<Original Articles>Effect of Ethanol on Pancreatic Lysosomes in Rats : A Possible Mechanism for Alcoholic Pancreatitis

AUTHOR(S):

HIRANO, TETSUYA; MANABE, TADAO

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Effect of Ethanol on Pancreatic Lysosomes in Rats: A Possible Mechanism for Alcoholic Pancreatitis

Tetsuya Hirano and Tadao Manabe

First Department of Surgery, Faculty of Medicine, Kyoto University, Kyoto 606, Japan

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Abstract

Four-hour infusion of ethanol (0.5 g/kg・hr) and additional 2-hour infusion of caerulein (0.2 μg/kg・hr) and secretin (0.2 CU/kg・hr) caused hyperamylasemia, pancreatic edema and pancreatic histological changes such as interstitial edema and acinar cell vacuolization in the rat. Such a combined treatment also induced a redistribution of lysosomal enzyme cathepsin B, from the lysosomal fraction to the zymogen fraction in subcellular fractionation. Only ethanol infusion caused marginal changes in all these parameters. Moreover, 4-hour pre-infusion of ethanol caused a significant increase in pancreatic cathepsin B output stimulated by caerulein (0.2 μg/kg・hr) and secretin (0.2 CU/kg・hr) compared with the control rats. This pretreatment of ethanol also caused a significant increase in cathepsin B/amylase output ratio. These results indicate that ethanol administration, if combined with exocrine stimulation, causes a colocalization of lysosomal hydrolases with digestive enzymes in the same subcellular compartment as well as in the pancreatic ductal space, and also suggest one possible mechanism for alcoholic pancreatitis both within and outside the acinar cells, since cathepsin B can activate trypsinogen.

Introduction

Alcohol has been reported to be one etiological factor in the pathogenesis of human pancreatitis[1,2], and abnormal histological findings in the pancreatic ductal system of alcoholic pancreatitis have also been reported[3]. However, little is know about the mechanism whereby alcohol induces pancreatic acinar and ductal injuries. Moreover, other factors such as gut hormones seem to be closely involved in the pathogenesis of alcoholic pancreatitis[4,5], and these additional factors seem to be needed to make the pancreas vulnerable to inflammation.

In this study, we evaluated the effect of ethanol on the exocrine pancreas, particularly in combination with pancreatic secretagogues such as caerulein and secretin, from several parameters including subcellular distribution of lysosomal enzymes and pancreatic secretion of lysosomal enzymes.

Key words: Ethanol, Lysosomal enzyme, Cathepsin B, Redistribution of lysosomal enzyme, Pancreatitis, Caerulein, Secretin

索引用語: エタノール，ライソゾーム酵素，カテプシンB，ライソゾーム酵素の再分布，膵炎，セルレイン，セクレチン

Present address: First Department of Surgery, Faculty of Medicine, Kyoto University, 54 Shogoin-Kawaracho, Sakyoku, Kyoto 606, Japan

Material and Method

Thirty seven male Wistar rats weighing about 300 g (Shizuoka Experimental Animals, Shizuoka, Japan) were used. All the rats were kept in light-dark cycle regulated (light; 5:00–17:00) and air-conditioned (23±3°C) animal quarters in our institute before the experiments, and were given free acess to tap water and diet (Oriental Rodent Chow, Tokyo, Japan). They were maintained throughout the study in accordance with the guidelines of the Committee on Animal Care of Kyoto University, and this study was approved by the committe. All the experiments were started at between 9:00 AM and 10:00 AM to rule out the effect of circadian rhythm on the exocrine pancreas.

After a 16-hour fast, under general anaesthesia with intraperitoneal injection of sodium pentobarbital (Nembtal®, Abbott Co., North Chicago, IL, U. S. A.) (30 mg/kg), a PE-50 catheter (Clay Adams, Parsippany, NJ, U. S. A.) was placed in the superior vena cava through the external jugular vein. At this point, all the rats were divided into the following two groups:

(a) Alcohol (ALC) group (n=21). Ethanol (Kanto Chemical Co., Tokyo, Japan) was infused at a dose of 0.5 g/kg · hr for 4 hours in heparinized (30 U/ml) saline at a rate of 0.58 ml/hr by an infusion pump (Truth Type B-6 Nakagawaseikodo, Tokyo, Japan).

(b) Control (CONT) group (n=16). Only heparinized saline was infused at a rate of 0.58 ml/hr for 4 hours.

In the ALC group, 8 rats (ALC+CER+SEC) were infused with both caerulein (0.2 μg/kg · hr) (Ceosunin Injection®, Kyowa Hakko Co., Tokyo, Japan) and secretin (0.2 CU/kg · hr) (Sigma Chemical Co., St. Louis, MO, U. S. A.) for additional 2 hour in heparinized saline at the same rate, and 7 rats (ALC+SAL) were infused only with heparinized saline for additional 2 hours.

In the CONT group, 5 rats (CER+SEC) were infused with both caerulein and secretin for additional 2 hour as in the ALC+CER+SEC group, and 5 rats (SAL) were infused only with heparinized saline for additional 2 hours.

During these 6-hour infusions, anaesthesia was maintained and the rats put on heating pad at 40°C to keep their core temperature.

At the selected times after these 6-hour infusions, rats were killed painlessly by a large dose of intravenous pentobarbital (80 mg/kg). After blood samplings from the inferior vena cava for the determination of serum amylase levels, the pancreas was removed quickly. One small portion of the pancreas from the splenic portion was fixed overnight by immersion in phosphate-buffered (pH 7.4) 10% neutral formalin, and pancreatic histological changes such as interstitial edema, acinar cell vacuolization and inflammatory cell infiltration were examined by a blinded observer on a 0 (no changes) to 4+ (maximum changes) scale.

About one third of the pancreatic tissue was used for the quantification of pancreatic water content by comparing the weight immediately after removal (wet weight) to that of the same sample after incubation at 150°C for 48 hours (dry weight) in a dessicator (Sanyo Drying Oven®, Sanyo, Tokyo, Japan).

The remaining portions of the pancreas were used for subcellular fractionation. The excised and trimmed pancreas was homogenized in cold (4°C) 5 mM MOPS (3-(N-morpholino) propanesulphonic acid) (Sigma Chemical Co.) buffer (pH 6.5) containing 1 mM $MgSO_4$ and 250 mM sucrose, and divided into its various subcellular fractions by differential centrifugations as described previously[6,7]. Briefly, the homogenate was centrifuged (150×g at 4°C for 10 min) to remove un-

broken cells and debris, and the resulting supernatant was cetrifuged (1300×g at 4°C for 15 min) to obtain a zymogen granule-rich pellet (1.3 KP) and a supernatant. This supernatant was centrifuged (12000×g at 4°C for 12 min) to pellet a lysosome-rich fraction (12KP) and a supernatant which is considered to be microsomal and soluble fraction (12KS). In each of these fractions, as a lysosomal enzyme, cathepsin B activity was measured and was expressed as a percentage of the total activity as an index of distribution of lysosomal enzymes in pancreatic acinar cells.

For the new rats of both the ALC and CONT group, next in-vivo secretion expreiments were performed. 8 rats in the ALC group (ALC+CER+SEC) and 6 rats in the CONT group (CER+SEC) were used. About one hour before the completion of these 4-hour ethanol or saline pre-treatment, laparotomy was performed and catheterization (PE-10) to the common hepatic duct for the biliary bypass to the duodenum was performed. Another catheterization(PE-10) to the pancreatico-biliary duct just adjacent to the duocenum was performed to collect pancreatic juice. After about 30-minute stabilization and also after 4-hour ethanol or saline pre-treatment, both ceerulein (0.2 μg/kg・hr) and secretin (0.2 CU/kg・hr) were infused in heparinized saline at a rate of 0.58 ml/hr for 2 hours to stimulate pancreatic secretion. Pancreatic juice was collected in preweighted eppendorf tubes on ice by an hour (fractions; F_1 and F_2). Pancreatic juice volume was calculated by direct weighting defining the specific gravity of juice as 1.0, and was expressed as ml/kg・hr. In collected pancreatic juice fractions, both amylase and cathepsin B activity were measured, and both the outputs were expressed as U/kg・hr.

Amylase activity was measured with blue starch (Shionogi Amylase A-Test, Shionogi Co., Osaka, Japan) as the substrate[8]. Cathepsin B activity was measured with N-benzyloxycarbonyl-arginyl-arginine-β-naphthylamide (Bachem Feinchemikallien AG, Budendorf, Switzerland) as the substrate[9].

The results represent the means±SEM for each value (n) obtained using a different animal. The significance of changes was eveluated using Student's t-test when the data consisted of two groups only, or by analysis of variance (ANOVA) when comparing three or more groups. If ANOVA indicated significant differences, the data was analyzed by using the TUKEY's method as a post hoc test for differences between the groups. $P<0.05$ was considered to be significant.

Results

Four-hour pre-infusion of ethanol and additional 2-hour infusion of caerulein and seretin (ALC+CER+SEC group) caused significant rises in serum amylase levels and in pancreatic water content compared with the control group (SAL group and CER+SEC group). Only ethanol infusion (ALC+SAL group) caused no significant changes. Histologically, samples from the ALC+CER+SEC group showed a mild, but significatnt, interstitial edema and slight, not significant, acinar cell vacuolization compared with the control group (SAL and CER+SEC group). Only ethanol infusion (ALC+SAL group) caused slight, not significant, interstitial edema (Table 1).

In the subcellular fractionation, 4-hour pre-infusion of ethanol and 2-hour infusion of both caerulein and secretin (ALC+CER+SEC group) caused a significnt decrease of cathepsin B activity in the lysosomal fraction (12KP; 40±3%) and a significant increase in the zymogen fraction (1.3KP; 42±3%) compared with the control group (SAL: 12KP; 59±2%, 1.3KP; 26±2%, CER+SEC: 12KP; 55±2%, 1.3KP; 28±2%), indicating a redistribution of lysosomal enzyme in pancreatic acinar cells. Only ethanol infusion (ALC+SAL group) still caused a partial significant redistribu-

Table 1 Effect of ethanol infusion in combination with infusion of pancreatic secretagogues on serum amylase levels, pancreatic water content and pancreatic histological changes in the rat (means ±SEM)

Group	n	Serum amylase levels (U/ml)	Pancreatic water content (% of wet weight)	Pancreatic histological changes		
				Interstitial edema	Acinar cell vacuoloization	Inflammatory cell infiltration
ALC+ CER+SEC	7	13±2*	83±2*	2+*(1~2) [1.6±0.2]	1+(0~1) [0.6±0.2]	0(0~1) [0.3±0.2]
ALC+SAL	6	8±1	78±2	1+ (0~1) [0.7±0.2]	0(0~1) [0.3±0.2]	0(0) [0]
CER+SEC	5	6±1	75±1	0(0) [0]	0(0) [0]	0(0) [0]
SAL	5	6±1	74±	0(0) [0]	0(0) [0]	0(0) [0]

ALC+CER+SEC: 4-hour pre-infusion of ethanol (0.5 g/kg · hr) and additional 2-hour infusion of caerulein (0.2 μg/kg · hr) and secretin (0.2 CU/kg · hr), ALC+SAL: 4-hour pre-infusion of ethanol and additional 2-hour infusion of heparinized saline, CER+SEC: 4-hour pre-infusion of heparinized saline, and additional 2-hour infusion of caerulein and secretin, SAL: 6-hour infusion of heparinized saline, As comapred with the CER+SEC and SAL group, *$p<0.05$.

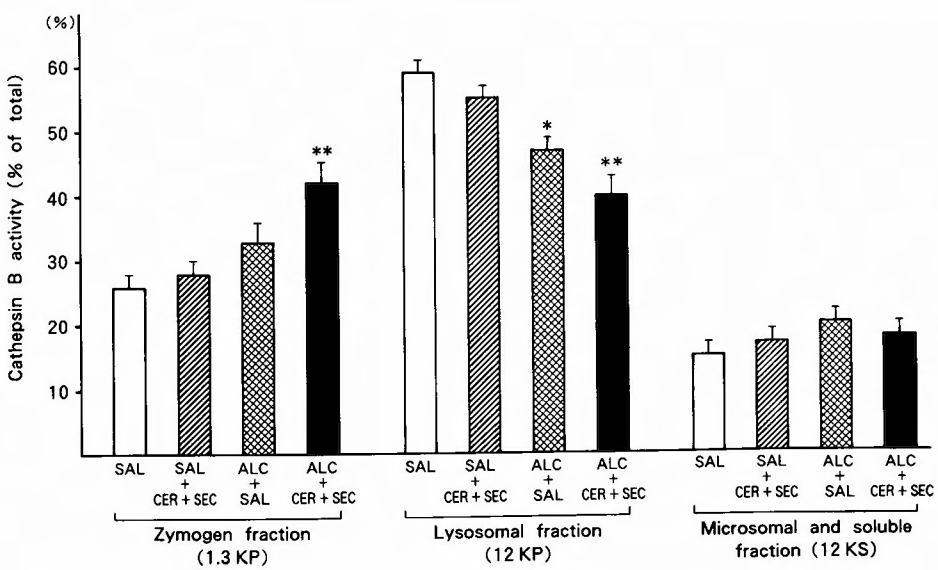

Fig. 1 Effect of ethanol infusion in combination with infusion of pancreatic secretagogues on subcellular distribution of lysosomal enzyme in the rat pancreatic acinar cells.
ALC+CER+SEC; 4-hour pre-infusion of ethanol (0.5 g/kg · hr) and additional 2-hour infusion of caerulein (0.2 μg/kg · hr) and secretin (0.2 CU/kg · hr) (n=7), ALC+SAL; 4-hour pre-infusion of ethanol and additional 2-hour infusion of heparinized saline (n=6), SAL+CER+SEC; 4-hour pre-infusion of heparinized saline and additional 2-hour infusion of caerulein and secretin (n=5), SAL; 6-hour infusion of heparinized saline (n=5), As compared with the SAL and SAL+CER+SEC group, *$p<0.05$ and **$p<0.02$.

tion (12KP; 47±2%, 1.3KP; 33±3%) (Figure 1).

In in-vivo secretion experiments stimulated by pancreatic secretagogues, there were no significant differences in pancreatic juice volume (ALC+CER+SEC group; 1.13±0.12 and 1.09±0.14 ml/kg・hr, CER+SEC group; 1.18±0.10 and 1.14±0.11 ml/kg・hr), nor in amylase output (ALC+CER+SEC group; 7976±752 and 7249±822 U/kg・hr, CER+SEC group; 8527±625 and 8361±673 U/kg・hr). However, 4-hour pre-infusion of ethanol (ALC+CER+SEC group) caused a significant rise in cathepsin B output into pacreatic juice (35±3 and 38±3 U/kg・hr) stimulated by caerulein and secretin compared with the control group (CER+SEC group; 19±2 and 17±2 U/kg・hr) (Figure 2a, b, c).

Reflecting these data, the cathepsin B output/amylase output ratio in the ALC+CER+SEC group was significantly higher (0.0044±0.0008 and 0.0051±0.0010) than in the CER+SEC group (0.0023±0.0006 and 0.0020±0.0009) (Figure 3).

Discussion

Four-hour pre-infusion of ethanol and additional 2-hour infusion of caerulein and secretin caused a mild, but significant, hyperamylasemia and pancreatic edema, and marginal, but also significant, histological changes such as interstitial edema. Moreover, subcellular fractionation study showed that such a combined treatment caused a redistribution of lysosomal enzyme from the lysosomal fraction to the heavier zymogen fraction, which was considered to represent a colocalization of lysosomal hydrolases with digestive enzymes in the same subcellular compartment. This col-

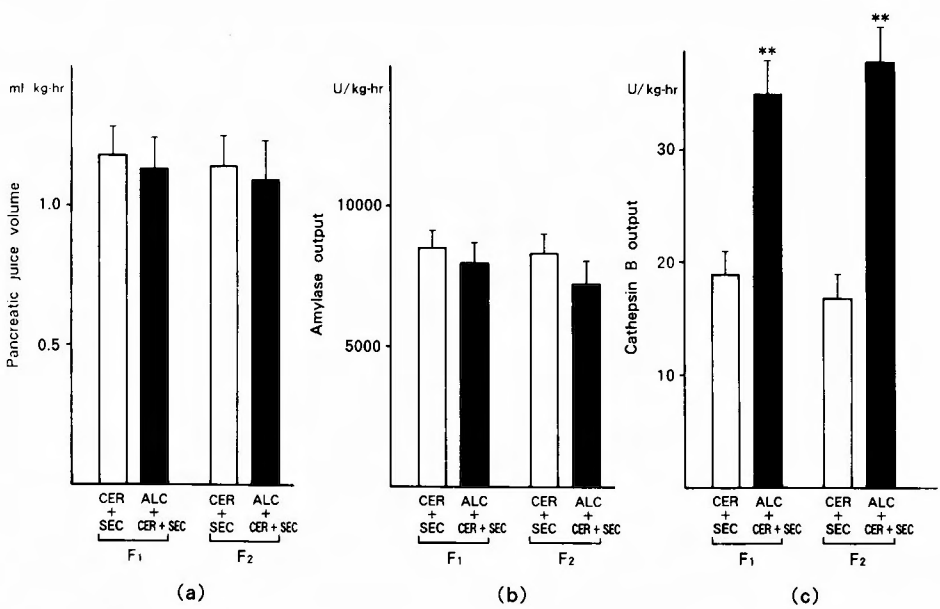

Fig. 2 Effect of ethanol infusion in combination with infusion of pancreatic secretagogues on pancreatic juice volume (a), amylase output (b) and cathepsin B output (c) in the rat.
ALC+CER+SEC; 4-hour pre-infusion of ethanol and additional 2-hour stimulation with caerulein and secretin (n=8), CER+SEC; 4-hour pre-infusion of saline and 2-hour stimulation with caerulein and secretion (n=6), As compared with the CER+SEC group, **$p<0.02$.

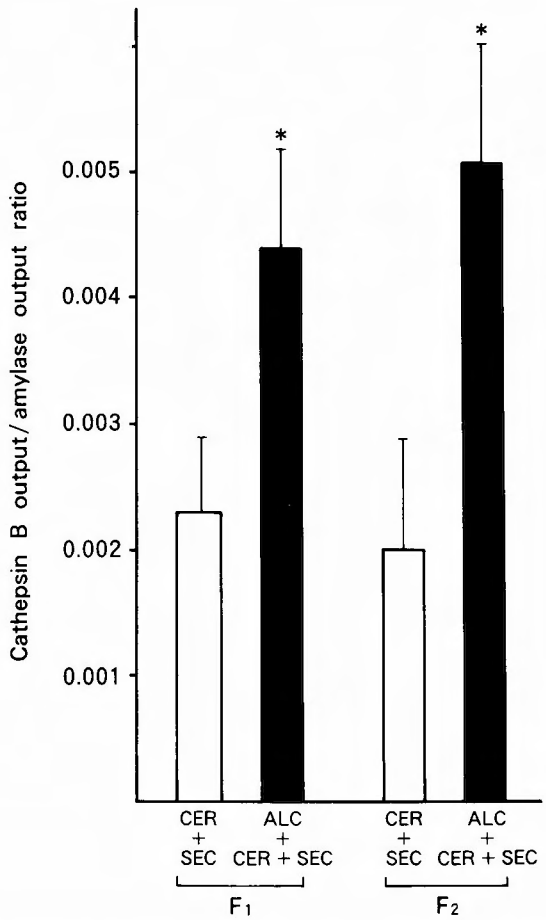

Fig. 3 Effect of ethanol infusion in combination with infusion of pancreatic secretagogues on cathepsin B output/amylase output ratio in the rat.
The symbols in the groups have the same meanings as in Figure 2. As compared with the CER + SEC group, $^*p < 0.05$.

ocalization phenomenon seems to play an important role in the pathogenesis of pancreatic injuries, since lysosomal enzyme, cathepsin B, can activate trypsinogen[10] and trypsin can activate other many digestive enzymes.

Although we have not examined the presence of free trypsin activity in the pancreas in this study, this colocalization phenomenon could lead to an intracellular activation of trypsinogen under appropriate conditions, which will give an opportunity for cathepsin B to exert its activity; such as acidification of these colocalized compartments.

On the other hand in this study, 4-hour pre-infusion of ethanol caused a significant increase in cathepsin B output into pancreatic juice stimulated by pancreatic secretgogues (caerulein and secretin), and also caused a significant increases in cathepsin B output/amylase output ratio compared with the control group. Previously we have reported the gut-hormone regulated secretion of lysosomal enzymes into pancreatic juice and colocalization of lysosomal enzymes with digestive en-

zymes in the same subcellular compartment; zymogen granules in the normal pancreatic acinar cells[11,12]. Thus our present data suggest the missorting of lysosomal enzymes induced by ethanol pre-treatment plus exocrine stimulation.

This augmented colocalization phenomenon of lysosomal enzymes with digestive enzymes in the pancreatic ductal space seems to play an important role in the pathogenesis of pancreatic ductal injuries. Although we have not examined the presence of free trypsin activity in pancreatic juice, nor pH's of pancreatic juice, nor visicosity of pancreacit juice in this study this augmented colocalization phenomenon could lead to an intraductular activation of trypsinogen under appropriate conditions.

The results in this study indicate that sustained high blood ethanol levels, when superimposed by exocrine stimulation with pancreatic secretagogues, will induce pancreatic inhuries including hyperamylasemia and pancreatic edema, and also suggest one possible mechanism for alcoholic pancreatitis probably via unbalaned protein concentration in pancreatic juice, or changed visicosity of pancreatic juice, or inappropriate activation of digestive enzymes in the ductal space and in the acinar cells, since only ethanol by itself can induce onyl marginal changes in the pancreas.

The effect of ethanol, particularly in combination with pancreatic secretagogues, on the biosynthesis and sorting of lysosomal enzymes in pancreatic acinar cells with need to be studied in the future study.

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和文抄録

ラットにおけるエタノールの膵ライソゾームにおよぼす影響
—アルコール膵炎のメカニズムについて—

京都大学医学部第一外科学教室

平野　鉄也，真辺　忠夫

4時間のエタノール静脈内投与 (0.5 g/kg・hr) とさらに2時間のセルレイン (0.2 μg/kg・hr) とセクレチン (0.2 CU/kg・hr) 投与により，ラットにおいて，高 amylase 血症，膵浮腫，組織学的に膵腺房細胞の空胞化が出現した．これらの処置により，ライソゾーム酵素である cathepsin B のライソゾーム分画よりチモーゲン分画への再分布も観察されるとともにセルレインとセクレチン刺激下での膵液中への cathepsin B 分泌量も増加した．エタノール単独群では軽度な変化のみ観察された．これらの結果は，エタノール投与と膵外分泌刺激により，膵腺房細胞中および膵管腔でのライソゾーム酵素と膵消化酵素の共存を意味するものであり，cathepsin B は trypsinogen を活性化し得ることより，アルコール性膵炎の解明にひとつの可能性を示唆するものと考えられた．