in Isolated Perfused Rat Pancreas

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The distribution and metabolism of elcatonin ([Asu^{1,7}]-eel calcitonin) were studied in isolated perfused rat pancreas. During the perfusion of ¹²⁵I-elcatonin, the radioactivity in the effluent rapidly attained equilibrium with the perfusate, although a considerable quantity of degradation products was observed in the effluent. The distribution space of ¹²⁵I-elcatonin was considered to be limited to the extracellular space. When unlabeled elcatonin was perfused, immunoreactive elcatonin concentration in the effluent also reached a rapid equilibrium at a lower level than that in the perfusate. These results suggest that the pancreas can degrade the entering elcatonin fairly well. The inclusion of insulin or aprotinin in the perfusate significantly suppressed elcatonin metabolism by the pancreas. Although a study of elcatonin inactivation by several proteases did not clarify the inhibitory mechanism of insulin, serine proteases were considered to be involved in the inactivation of elcatonin by the pancreas.

Keywords—elcatonin; calcitonin; aprotinin; insulin; drug metabolism; isolated perfused rat pancreas; gel filtration; radioimmunoassay

The pharmacological action of drugs is closely related to their metabolic fate in certain target tissues. Calcitonin, a basic peptide hormone composed of 32 amino acids, has been demonstrated to inhibit exocrine pancreatic secretion, as well as gastric acid and gastrin secretion.¹⁾ These actions indicate that calcitonin may be an effective drug for the treatment of acute pancreatitis.²⁾ However, the disposition of calcitonin in the pancreas has not been elucidated.

In our previous paper,³⁾ we reported the distribution and metabolism of secretin and aprotinin in the isolated perfused rat pancreas, and demonstrated the importance of the molecular structure of peptides with regard to their metabolic fate in the target tissues. Elcatonin ([Asu^{1,7}]-eel calcitonin), a stable analog of eel calcitonin, contains a cyclic structure in the molecule involving an ethylene linkage of aminosuberic acid, instead of a disulfide bond in the natural peptide between the N-terminal 1 and 7 positions.⁴⁾ In the present study, by using an organ perfusion technique, we have investigated the distribution and metabolism in the pancreas of elcatonin as a model peptide of calcitonin, and compared the findings with our previous results on secretin, a typical unstable peptide with a linear structure, and aprotinin, a stable peptide with a globular structure maintained by disulfide bonds.³⁾ We have also studied the effects of various peptides on elcatonin metabolism by the perfused pancreas to obtain information about metabolic interactions of peptides at the organ level.

Materials and Methods

Materials—Elcatonin (4000 U/mg) and its antiserum (7704R1) were kindly donated by Toyo Jozo Co. Carrier-free Na¹²⁵I was obtained from the Radiochemical Centre; leucine aminopeptidase (Type III-CP), carboxypeptidase A (Type I), trypsin (Type IX), chymotrypsin (Type II), and bovine albumin fraction V from Sigma Chemical Co.; Sephadex G-25, Blue dextran 2000, and dextran T-70 from Pharmacia Fine Chemicals; porcine monocom-

ponent insulin and porcine glucagon from Novo Industri A/S; aprotinin from Bayer A.G.; salmon calcitonin from Sandoz A.G.; porcine calcitonin from Armour Pharmaceutical Co.; human calcitonin from the Protein Research Foundation; tetracosactide acetate from Daiichi Seiyaku Co. and pancreozymin from Boots Pure Drug Co. Radioiodinated elcatonin was prepared by the method of Hunter and Greenwood,⁵⁾ and purified by gel filtration on Sephadex G-25 in 0.1 M acetic acid. The labeled elcatonin fraction was lyophilized and stored at -35°C . The specific activity was about 100 mCi/mg.

Perfusion of Pancreas—Wistar male rats, weighing 250–300 g, were anesthetized with pentobarbital and the pancreas was isolated and perfused as described previously.³⁾ The perfusion medium was Krebs–Ringer bicarbonate solution supplemented with glucose (5.8 mM), bovine serum albumin (0.5%) and dextran T-70 (4.6%). The perfusate was oxygenated with carbogen gas (O_2 95%, CO_2 5%) through a fiber-type oxygenator (microporous hollow fiber, Mitsubishi Rayon Co.) and maintained at pH 7.4. The perfusion was performed at the rate of 2 ml/min in a nonrecirculating system at 37°C . For conditioning the pancreas was initially perfused with Krebs–Ringer buffer for 15 min. The pancreatic juice was collected from the duodenal end of the common bile duct. The amylase activity in the pancreatic juice was determined by the method of Caraway.⁶⁾

Metabolism of Elcatonin in the Perfused Pancreas—When ^{125}I -labeled elcatonin (about 0.1 nM) was perfused, the inactivation of elcatonin was examined by gel filtration of a tissue sample extract and effluent on a 1.0×70 cm Sephadex G-25 column eluted with 3 M guanidine-HCl and 2.4 M formic acid at 4°C . The extraction of tissue-associated radioactivity was performed as described previously.³⁾ The column was calibrated with Blue dextran as a void volume marker (V_0), and with standard ^{125}I -elcatonin and ^{125}I -tyrosine as an internal volume marker (V_i). During the perfusion of unlabeled elcatonin, portal immunoreactive elcatonin concentration was determined by the method of Orimo *et al.*⁷⁾

Inactivation of Elcatonin by Proteases—Elcatonin (5.7 nM) was incubated with several proteases at 37°C for 10 min in the pancreas perfusion medium. The remaining elcatonin concentration was determined by radioimmunoassay.⁷⁾

Results and Discussion

The effect of elcatonin on the exocrine function of the perfused pancreas was examined prior to the study on the disposition of this peptide. Iwatsuki and Hashimoto⁸⁾ reported a suppressive effect of salmon calcitonin on enzyme secretion using blood-perfused canine pancreas. The isolated pancreas was initially perfused with 50 mU/ml pancreozymin for 20 min, and then the perfusate was supplemented with elcatonin (7 or 35 nM) for the following 20 min. Amylase output was significantly decreased by the concomitant perfusion of 35 nM elcatonin (Table I). The inhibitory effect on the exocrine secretion again indicates that elcatonin could be an effective drug for the treatment of acute pancreatitis.

Representative gel filtration profiles of radioactivity in portal effluent and extract of pancreas samples taken 10 min after the perfusion of ^{125}I -elcatonin are presented in Fig. 1. The intact fraction in the effluent was $66.1 \pm 4.4\%$ (mean \pm S.E.) of that in the perfusate, and $26.3 \pm 2.8\%$ of the radioactivity in the pancreas extract was eluted at the position of the intact fraction. Figure 2 shows the time course of the distribution of ^{125}I -elcatonin. The radioactivity in the effluent rapidly attained equilibrium. The intact concentration ratio of effluent to perfusate at 10 min was 0.63 ± 0.04 , and that of pancreas to perfusate was 0.18 ± 0.02 . The

TABLE I. Effect of Elcatonin on Amylase Output Induced by Pancreozymin in Isolated Perfused Rat Pancreas

	Amylase output ($\times 10^{-3}$ U/20 min) ^{a)}	
	without elcatonin 0–20 min	with elcatonin 20–40 min
Pancreozymin (50 mU/ml)	4.28 ± 0.66	3.78 ± 0.12
+ Elcatonin (7 nM)	4.92 ± 0.86	2.97 ± 0.58
+ Elcatonin (35 nM)	4.25 ± 0.70	$1.83 \pm 0.25^b)$

a) Mean \pm S.E. of three experiments.

b) Significantly different from pancreozymin alone (20–40 min), $p < 0.05$.

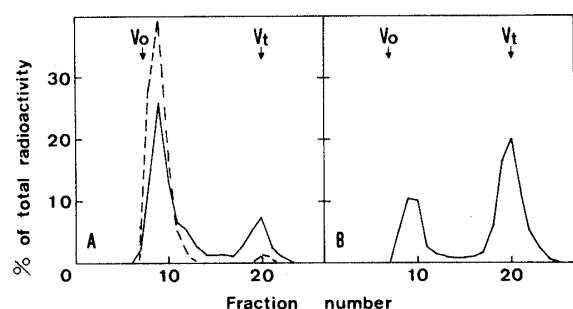


Fig. 1. Gel Filtration Profiles of Effluent and Tissue Extract during ^{125}I -Elcatonin Perfusion

Samples taken 10 min after the beginning of the perfusion of ^{125}I -elcatonin were subjected to gel filtration on a 1.0×70 cm Sephadex G-25 column. The broken line represents the gel filtration profile of the standard elcatonin. A, effluent; B, tissue extract.

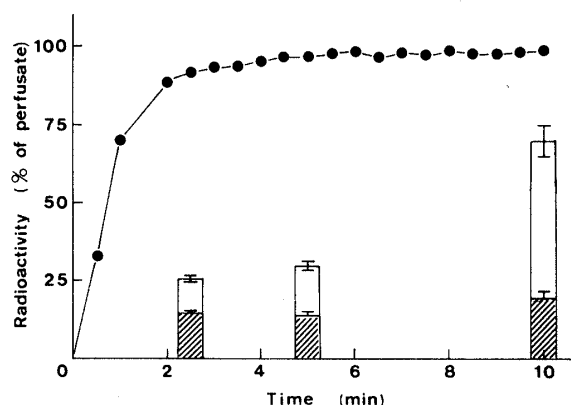


Fig. 2. Distribution of ^{125}I -Elcatonin in Isolated Perfused Rat Pancreas

Solid circles represent the total radioactivity in 1 ml of effluent, the total height of open columns is the total radioactivity per g of pancreas, and the hatched columns represent the intact elcatonin concentration, determined by gel filtration, in the pancreas. Data are expressed as percent of perfusate. Each point is the mean value of 3–7 experiments. The vertical lines give the S.E.

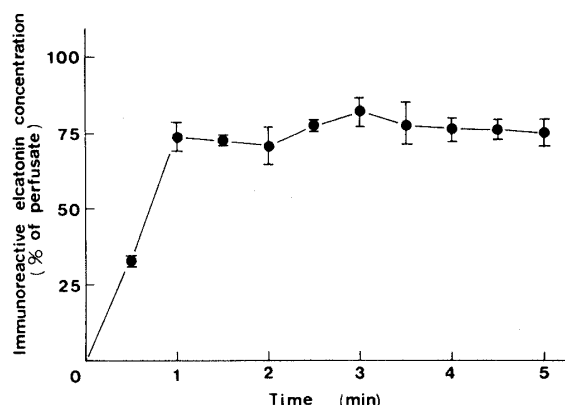


Fig. 3. Appearance of Immunoreactive Elcatonin in the Effluent from the Perfused Pancreas during the Infusion of Elcatonin (0.3 nM)

Each point represents the mean \pm S.E. of three experiments.

extracellular space of perfused pancreas was about 19% of the pancreatic volume,³⁾ and so the distribution space of ^{125}I -elcatonin was considered to be limited to the extracellular space. Although the radioactivity of small molecular fragments increased in the pancreas with time of perfusion, intact elcatonin concentration changed little throughout the perfusion period. The radioactivity in the pancreatic juice was low during perfusion for 10 min.

When unlabeled elcatonin (0.3 nM) was perfused, a rapid increase of immunoreactive elcatonin concentration in the effluent was followed by a rapid equilibration (Fig. 3). The concentration ratio of effluent to perfusate was 0.75 ± 0.01 after the steady state was established (1.5–5.0 min).

These results indicated that approximately 30% of the elcatonin entering the pancreas might be removed and metabolized. It has been reported that the kidney is the primary organ for clearance of calcitonin,⁹⁾ and our previous study using perfused rat kidney and renal cortical slices demonstrated the existence of high-affinity binding sites for elcatonin in rat kidney.¹⁰⁾ The perfused pancreas did not show such a specific accumulation of elcatonin. The biological half-life of elcatonin in the rat was reported to be 5 min,^{11a)} and the total body clearance was estimated to be about 30 ml/min/rat. The blood supply to the pancreas is relatively small (1.3% of cardiac output in the rat,¹²⁾ so that the inactivation of elcatonin in the pancreas would not be a major determinant of the rapid clearance of elcatonin in the whole body.¹¹⁾

The intact ratio of samples during the perfusion of elcatonin has been shown to be larger

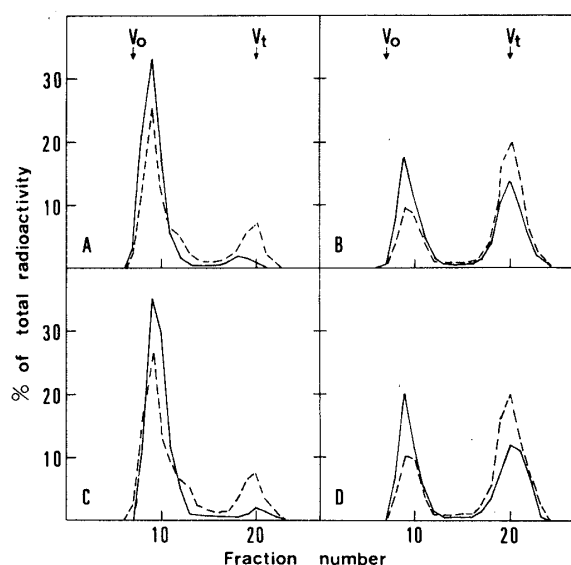


Fig. 4. Gel Filtration Profiles of Effluent and Tissue Extract during the Concomitant Perfusion of ^{125}I -Elcatonin with Aprotinin and Insulin

Samples taken 10 min after the beginning of the concomitant perfusion of ^{125}I -elcatonin with aprotinin (500 U/ml; A, B) or insulin (10 mU/ml; C, D) were subjected to gel filtration on a 1.0×70 cm Sephadex G-25 column. The broken lines represent the gel filtration profiles of samples obtained from control experiments. A, C, effluent; B, D, tissue extract.

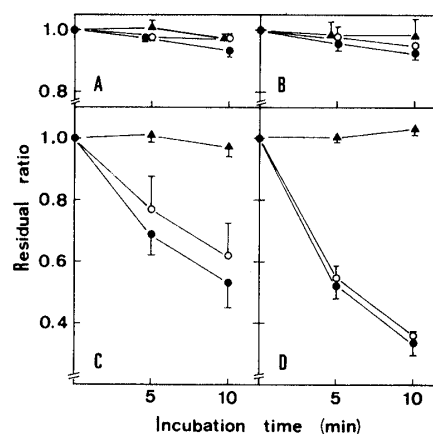


Fig. 5. Effects of Aprotinin and Insulin on Inactivation of Elcatonin by Proteases

Elcatonin (5.7 nM) was incubated with several proteases at 37°C in the presence of aprotinin (500 U/ml; \blacktriangle) or insulin (200 mU/ml; \circ). Solid circles represent the values in control experiments. Each point is the mean \pm S.E. of 3–4 experiments. A, leucine aminopeptidase (10 $\mu\text{g}/\text{ml}$); B, carboxypeptidase A (10 $\mu\text{g}/\text{ml}$); C, trypsin (0.1 $\mu\text{g}/\text{ml}$); D, chymotrypsin (10 $\mu\text{g}/\text{ml}$).

TABLE II. Effect of Various Peptides on the Metabolism of ^{125}I -Elcatonin in Isolated Perfused Rat Pancreas

Peptide	Intact elcatonin fraction (%) ^{a)}	
	Portal effluent	Pancreas extract
Control	66.1 ± 4.4	26.3 ± 2.8
Aprotinin (500 U/ml)	$81.4 \pm 2.4^b)$	$42.3 \pm 6.1^b)$
Insulin (10 mU/ml)	$88.5 \pm 2.7^b)$	$45.8 \pm 5.5^b)$
(100 mU/ml)	$88.3 \pm 4.0^b)$	$44.5 \pm 5.6^b)$
Elcatonin (100 nM)	55.1 ± 7.5	17.6 ± 6.2
Human calcitonin (30 nM)	65.3 ± 11.9	19.6 ± 11.5
Porcine calcitonin (100 nM)	68.9 ± 7.5	17.7 ± 2.3
Salmon calcitonin (300 nM)	69.2 ± 2.7	20.0 ± 1.0
Glucagon (3 μM)	72.1 ± 11.2	30.0 ± 6.8
Tetracosactide (3 μM)	75.0 ± 5.9	27.6 ± 8.7

a) Mean \pm S.E. of three to seven experiments.

b) Significantly different from control ($p < 0.05$). Perfusion time: 10 min.

than that of samples during the perfusion of secretin, a linear peptide.³⁾ However, a significant part of the elcatonin entering the pancreas was inactivated, in contrast to the stability of aprotinin, a globular peptide.³⁾ Chemical modification of disulfide bonds in aprotinin has been reported to render the molecule susceptible to degradation by the pancreas.³⁾ It can be considered that the cyclic structure in the elcatonin molecule probably contributes to its stability *in vivo*. Therefore, we studied the effects of various peptides, with or without a cyclic

linkage in their molecules, on elcatonin metabolism by the pancreas in the following experiments. The degradation of elcatonin was assessed from the gel filtration profiles of radioactivity of effluent and extract of tissue samples, obtained 10 min after the beginning of the perfusion with ^{125}I -elcatonin and various kinds of peptides. The addition of aprotinin or insulin to the perfusate significantly decreased the degradation products of ^{125}I -elcatonin (Fig. 4). Calcitonins of several species and linear peptides (tetracosactide and glucagon) did not modify the metabolism of ^{125}I -elcatonin (Table II).

It has been reported that the addition of aprotinin to injection solution decreases the extent of local inactivation of porcine calcitonin, when injected subcutaneously into chicks.¹³⁾ Insulin has been shown to block the degradation of ^{125}I -salmon calcitonin by human breast cancer cells.¹⁴⁾ However, the inhibitory mechanisms of insulin and aprotinin, both of which contain three disulfide bonds, on calcitonin metabolism have not been investigated. Therefore, the effects of aprotinin and insulin on elcatonin inactivation by several typical proteases were studied. Although exoproteases such as aminopeptidase and carboxypeptidase did not cause significant degradation of elcatonin, incubation with endopeptidases such as trypsin and chymotrypsin caused a rapid loss of immunoreactive elcatonin (Fig. 5). Inclusion of aprotinin (500 U/ml) almost completely blocked the degradation of elcatonin by trypsin or chymotrypsin, but the rate of elcatonin inactivation was not altered by the addition of insulin (200 mU/ml).

Aprotinin is known to inhibit the activity of trypsin-or kallikrein-like serine proteases by forming a stable complex with the proteases.¹⁵⁾ The inhibitory effect of aprotinin on elcatonin inactivation by the perfused pancreas may be due to the inhibition of serine proteases located on cell surface membranes in the pancreas. However, the suppressive effect of insulin could not be explained by the inhibition of these proteases. Insulin has been proved to regulate the function of the pancreas,¹⁶⁾ and its receptors exist in both the islets¹⁷⁾ and acinar cells of the pancreas.¹⁸⁾ Thus, a different process should be involved in the inhibitory action of insulin on elcatonin metabolism. These findings suggest that a better knowledge of metabolic interactions of peptides at the target tissues is required in order to permit a more rational therapeutic use of peptide drugs.

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