Arch Jpn Chir 61(3), 234～248, Mai., 1992

Effect of Short-Termed Pancreatico-Biliary Duct Obstruction on Lysosomal Enzyme in Rats: Protective Effect of a Potent New Protease Inhibitor, E-3123

Tetsuya Hirano, Tadao Manabe, Fumiaki Yotsumoto, Gakuji Ohshio and Yoshinori Nio

First Department of Surgery, Faculty of Medicine, Kyoto University, Kyoto, Japan
Received for Publication, Feb. 21, 1992

Abstract

To investigate the mechanism by which the pancreatic acinar cells are injured in animals with an obstructed common channel, we measured the amount of lysosomal enzymes and of amylase in the pancreatico-biliary juice in rats with pancreatico-biliary duct obstruction (PBDO). We tested the protective effect of a new potent synthetic protease inhibitor, E3123 (4-guanidinobenzoate methanesulfonate), on the exocrine pancreas in this model of PBDO and secretin infusion. Blockage of PBD for 4 hours and secretin (0.2 CU/kg・hr) infusion caused a significant rise in portal serum amylase and cathepsin B levels, pancreatic water content, and pancreatic amylase content, as well as redistribution of cathepsin B in acinar cells. These changes tended to continue for 12 hours after the removal of PBDO and disappeared at 24 hours. All the changes induced by PBDO with secretin infusion were no longer observed at 48 hours. The administration of 5 mg/kg・hr of E3123 during PBDO markedly attenuated all the parameters examined in this study. Thus, it had a significant protective effect on acinar cells in this model. E3123 in a dose of 2 mg/kg・hr had a partial, but significant, protective effect. These results indicate the possible usefulness of E3123 in the treatment of pancreatic duct obstructed pancreatitis.

Introduction

Both morphological and biochemical studies have shown that, in the early stage, two forms of experimental pancreatitis (diet-induced[1,2], and secretagogue-induced[3,4,5,6]) share the common attribute of co-localization of digestive enzymes and lysosomal hydrolases inside large cytoplasmic vacuoles[7], and redistribution of lysosomal enzymes from the lysosome-rich to the zymogen granule-rich fraction in the subcellular fractions of acinar cells. Since cathepsin B, a lysosomal enzyme, can activate trypsinogen[8,9,10], and trypsin can activate the other pancreatic digestive enzymes, the co-localisation of digestive enzymes with lysosomal hydrolases could lead to the activation of intracellular digestive enzymes, and be an important trigger in the development of acute pancreatitis inside the acinar cells. Thus, lysosomal enzymes seem to play an important role in the pathogenesis of

Key words: Pancreatico-biliary duct obstruction, Cathepsin B, Lysosomal fragility, E-3123.
索引語：総膵胆管結紮，カテプシンB，ライソゾーム脆弱性，E-3123
Present address: First Deparment of Surgery, Faculty of Medicine, Kyoto University, 54 Shogoin-Kawaracho, Sakyoku, Kyoto 606, Japan.

acute pancreatitis both inside and outside acinar cells[11].

Gallstone pancreatitis, which is the most common form of acute pancreatitis in humans, seems to be triggered by the passage of a stone through or its incarceration in the terminal bile duct [12, 13, 14, 15, 16, 17] It has also been suggested that such a stone might obstruct the pancreatic duct or both the pancreatic duct and the biliary duct, but the mechanism whereby pancreatic duct obstruction with or without biliary duct obstruction induces pancreatitis, has not been clarified. Our rat model of pancreatico-biliary duct obstruction seems to have many advantages in the investigation of the mechanism of common channel duct obstruction, because in rats, pancreatic ducts unite with the biliary duct, duct in its terminal portion causes obstruction of both pancreatic and biliary ducts.

A potent new synthetic protease inhibitor, E3123, 4-(2-succinimidoethylthio) phenyl 4-guanidinobenzoate methanesulfonate is the guanidino acid esters[18]. It has been found to inhibit strongly several kinds of proteases, such as trypsin, plasmin, phospholipase A_2, elastase and kallikrein, and its relatively low molecular weight (508, 59) encouraged us to evaluate its protective effect on acinar cells in acute pancreatitis. In this study, we examined the protective effect of E3123 on the secretion of lysosomal hydrolases into pancreatico-biliary juice and the distribution of lysosomal enzyme in the subcellular fractions of acinar cells after short-term pancreatico-biliary duct obstruction in rats.

Materials and Methods

In this study 288 male Wistar rats weighing about 250 g (Shizuoka Experimental Animals, Shizuoka, Japan) were used. They were kept in light-dark cycle regulated (light, 5:00–17:00) and air-conditioned (23±3°C) animal quarters in our university before the experiments and allowed to become acclimatized to standard laboratory conditions for at least 4 days. Anesthesia was induced by the intraperitoneal administration of sodium pentobarbital (25 mg/kg) and maintained by periodic intravenous injections of pentobarbital (10 mg/kg). A catheter (V-3 catheter Insul-Tab, Woburn, MA, U.S.A.) was passed through the right external jugular vein into the superior vena cava to serve as a venous line. The animals were then divided into the following 4 groups: 1) pancreatico-biliary duct obstruction (PBDO) plus secretin (Sigma Chemical, St. Louis, MO, U.S.A.) infusion (PBDO+S), 2) E3123 treatment (2 mg/kg · hr; E1), 3) E3123 treatment (5 mg/kg · hr; E2), 4) controls, laparotomy only (CONT).

In the 84 PBDO+S rats the abdomen was opened and the pancreatico-biliary duct (PBD) just adjacent to the duodenum was clamped with a metal clip, the abdomen was closed, anesthesia was maintained and secretin (0.2 CU/kg · hr) was infused for 4 hours. After PBDO for 4 hours, the metal clip on the PBD was removed, and 21 rats were assigned to each of the following experiments: 0, 12, 24, and 48 hours after the removal of the PBDO. After removal of the metal clip, the abdomen was closed, animals were given nothing by mouth and infused with heparinized (30 IU/ml) lactate-Ringer solution containing 10% glucose at a speed of 0.58 ml/hr with an infusion pump. The pancreatico-biliary ducts of 72 E1 rats were ligated as in the PBDO+S group, and secretin was infused (0.2 CU/kg · hr); E3123 was also infused (2 mg/kg · hr) throughout the PBDO. The PBDO of 72 E1 rats were blocked as in the PBDO+S group, secretin was infused and 5 mg/kg · hr of E3123 was infused throughout the period of PBDO. In these two groups, 18 rats were used for each of the following experiments: 0, 12, 24, and 48 hours after the removal of the PBDO. The PBD near the duodenum of 55 control rats (CONT), was manipulated gently with a cotton swab, the ab-

domen was closed, and anesthesia was maintained for 4 hours.

In each group, at each stage, the abdomen was reopened, and a catheter (PE 50, Clay Adams, Parsippany, NJ, U.S.A.) was inserted into the pancreatico-biliary duct just adjacent to the duodenum for the collection of pancreatico-biliary juice. After a 15 min period of stabilization, pancreatico-biliary juice was collected for one hour in preweighed eppendorf tubes on ice for baseline calculations; then caerulein (0.2 μg/kg・hr) was infused intravenously for one hour to stimulate pancreatic secretion. The activities of amylase and of cathepsin B and N-acetyl-β-D-galactosidase as lysosomal enzymes in each fraction were measured.

At each stage (0, 12, 24 and 48 hours after removal of PBDO) other animals in each group were killed with a large dose of pentobarbital, blood was taken from the portal trunk, and portal amylase and cathepsin B levels were determined.

The pancreas was removed from rats in each group at each stage. A small part was used for the quantitation of the pancreatic water content by comparison of the weight immediately after removal (wet weight) with the weight after desiccation at 150°C for 48 hours in a desiccator.

Other small portions of the pancreas were fixed overnight by immersion in phosphate-buffered 10% neutral formalin. After paraffin embedding, sectioning and staining with hematoxylin-eosin, the sections were examined light microscopically by a blinded observer for acinar cell changes, such as interstitial edema and vacuolization, and for inflammatory cell infiltration.

About one forth of the pancreas was used for the determination of pancreatic amylase and cathepsin B content. This portion was homogenized in cold phosphate-buffered saline (pH 7.4) containing 0.5% Triton X-100 (Fisher Scientific) in a polytron homogenizer and unbroken cells and debris were removed by low speed centrifugation (150×g, 15 min, 4°C). Amyalse activity, cathepsin B activity and deoxyribonucleic acid (DNA) concentration in the resulting supernatant were measured. The pancreatic amylase and cathepsin B content were expressed as U/mg・DNA.

The remaining half of the pancreas was used for subcellular fractionation and the determination of the distribution of cathepsin B in acinar cells (19). Pancreatic fragments were homogenized in 6 ml of cold 5 mM MOPS (3-(N-morpholino) propanesulfonic acid) buffer (pH 6.5) containing (mM) $MgSO_4$ (1) and sucrose (250) with three up-and-down strokes of a Dounce homogenizer. The resulting homogenate was centrifuged (150×g, 10 min, 4°C) to pellet debris and unbroken cells. The supernatant was centrifuged (1300×g, 15 min, 4°C) to obtain a zymogen granule-rich pellet, and the supernatant was centrifuged again (12000×g, 12 min, 4°C) to yield a lysosome- and mitochondria-rich pellet and a supernatant, which was considered to be the microsomal and soluble fraction. The various pellets obtained during fractionation were resuspended individually in 2 ml of cold (4°C) 4 mM MOPS solution, and cathepsin B and amylase activities in each fraction were measured and expressed as a percentage of the total activity, an index of the distribution of lysosomal enzyme and pancreatic digestive enzyme in acinar cells.

Amylase activity was measured by the method of Bernfeld[20]. Cathepsin B activity was measured fluorometrically by the method of McDonald and Ellis[21] N-acetyl-β-D-galactosaminidase activity was measured fluorometrically by the method of Rinderknecht and co-workers [22] Deoxyribonucleic acid (DNA) concentration was measured fluorometrically by the method of Labarca and Paigen[23]. E3123 was kindly donated by Eisai Pharmaceutical Company, Tokyo, Japan.

The results in this communication represent the mean±SEM for n determinations. Differences between groups were evaluated by analysis of variance and the Tukey's procedure, and

Table 1 Effect of pancreatico-biliary duct obstruction (PBDO) for 4 hours and secretin infusion (0.2 CU/kg·hr) on baseline volume of pancreatico-biliary juice in rats

Group	n	Pancreatico-biliary juice volume at the basal phase (ml/kg·hr)			
		4 hours after PBDO with secretin infusion	12 hours after removal of PBDO	24 hours after removal of PBDO	48 hours after removal of PBDO
PBDO+S	7	2.52±0.14	2.92±0.22	4.35±0.26	3.82±0.29
E_1	6	2.97±0.20	3.18±0.14	3.58±0.13*	3.70±0.18
E_2	6	3.24±0.19*	3.53±0.27	3.43±0.24*	3.25±0.23
CONT	5	3.42±0.18*	3.36±0.23	3.28±0.15*	3.59±0.22

The volume of pancreatico-biliary juice is expressed as ml/kg·hr and as mean±SEM. PBDO+S, pancreatico-biliary duct obstruction and secretin (0.2 CU/kg·hr) infusion group; E_1 and E_2 groups, pancreatico-biliary duct obstruction and secretin infusion plus E3123 infusion (E_1, 2 mg/kg·hr; E_2, 5 mg/kg·hr) during the PBDO; CONT, controlled group, laparotomy only; *, $p<0.05$ compared with PBDO+Secretin group.

Table 2 Effect of pancreatico-biliary duct obstruction (PBDO) for 4 hours and secretin infusion (0.2 CU/kg·hr) on volume of pancreatico-biliary juice in rats stimulated with caerulein (0.2 CU/kg·hr)

Group	n	Pancreatico-biliary juice volume stimulated by caerulein (0.2 μg/kg·hr) (ml/hr·hr)			
		4 hours after PBDO with secretin infusion	12 hours after removal of PBDO	24 hours after removal of PBDO	48 hours after removal of PBDO
PBDO+S	7	2.91±0.28	3.86±0.35	5.87±0.31	5.24±0.26
E_1	6	3.93±0.36	4.48±0.30	4.52±0.33*	4.73±0.29
E_2	6	4.78±0.24*	4.89±0.42	4.62±0.37*	4.94±0.35
CONT	5	4.66±0.32*	4.82±0.39	4.49±0.34*	5.06±0.38

The volume of pancreatico-biliary juice is expressed as ml/kg · hr at each stage. The symbols are the same as in Table 1. *, $p<0.05$ compared with PBDO+Secretin group.

significant differences were defined as those associated with a probability value (P) of less than 0.05.

Results

After 4 hours of pancreatico-biliary duct obstruction (PBDO) and secretin infusion, the volume of pancreatico-biliary juice was significantly smaller than in the control group, and this difference continued for 12 hours. However, the volume of juice 24 hours after the removal of the PBDO was significantly greater than in the control group, and at 48 hours there was no significant difference between the two groups. The rats which received 5 mg/kg · hr of E3123 during PBDO (E2 group) showed significantly smaller changes in pancreatico-biliary juice volume. E3123 (2 mg/kg · hr, E1 group) also had some protective effect, but it was not significant (Table 1).

The volume of pancreatico-biliary juice in rats stimulated with caerulein showed the same changes in the baseline phase. After 4 hours of PBDO and secretin (S) infusion the volume of pancreatico-biliary juice was significantly decreased and remained low for 12 hours after the removal of PBDO, but at 24 hours, it was significantly greater than in the control group (CONT), and at 48 hours the volume in the 2 groups was approximately the same. E3123 in a dose of 5 mg/kg · hr lessened significantly the decrease in pancreatico-biliary juice output, but in a dose of 2 mg/kg · hr E3123 had no significant effect (Table 2).

PBDO for 4 hours and secretin infusion caused a significant decrease in amylase output into the pancreatico-biliary juice in the baseline phase (845±129 U/kg・hr; control group, 1714±241 U/kg・hr). At 12 hours after removal of the PBDO, the amylase output had risen to 1359±238 U/kg・hr (controls, 1683±252 U/kg・hr), and at 24 hours it was 2492±284 U/kg・hr (controls, 1441±175 U/kg・hr), but at 48 hours there was no significant difference between the two groups. The administration of 5 mg/kg・hr of E3123 significantly prevented the decrease in amylase output (1467±225 U/kg・hr after 4 hours of PBDO and secretin infusion, 1534±203 U/kg・hr at 12 hours after removal of the PBDO, 1316±195 U/kg・hr at 24 hours, and 1601±235 U/kg・hr at 48 hours). E3123 in a dose of 2 mg/kg・hr had a partial but not significant protective effect (1138±186 U/kg・hr after 4 hours of PBDO and secretin infusion, 1394±239 U/kg・hr at 12 hours after removal of the PBDO, 1403±258 U/kg・hr at 24 hours, and 1473±248 U/kg・hr at 48 hours PBDO). The output of two lysosomal enzymes, cathepsin B and N-acetyl-β-D-galactosaminidase, was similar to the amylase output. PBDO for 4 hours and secretin infusion caused a significant decrease in the output of both cathepsin B (4±1 U/kg・hr) and N-acetyl-β-D-galactosaminidase (7±2 U/kg・hr), and E3123 in a dose of 5 mg/kg・hr significantly reduced the decrease (cathepsin B, 9±1 U/kg・hr; N-acetyl-β-D-galactosaminidase, 17±2 U/kg・hr). At 12 hours after removal of the PBDO there were no significant differences among the 4 groups in either cathepsin B output (PBDO+S, 8±2 U/kg・hr, E_1, 7±2 U/kg・hr, E_2, 10±2 U/kg・hr; CONT, 12±2 U/kg・hr) or N-acetyl-β-D-galactosaminidase output (PBDO+S, 15±3 U/kg・hr; E_1 16±3 U/kg・hr; E_2, 19±2 U/kg・hr; CONT, 20±2 U/kg・hr). However, at 24 hours both cathepsin B (21±3 U/kg・hr) and N-acetyl-β-D-galactosaminidase outputs (32±4 U/kg・hr) were significantly greater than in the control group (cathepsin B, 10±2 U/kg・hr; N-acetyl-β-D-galactosaminidase, 18±2 U/kg・hr), the E_1 group (cathepsin B, 9±2 U/kg・hr; N-acetyl-β-D-galactosaminidase, 17±2 U/kg・hr), or the E_2 group (cathepsin B, 11±1 U/kg・hr; N-acetyl-β-D-galactosaminidase, 19±3 U/kg・hr). By 48 hours, there were no significant differences among the 4 groups: cathepsin B (PBDO+S, 14±2 U/kg・hr; E_1, 12±2 U/kg・hr; E_2, 13±3 U/kg・hr; CONT, 9±2 U/kg・hr); N-acetyl-β-D-galactosaminidase (PBDO+S, 23±3 U/kg・hr E_1, 17±2 U/kg・hr; E_2, 18±2 U/kg・hr; CONT, 20±2 U/kg・hr) (Fig. 1).

The output of amylase, and lysosomal enzymes into the pancreatico-biliary juice of rats stimulated with caerulein showed the same changes in the baseline phase. PBDO and secretin infusion for 4 hours caused a significant decrease in amylase output (4391±776 U/kg・hr; controls, 8823±829 U/kg・hr), and this reduction in output continued for 12 hours after removal of the PBDO (5957±612 U/kg・hr; CONT, 7925±902 U/kg・hr). At 24 hours, the amylase output in rats stimulated with caerulein was significantly greater than in the controls (12249±1038 U/kg・hr vs. 8443±773 U/kg・hr). By 48 hours, there were no significant differences between the PBDO+S group (10247±973 U/kg・hr) and the control group (8356±758 U/kg・hr). The administration of 5 mg/kg・hr of E3123 during PBDO significantly raised the output of amylase into pancreatico-biliary juice (7258±784 U/kg・hr) in the PBDO+S group, but in a dose of 2 mg/kg・hr it had no protective effect. The outputs of both cathepsin B and N-acetyl-β-D-galactosaminidase in rats stimulated with caerulein were impaired significantly by 4 hours of PBDO+S (cathepsin B, 14±3 U/kg・hr; N-acetyl-β-D-galactosaminidase, 25±3 U/kg・hr, controls; cathepsin B, 33±4 U/kg・hr; N-acetyl-β-D-galactosaminidase, 44±4 U/kg・hr), and these differences continued for 12 hours after removal of the PBDO (PBDO+S: cathepsin B, 22±3 U/kg・hr; N-acetyl-β-D-galactosaminidase, 38±4 U/kg・hr; CONT: cathepsin B, 31±3 U/kg・hr; N-acetyl-β-D-galac-

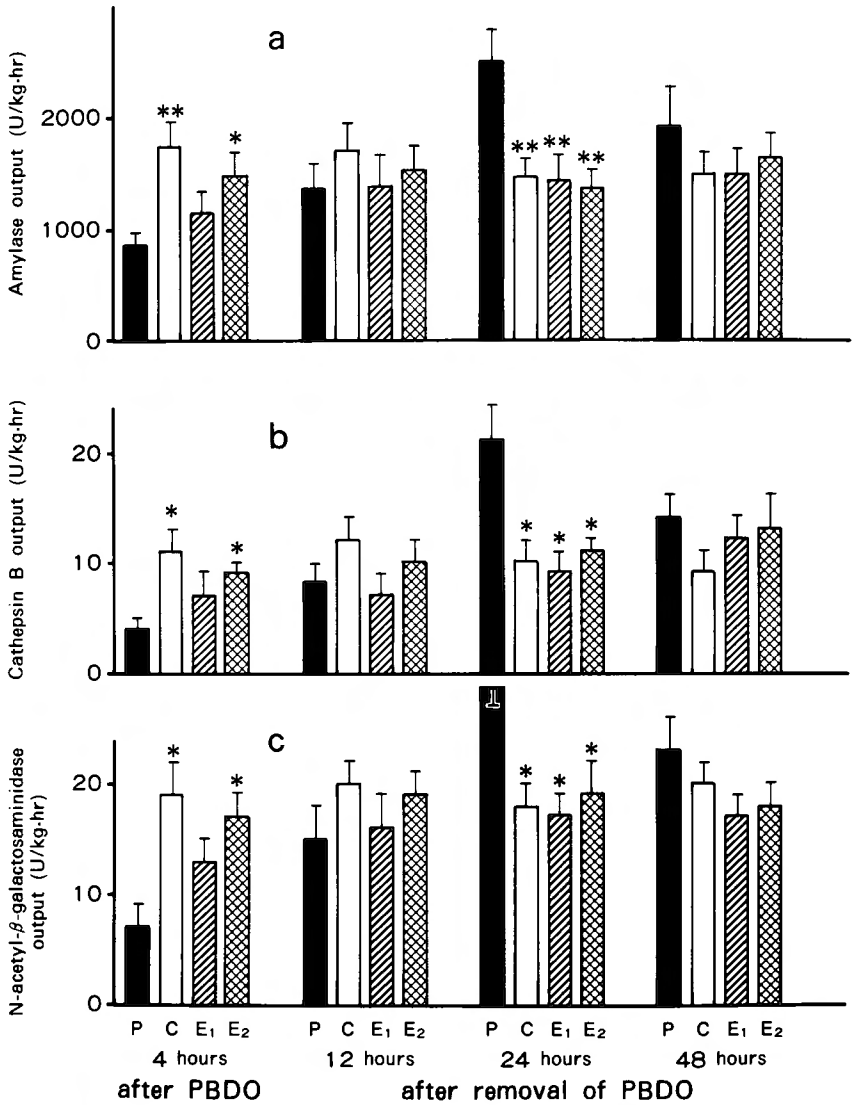

Fig. 1 Effect of pancreatico-biliary duct obstruction and secretin infusion on output of amylase (a), cathepsin B (b) and N-acetyl-β-D-galactosaminidase (c) into pancreatico-biliary juice at the basal phase in rats
P: pancreatico-biliary duct obstruction for 4 hours and secretin (0.2 CU/kg·hr) infusion (PBDO+S) group (n=7), C: control group, laparotomy only (n=5), E_1: PBDO and S plus 2 mg/kg·hr of E3123 (n=6), E_2: PBDO and S plus 5 mg/kg·hr of E3123 (n=6), Mean±SEM, * $p<0.05$, ** $p<0.02$ compared with P.

tosaminidase, 46±5 U/kg · hr).

However, 24 hours after removal of the PBDO, both cathepsin B (47±5 U/kg · hr) and N-acetyl-β-D-galactosaminidase (65±5 U/kg · hr) outputs were significantly greater than in the controls (cathepsin B, 29±3 U/kg · hr; N-acetyl-β-D-galactosaminidase, 41±3 U/kg · hr). At 48 hours there were no significant differences between the PBDO+S group (cathepsin B, 38±4 U/kg · hr; N-acetyl-β-D-galactosaminidase, 51±5 U/kg · hr) and the control group (cathepsin B, 34±3

U/kg・hr; N-acetyl-β-D-galactosaminidase, 40±3 U/kg・hr). The administration of E3123 in a dose of 5 mg/kg・hr significantly improved the output of both cathepsin B (29±3 U/kg・hr) and N-acetyl-β-D-galactosaminidase (40±4 U/kg・hr) reduced by 4 hours of PBDO and secretin infusion,

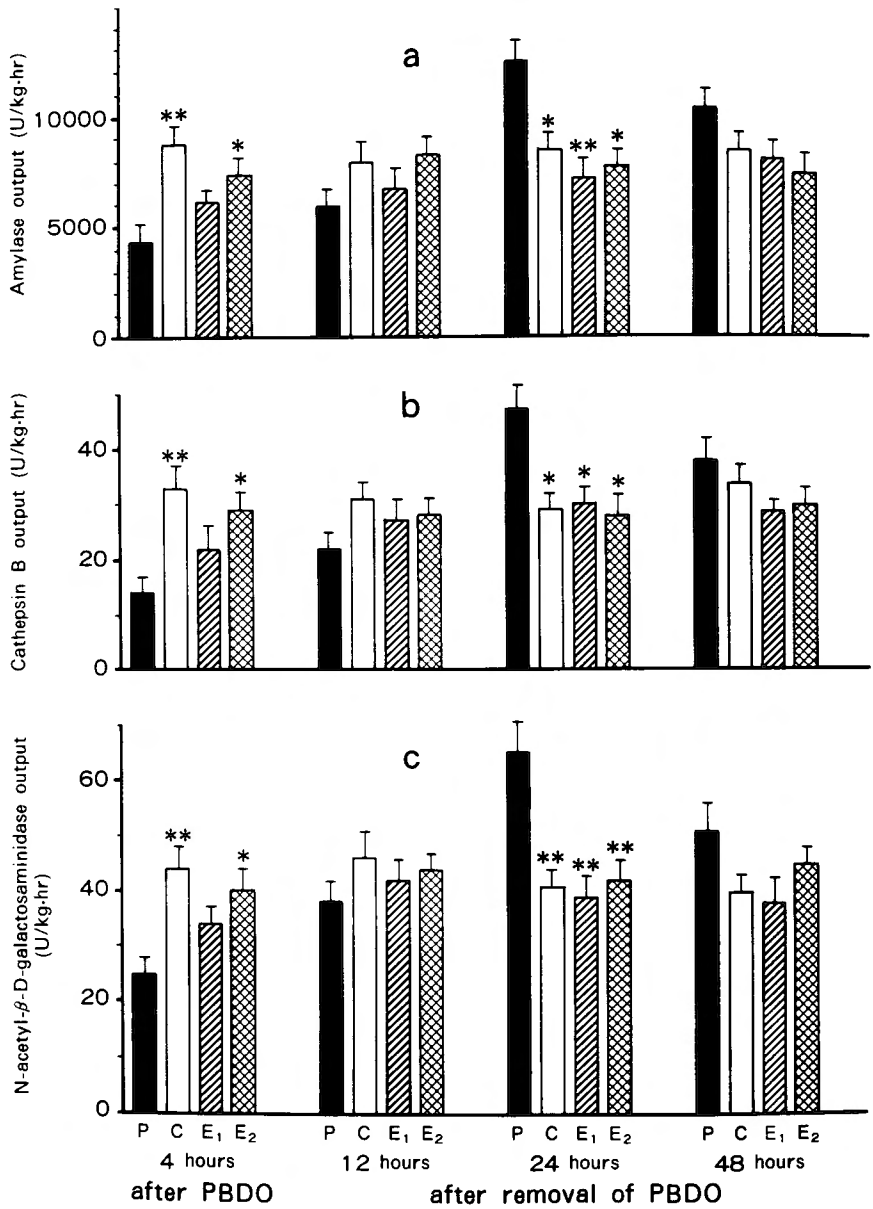

Fig. 2 Effect of pancreatico-biliary duct obstruction and secretin infusion on output of amylase (a), cathepsin B (b), and N-acetyl-β-D-galactosaminidase (c) into pancreatico-biliary juice in rats after stimulation with caerulein (0.2 μg/kg・hr).

P: PBDO+S group (n=7), C: control group (n=5), E_1: PBDO and S plus 2 mg/kg・hr of E3123 (n=6), E_2: PBDO and S plus 5 mg/kg・hr of E3123 (n=6), Mean±SEM, * $p<0.05$, ** $p<0.02$ compared with P.

Table 3 Effect of pancreatico-biliary duct obstruction (PBDO) for 4 hours and secretin infusion (0.2 CU/kg·hr) on portal serum amylase levels in rats.

Group	n	Portal serum amylase levels (U/ml)			
		4 hours after PBDO+Secretin infusion	12 hours after removal of PBDO	24 hours after removal of PBDO	48 hours after removal of PBDO
PBDO+S	7	62±8	26±4	19±2	15±2
E_1	6	31±5*	16±2*	12±2	14±2
E_2	6	18±3**	13±2*	16±2	11±2
CONT	5	12±2***	15±2*	17±2	10±2

PBDO+S, pancreatico-biliary duct obstruction and secretin infusion group; E_1 and E_2, PBDO+S plus infusion of 2 mg/kg·hr or 5 mg/kg·hr of E3123 during PBDO; CONT, control group, laparotomy only; *, $p<0.05$; **, $p<0.02$; ***, $p<0.01$ compared with PBDO+S group.

Table 4 Effect of pancreatico-biliary duct obstruction (PBDO) for 4 hours and secretin (0.2 CU/kg·hr) infusion on portal serum cathepsin B activity levels in rats

Group	n	Portal serum cathepsin B levels (U/ml)			
		4 hours after PBDO+Secretin infusion	12 hours after removal of PBDO	24 hours after removal of PBDO	48 hours after removal of PBDO
PBDO+S	7	7.5±0.7	3.2±0.5	2.6±0.3	2.3±0.3
E_1	6	3.6±0.5*	2.8±0.3	2.4±0.2	2.0±0.3
E_2	6	2.9±0.4*	2.7±0.3	2.2±0.3	1.7±0.2
CONT	5	2.6±0.3*	2.4±0.2	2.1±0.3	1.8±0.2

PBDO+S, pancreatico-biliary duct obstruction with secretin infusion group; E_1 and E_2, PBDO+S infusion of 2 mg/kg·hr or 5 mg/kg·hr during PBDO; CONT, control group, laparotomy only; *, $p<0.02$ compared with PBDO+S groups.

Table 5 Effect of pancreatico-biliary duct obstruction (PBDO) for 4 hours and secretin (0.2 CU/kg·hr) infusion on pancreatic water content in rats.

Group	n	Pancreatic water content (% of the total weight)			
		4 hours after PBDO+Secretin infusion	12 hours after removal of PBDO	24 hours after removal of PBDO	48 hours after removal of PBDO
PBDO+S	7	86±3	82±3	78±2	73±2
E_1	6	81±3	75±2	73±2	76±2
E_2	6	77±2*	74±2	75±2	76±1
CONT	5	74±2*	76±2	73±1	74±1

PBDO+S, pancreatico-biliary duct obstruction and secretin infusion group; E_1 and E_2, PBDO+S plus infusion of 2 mg/kg·hr or 5 mg/kg·hr of E3123 during PBDO; CONT, control group, laparotomy only; *, $p<0.05$ compared with PBDO+S group.

but in a dose of 2 mg/kg · hr it had no significantly protective effect (Fig. 2).

Pancreatico-biliary duct obstruction (PBDO) and secretin infusion for 4 hours caused a marked rise in the portal serum amylase level, and 12 hours after removal of the PBDO, the portal serum amylase level was still significantly higher than in the control group. The administration of E3123, both 2 mg/kg · hr and 5 mg/kg · hr, significantly reduced the hyperamylasemia induced by PBDO+secretin infusion; 5 mg/kg · hr had the greatest effect. At 24 hours and 48 hours, there

were no significant differences among the 4 groups (Table 3). The portal serum lysosomal enzyme (cathepsin B) levels showed almost the same changes as the amylase levels. After 4 hours of PBDO+secretin infusion there was a significant rise in the portal serum cathepsin B level, but 12 hours after removal of the PBDO, there were no significant differences among 4 groups. Both 2 mg/kg・hr and 5 mg/kg・hr of E3123 had, to almost the same degree, a protective effect against the rise in the portal serum cathepsin B level. The portal serum cathepsin B level tended to return to the control value before the amylase level (Table 4).

PBDO and secretin infusion for 4 hours caused a slight, but significant, increase in the pan-

Table 6 Effect of pancreatico-biliary duct obstruction (PBDO) and secretin (0.2 CU/kg·hr) infusion on pancreatic histology in rats

Group	n	Interstitial edema (0–+4)			
		4 hours after PBDO+Secretin	12 hours after removal of PBDO	24 hours after removal of PBDO	48 hours after removal of PBDO
PBDO+S	7	+3 (2–3) [2.6±0.2]	+1 (1–2) [1.3±0.2]	0 (0–1) [0.4±0.2]	0 (0–1) [0.1±0.1]
E_1	6	+1 (0–2)* [1.0±0.3]	0 (0–1)* [0.3±0.2]	0 (0) [0]	0 (0) [0]
E_2	6	0 (0–1)** [0.3±0.2]	0 (0–1)* [0.2±0.2]	0 (0) [0]	0 (0) [0]
CONT	5	0 (0)*** [0]	0 (0)** [0]	0 (0) [0]	0 (0) [0]

Group	n	Acinar cell vacuolization (0–+4)			
		4 hours after PBDO+Secretin	12 hours after removal of PBDO	24 hours after removal of PBDO	48 hours after removal of PBDO
PBDO+S	7	+2 (1–2) [1.6±0.2]	0 (0–1) [0.4±0.2]	0 (0–1) [0.1±0.1]	0 (0) [0]
E_1	6	+1 (0–1)* [0.7±0.2]	0 (0–1) [0.3±0.2]	0 (0) [0]	0 (0) [0]
E_2	6	0 (0–1)** [0.3±0.2]	0 (0–1) [0.1±0.1]	0 (0) [0]	0 (0) [0]
CONT	5	0 (0)*** [0]	0 (0)** [0]	0 (0) [0]	0 (0) [0]

Group	n	Inflammatory cell infiltration			
		4 hours after PBDO+Secretin	12 hours after removal of PBDO	24 hours after removal of PBDO	48 hours after removal of PBDO
PBDO+S	7	+1 (0–1) [0.6±0.2]	0 (0–1) [0.3±0.2]	0 (0–1) [0.1±0.1]	0 (0) [0]
E_1	6	0 (0–1) [0.3±0.2]	0 (0–1) [0.1±0.1]	0 (0) [0]	0 (0) [0]
E_2	6	0 (0–1) [0.1±0.1]	0 (0) [0]	0 (0) [0]	0 (0) [0]
CONT	5	0 (0) [0]	0 (0) [0]	0 (0) [0]	0 (0) [0]

Histological changes were graded blindly on a scale from 0 (no change) to +4 (maximum changes), and the values were expressed as the mean rounded to the nearest whole number. ( ), range of true values; [ ], mean±SEM of true values; *, $p<0.05$; **, $p<0.02$; ***, $p<0.01$ compared with PBDO+S group.

creatic water content, (86±3%; control group, 74±2%). This increased pancreatic water content was barely detected macroscopically. At 12 hours after removal of the PBDO, this increased water content was less evident, and at 24 and 48 hours, there were no significant differences among the 4 groups. The administration of 5 mg/kg · hr of E3123 significantly prevented this edema, but 2 mg/kg · hr had only a slight protective effect (Table 5).

Histological findings after 4 hours of PBDO+secretin infusion were: moderate interstitial edema, mild acinar cell vacuolization, and slight inflammatory cell infiltration. These changes were significant in comparison with the findings in the control group. At 12 hours after removal of the PBDO, these changes were less evident, and at 24 hours and 48 hours, there were no significant differences among the 4 groups. The administration of both 2 mg/kg · hr and 5 mg/kg · hr of E3123 significantly prevented these histological changes in a dose-dependent manner (Table 6).

PBDO+secretin infusion for 4 hours caused a significant increase in pancreatic amylase content, suggesting congestion of pancreatic digestive enzymes during PBDO. At 12 hours after removal of the PBDO, the amylase content in the PBDO+secretin group was still higher than in the control group, but not significantly so. The administration of E3123 (both 2 mg/kg · hr and 5 mg/kg · hr) had a significant protective effect against the rise in pancreatic amylase content (Table 7).

PBDO+secretin infusion for 4 hours caused a slight, but not significant increase in the pancreatic cathepsin B content. At the other stages, too, there were no significant differences among the 4 groups.

PBDO and secretin infusion for 4 hours caused a significant increase of cathepsin B activity in zymogen pellets (47±4%) and a significant decrease of cathepsin B activity in the lysosomal fraction (30±3%); control group (zymogen fraction, 22±2%; lysosomal fraction, 56±3%). These changes indicate a marked transfer of cathepsin B activity from the lysosomal fraction to the heavier zymogen fraction-a redistribution of cathepsin B in the subcellular fractions of acinar cells and a colocalization of lysosomal enzymes with digestive enzymes. This distribution continued for 12 hours after removal of the PBDO (zymogen fraction, 38±3%; lysosomal fraction, 39±4%; control group:

Table 7 Effect of pancreatico-biliary duct obstruction (PBDO) and secretin (0.2 CU/kg·hr) infusion on pancreatic amylase and cathepsin B content in rats

Pancreatic content	Group	n	Stage			
			4 hours after PBDO+Secretin	12 hours after removal of PBDO	24 hours after removal of PBDO	48 hours after removal of PBDO
Pancreatic amylase content (U/mg·DNA)	PBDO+S	7	774±54	592±49	554±33	532±44
	E_1	6	628±49*	574±51	536±40	516±34
	E_2	6	587±38*	562±49	523±35	541±27
	CONT	5	513±42**	543±56	508±39	497±45
Pancreatic cathepsin B content (U/mg·DNA)	PBDO+S	7	1953±219	1828±193	1775±158	1724±206
	E_1	6	1881±212	1756±161	1488±173	1425±169
	E_2	6	1706±144	1558±152	1599±184	1527±186
	CONT	5	1462±173	1618±189	1584±165	1493±148

Amylase and cathepsin B content are expressed as U/mg·DNA. PBDO+S, pancreatico-biliary duct obstruction and secretin infusion group; E_1 and E_2, PBDO+S plus infusion of 2 mg/kg·hr or 5 mg/kg·hr of E3123 during PBDO; CONT, control group, laparotomy only; *, $p<0.05$; **, $p<0.02$ compared with PBDO+S group.

zymogen fraction, 24±2%; lysosomal fraction, 55±3%). At 24 and 48 hours after removal of the PBDO, there were no significant differences between the PBDO+S group and the control group, indicating that the redistribution of cathepsin B activity had ended. E3123 in a dose of 5 mg/kg・hr almost completely prevented the redistribution of cathepsin B activity (zymogen and lysosomal fractions: 25±2% and 51±3% at 4 hours after PBDO+secretin infusion, 23±3% and 54±3% at 12 hours, 22±2% and 55±3% at 24 hours, and 25±2% and 55±3% at 48 hours after removal of the PBDO, respectively). E3123 in a dose of 2 mg/kg・hr provided partial protection (zymogen and lysosomal fractions: 33±3% and 46±3% at 4 hours after PBDO+secretin infusion, 28±2% and 52±3% at 12 hours, 25±2% and 54±3% at 24 hours, and 23±2% and 52±3% at 48 hours after removal of PBDO, respectively). In the microsomal and soluble fraction, there were no significant differences at any stage among these 4 groups (Fig. 3). PBDO and secretin infusion for 4 hours caused a significant rise in amylase activity in the microsomal and soluble fraction (58±4%; control group, 40±4%), suggesting increased fragility of zymogen-containing granules in the process of subcellular fractionation. Amylase activity in the zymogen pellets (26±3%) was lower than in the control group (36±3%), but not significantly so. The administration of E3123 in a dose of 5 mg/kg・hr significantly prevented this increased fragility of zymogen granules (44±3%), but at 12, 24 and 48 hours after removal of the PBDO, there were no significant differences in amylase activity in either fraction between the PBDO+S group and the control group. E3123 in a dose of 2 mg/kg・hr had no significant protective effect (Fig. 4).

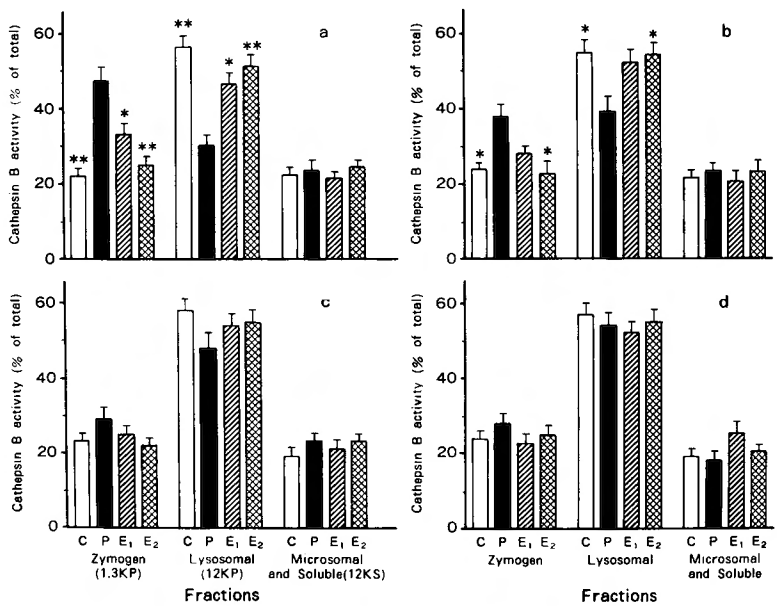

Fig. 3 Effect of pancreatico-biliary duct obstruction and secretin infusion on distribution of cathepsin B activity in subcellular fractions of pancreatic acinar cells in rat. a: 4 hours after PBDO+secretin infusion, b: 12 hours after removal of PBDO, c: 24 hours after removal of PBDO, d: 48 hours after removal of PBDO
C: control group (n=5), P: PBDO+S group (n=7), E_1: PBDO and S plus 2 mg/kg・hr of E3123 (n=6), E_2: PBDO and S plus 5 mg/kg・hr of E3123 (n=6), Mean±SEM, * $p<0.05$, ** $p<0.02$ compared with P.

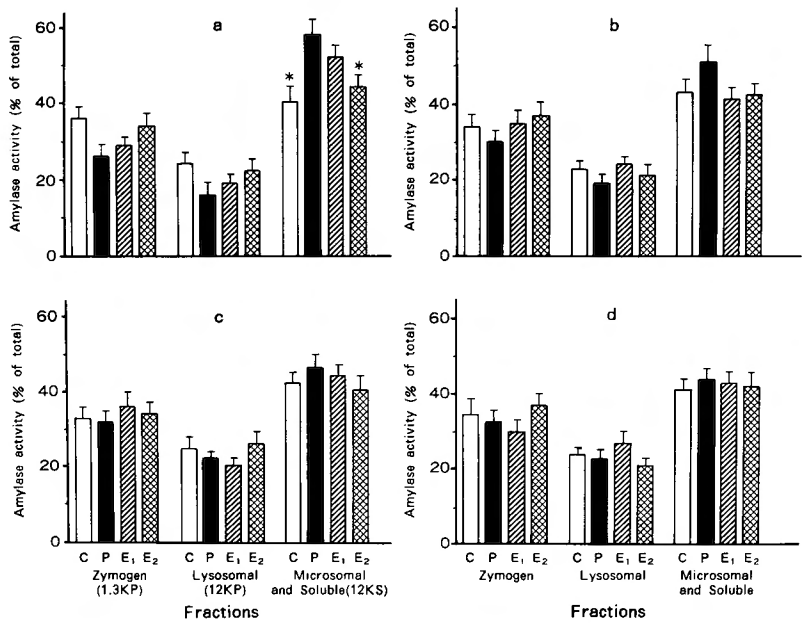

Fig. 4 Effect of pancreatico-biliary duct obstruction and secretin infusion on distribution of amylase activity in subcellular fractions of pancreatic acinar cells in rats. a: 4 hours after PBDO+secretin infusion, b: 12 hours after removal of PBDO, c: 24 hours after removal of PBDO, d: 48 hours after removal of PBDO C: control group (n=5), P: PBDO+S group (n=7), E_1: PBDO and S plus 2 mg/kg·hr of E3123 (n=6), E_2: PBDO and S plus 5 mg/kg·hr of E3123 (n=6), Mean±SEM, * $p<0.05$, ** $p<0.02$ compared with P.

Discussion

Gallstone pancreatitis in humans appears to be precipitated by the passage of a stone through or its incarceration in the terminal portion of the common bile duct[12, 17] Pancreatico-biliary duct obstruction with secretin infusion in the rat model used in this study, in which both bile and pancreatic ducts were obstructed and hypersecretion was induced by secretin stimulation, seems to provide suitable conditions for a demonstration of the early events of gallstone pancreatitis. The studies reported in this communication may also provide an important clue to the understanding of the events leading to pancreatitis, since they show that in the normal physiological stage, secretion of lysosomal enzymes into pancreatico-biliary juice occurs along with that of classical digestive enzymes, such as amylase. This finding indicated that a considerable amount of cathepsin B present in the pancreatico-biliary juice or in the "common channel" can lead to the activation of pancreatic digestive enzymes in the pancreatic duct system, since cathepsin B can activate trypsinogen, and trypsin can activate other digestive enzymes.

In both caerulein-induced[3, 4, 6] and diet-induced[1] pancreatitis marked enlargement of zymogen-containing organelles in the cell apex and colocalization of lysosomal hydrolases and digestive enzymes within large acidic cytoplasmic vacuoles have been observed. In this study, too, subcellular fractionation experiments showed that pancreatico-biliary duct obstruction with secretin infusion leads to a redistribution of cathepsin B activity and that, as a result, lysosomal hydrolase becomes col-

ocalized in a fraction that is rich in digestive enzymes. This colocalization phenomenon might under appropriate conditions, result in the intra-acinar cell activation of potentially dangerous digestive enzymes. In our present study, pancreatico-biliary duct obstruction and secretin infusion caused a rise in portal serum amylase levels, lysosomal enzymes levels, moderate microscopic edema, mild acinar cell vacuolization and inflammatory cell infiltration.

Another important finding in our present study was that the output of both amylase and lysosomal enzymes into the pancreatico-biliary juice rose during recovery after pancreatico-biliary duct obstruction, when the redistribution of cathepsin B in acinar cells had almost disappeared. Since short-term (4 hours) pancreatico-biliary duct obstruction and secretin infusion causes a rise in the pancreatic amylase content, indicating congestion of digestive enzymes in acinar cells during pancreatico-biliary duct obstruction, the increase in the secretion of amylase and lysosomal enzymes appears to play an important role in the maintenance of normal acinar cell organization during recovery after pancreatico-biliary duct obstruction by preventing the congestion of pancreatic digestive enzymes. We cannot explain clearly at present the rise in lysosomal enzyme output during recovery, because one compartment of these lysosomal enzymes seems to be derived from the liver and the other from the pancreas, but one possible explanation is that pancreatico-biliary duct obstruction with hypersecretion causes more lysosomal enzymes to enter zymogen granules than in the normal stage. The increased secretion of both digestive enzymes and lysosomal hydrolase into pancreatico-biliary juice and the pancreatic ductal space and the redistribution of lysosomal enzyme in the pancreatic acinar cells may have special importance in the etiology of gallstone pancreatitis in humans. Gallstone attacks are often repeated, and after the first obstruction induced by a gallstone, if the secretion of a pancreatic secretagogue, such as cholecystokinin or secretin, is stimulated by food intake, colocalized digestive enzymes and lysosomal hydrolases can be secreted in increased amounts into the pancreatico-biliary juice together; but when the pancreatico-biliary duct system is obstructed by another stone or another attack, or if edema of the sphincter of Oddi persists, there is no way for these digestive enzymes and lysosomal hydrolases to drain into the pancreatico-biliary juice, and within the acinar cells, may be another redistribution of lysosomal enzymes.

Potential modulators of the severity of pancreatitis might be alterations in the pancreatic microcirculation[24], and changes in pancreatic ductal permeability induced by oral agents[25], which might permit mild pancreatitis to progress to a more severe form. Although the factors responsible for such a process are not yet clearly understood, the secretion of lysosomal enzymes, which can potentially activate pancreatic digestive enzymes, into pancreatico-biliary juice in bulk amounts seems to favor the conversion of the mild edematous form into severe hemorrhagic and necrotic pancreatitis, and is a possible trigger of pancreatitis in the "common channel" theory.

E3123 is a new synthetic agent composed of guanidino acid esters, which inhibits potently a number of proteases, such as trypsin, phospholipase A_2, kallikrein, plasmin, thrombin, etc.[18]. Its potent inhibitory ability and relatively low molecular weight encouraged us to evaluate its protective effects in this model. E3123 showed strong protective effects against almost all the changes induced by pancreatico-biliary duct obstruction and secretin infusion in a dose-dependent manner (2 mg/kg · hr and 5 mg/kg · hr). This new type of synthetic protease inhibitor may be useful in the treatment of acute pancreatitis, such as that due to gallstones, whereas another protease inhibitor, aprotinin, has been reported to have almost no protective effect against acute pancreatitis[26].

Acknowledgements

This study was supported by grants for scientific research (B-03454319 and C-01570754) from the Ministry of Education, Science and Culture, and a grant from the Ministry of Health and Welfare of Japan.

We thank Ms. Yoko Manabe for her beautiful typing of the manuscript and Ms. Kimiko Hirano for helping in its preparation.

References

1) Koike H, Steer ML, Meldolesi J: Pancreatic effects of ethionine; blockage of exocytosis and appearance of crinophagy and autophagy precede cellular necrosis. Am. J. Physiol. **242**: G297–307, 1982.
2) Ohshio G, Saluja AK, Leli U, Sengupta A, Steer ML: Esterase inhibitors prevent lysosomal enzyme redistribution in two noninvasive models of experimental pancreatitis. Gastroenterology **96**: 853–859, 1989.
3) Saito I, Hashimoto S, Saluja A, Steer ML, Meldolesi J: Intracellular transport of pancreatic zymogens during caerulein supramaximal stimulation. Am. J. Physiol. **253**: G517–526, 1987.
4) Saluja A, Hashimoto S, Saluja M, Powers RE, Meldolesi J, Steer ML: Subcellular redistribution of lysosomal enzymes during caerulein-induced pancreatitis. Am. J. Physiol. **253**: G508–516, 1987.
5) Saluja A, Saito I, Saluja M, Houlihan M, Powers RE, Meldolesi J, Steer ML: In vivo rat pancreatic acinar cell function during supramaximal stimulation with caerulein. Am. J. Physiol. **249**: G702–710, 1985.
6) Watanabe O, Baccino FM, Steer ML, Meldolesi J: Supramaximal caerulein stimulation and ultrastructure of rat pancreatic acinar cell; early morphological changes during development of experimental pancreatitis. Am. J. Physiol. **246**: G457–467, 1984.
7) Steer ML, Meldolesi J: The cell biology of experimental pancreatitis. N. Engl. J. Med. **316**: 144–150, 1987.
8) Figarella C, Miszczuk-Jamska B, Barrett AJ: Possible lysosomal activation of pancreatic zymogens. Activation of both human trypsinogen by cathepsin B and spontaneous acid activation of human trypsinogen I Biol. Chem. Hoppe-Seyler **369 (suppl)**: 293–298, 1988.
9) Greenbaum LM, Hirshkowitz A: Endogenous cathepsin activation of trypsinogen in extracts of dog pancreas. Proc. Soc. Exp. Biol. Med. **107**: 74–76, 1961.
10) Rinderknecht H: Activation of pancreatic zymogens. Normal activation, premature intrapancreatic activation, protective mechanism against inappropriate activation. Dig. Dis. Sci. **31**: 314–321, 1986.
11) Steer ML, Meldolesi J, Figarella C: Pancreatitis. The role of lysosomes. Dig. Dis. Sci. **29**: 934–938, 1984.
12) Acosta JL, Ledesma CL: Gallstone migration as a cause of acute pancreatitis. N. Engl. J. Med. **290**: 484–487, 1974.
13) Armstrong, CP, Taylor TV, Jeacock J, Lucas S: The biliary tract in patients with acute gallstone pancreatitis. Br. J. Surg. **72**: 551–555, 1985.
14) Dworken HJ: Recent experience with spontaneous disappearing gallstones. Gastroenterology **38**: 76–86, 1960.
15) Frei GJ, Grei VT, Thirlby RC, McClelland RN: Biliary pancreatitis clinical presentation and surgical management. Am. J. Surg. **151**: 170–175, 1986.
16) Molander DW, Bell ET: Relation of cholelithiasis to acute hemorrhagic pancreatitis. Arch. Pathol. **41**: 17–18, 1946.
17) Opie EL: The etiology of acute hemorrhagic pancreatitis. Bull. Johns Hopkins Hosp. **12**: 182–188, 1901.
18) Iwatsuki K, Horiuchi A, Yonekura H, Chiba S: Ischemia-induced acute pancreatitis in the dog and its amelioration by a new synthehtic protease inhibitor, E3123. Asia Pacific J. Pharmacol. **5**: 113–119, 1990.
19) Tartakoff A, Jamieson JE: Fractionation of guinea pig pancreas. Methods Enzymol. **31**: 41–59, 1974.
20) Bernfeld P: Amylase α and β. Methods Enzymol. **1**: 149–158, 1955.
21) Mcdonald JK, Ellis S: On the substrate specificity of cathepsin B_1 and B_2 including a new fluorogenic substrate for cathepsin B_1. Life. Sci. **17**: 1269–1276, 1975.
22) Rinderknecht H, Renner IG, Koyama HH: Lysosomal enzymes in pure pancreatic juice from normal healthy volunteers and chronic alcohlics. Dig. Dis. Sci. **24**: 180–186, 1979.
23) LaBarca C, Paigen R: A simple, rapid and sensitive DNA assay procedure. Anla. Biochem. **102**: 334–352, 1980.
24) Donaldson LA, Williams RW, Shenk Jr WG: Experimental pancreatitis; Effects of plasma and dextran on pancreatic blood flow. Surgery **84**: 313–321, 1978.

25) Wedgwood KR, Adler G, Kern H, Reber HA: Effects of oral agents on pancreatic duct permeability. A model of acute alcohlic pancreatitis. Dig. Dis. Sci. 31: 1081–1088, 1986.
26) Lankisch PG: Acute and chronic pancreatitis; An update on management. Drugs 28: 554–564, 1984.

和文抄録

ラットにおける短期間総膵胆管結紮のライソゾーム酵素に及ぼす影響：新合成プロテアーゼインヒビター E-3123 の保護効果について

京都大学医学部 第一外科学教室
平野 鉄也，真辺 忠夫，四元 文明，大塩 学而，仁尾 義則

共通管の閉塞が膵外分泌系に与える影響を検討する目的にて，ラットでの総膵胆管閉塞後の膵胆汁液中でのライソゾーム酵素とアミラーゼの分泌量を測定した．同時に，新合成 protease inhibitor である E-3123 (4-guanidinobenzoate methanesulfonate) の保護効果をも検討した．4時間の総膵胆管閉塞と secretin (0.2 CU/kg・hr) の投与により，門脈血中アミラーゼ値，カテプシンB値，膵水分量および膵アミラーゼ含有量の有意な上昇とともに膵腺房細胞内でのカテプシンBの再分布が観察された．これらの変化は，閉塞解除後も12時間は持続する傾向を示したが，24時間後にはほぼ消失した．E-3123 を 5 mg/kg・hr にて閉塞期間中投与することにより，これらすべての変化が有意に著明に改善し，膵外分泌系への保護効果を示したが，2 mg/kg・hr の用量にても 5 mg/kg・hr には劣るが有意な保護効果を示した．これらの結果は共通管閉塞性膵傷害の治療に E-3123 が有用であることを示唆させた．