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EFFECTS OF ETHANOL FEEDING ON COLLAGEN SYNTHESIZING AND DEGRADING ENZYMES IN RAT PANCREAS

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Abstract—Collagen metabolism in the pancreas was investigated in male Wistar strain rats after 7 weeks of ethanol feeding. Compared with control rats, the ethanol-fed rats had a normal hydroxyproline content in the pancreas. However, prolyl hydroxylase activity and collagenolytic cathepsin activity were increased, though collagenase activity did not change. Both prolyl hydroxylase activity and collagenolytic cathepsin activity were inversely correlated with amylase activities. These findings were also confirmed in ethanol-pyrazole treated rats. These results suggest that the ethanol-induced pancreatic injury, even at an early stage, accelerates the collagen metabolism in the pancreas.

Chronic ethanol consumption is known to be associated with chronic diseases of the liver and pancreas. Several studies revealed that hepatic collagen synthesis and the degradation system were accelerated in the early stage of ethanol feeding, probably through the oxidative metabolism of ethanol [1–3]. However, the pathogenic mechanism whereby excessive intake of ethanol is related to injury of the pancreas, eventually leading to pancreatic fibrosis, remains to be elucidated. We previously showed that glycyl-prolyl dipeptidyl-aminopeptidase activity, which is regarded as a kind of collagen-peptidase, was significantly increased in rat pancreas after 4 weeks of ethanol feeding [4]. The present study was designed to evaluate the effect of chronic ethanol ingestion on collagen metabolism in the rat pancreas, and to assess whether the oxidative or nonoxidative metabolism of ethanol participates in pancreatic collagen metabolism.

MATERIALS AND METHODS

Male Wistar strain rats each weighing about 200 g were used. All animals received a nutritionally adequate liquid diet (Kurea Co., Osaka, Japan) as described previously [3, 4]. The control diet supplied 18% of the calories as protein, 35% as fat and 47% as carbohydrate (control group). The ethanol diet was similar to the control diet except that ethanol was substituted isocalorically for carbohydrate to provide 36% of the calories (ethanol group). In two additional groups, pyrazole (Nakarai Chemicals Ltd., Kyoto, Japan) was given 2 mmol/kg b.wt. daily in each liquid diet to control rats (pyrazole group) and ethanol-fed rats (ethanol-pyrazole group) [5]. The animals were fed for 7 weeks, and after fasting overnight all rats were killed under ether anesthesia by exsanguination from the aorta. The pancreas was quickly removed, washing in ice-cold 0.24 M sucrose and dissected free of fat and connective tissue.

Pancreatic prolyl hydroxylase activity was assayed by the method of Hutton *et al.* [6], using ^{3}H-labeled protocollagen as substrate. Briefly, the protocollagen was prepared from the 9-day-old decapitated chick embryos labeled with 3,4-[^{3}H]-proline (36.8 Ci/mmol: Amersham Co., U.S.A.). The specific activity of the prepared protocollagen was 56×10^{4} cpm/ml of protocollagen solution. To 0.2 ml of the substrate solution was added 0.2 ml of the supernatant of 10% homogenized pancreas, mixed with 1 ml of 0.1 M Tris–HCl buffer at pH 7.5 containing 1.25 mM *l*-ascorbic acid, 0.3 mM α-ketoglutarate, 0.08 mM $FeSO_4$, 0.4 mg catalase (Sigma Chemical Co., St Louis, MO), 2 mg bovine serum albumin and 0.1 mM dithiothreitol, and then the mixture was incubated for 60 min at 37°. The enzyme reaction was stopped by the addition of 0.4 ml of 50% TCA. The tritiated water of the reaction system was separated by the vacuum distillation method; 0.5 ml tritiated water was added to 10 ml of Aqueous Counting Scintillant (ACS-II: Amersham Co.) and was then counted in a liquid scintillation counter (Packard Tri-Carb 4640). Under this condition, the formation of $^{3}H_2O$ rose linearly up to 4 mg of protein content of the enzyme preparation. Prolyl hydroxylase activity was expressed as cpm/mg protein/hr.

Pancreatic collagenase activity was assayed, using ^{14}C-labeled neutral soluble rat skin collagen as substrate [3, 7]. Briefly, the specific activity of the prepared collagen was 2375 cpm/mg of collagen. The pancreas specimen was homogenized in 0.05 M Tris–HCl buffer at pH 7.8, containing 0.2 M NaCl and 5 mM $CaCl_2$. 0.2 ml of 10% homogenate was preincubated at 37° for 30 min with 0.1 ml of the same buffer containing 3 mM 4-aminophenylmercuric acetate, and then 0.2 ml of 2% ^{14}C-collagen solution was added and incubated for 18 hr at 35°. The enzyme reaction was stopped by the addition of 20 μl of 80 mM *o*-phenanthroline dissolved in 50% dioxane. After the addition of 0.5 ml dioxane, the reaction mixture was centrifuged at 10,000 *g* for 10 min at 4°. The radioactivity of the supernatant (0.5 ml) in Bray's solution was measured by a liquid scintillation

counter. Collagenase activity was expressed as cpm/mg protein/hr.

Pancreatic collagenolytic cathepsin activity was assayed using ^{14}C-labeled acid soluble skin collagen as the substrate [3, 8]. Briefly, the specific activity of the prepared collagen was 3960 cpm/mg of collagen. Pancreas specimens were homogenized in 0.1 M sodium acetate buffer at pH 4.0, containing 2 mM cystein and 2 mM EDTA. A 0.2% solution of the labeled acid soluble collagen (0.5 ml) was mixed with 0.5 ml of 10% pancreas homogenate. After incubation for 18 hr at 35° 1 ml of absolute ethanol was added. The mixture was centrifuged as 20,000 *g* for 20 min at 4°. The radioactivity of the supernatant (0.5 ml) in Bray's solution was measured by a liquid scintillation counter. Collagenolytic cathepsin activity was expressed as cpm/mg protein/hr.

Pancreatic amylase activity was measured using the Phadebas amylase test (Pharmacia Diagnostics Co., Sweden). Pancreatic contents of hydroxyproline and protein were determined by the method of Bergmann *et al.* [9] and Lowry *et al.* [10], respectively.

RESULTS

As shown in Table 1, the hydroxyproline content in the normal pancreas was 6.04 ± 0.38 (±SEM) μg/mg protein, indicating that the normal pancreas contains about 4.2 mg of collagen per g of wet tissue. Activities of amylase, prolyl hydroxylase, collagenase and collagenolytic cathepsin in the normal pancreas were 97.5 ± 10.3 × IU/mg protein, 271 ± 30 cpm/mg protein/hr, 1.65 ± 0.57 cpm/mg protein/hr and 1.57 ± 0.07 cpm/mg protein/hr, respectively. Ethanol feeding resulted in a significant decrease in body weight up to 70% of the control level ($P < 0.01$). Pancreas weight could not be measured, because all pancreatic tissue was not obtained. In the ethanol group, pancreatic amylase activity decreased up to 14% of the control value ($P < 0.01$). Pancreatic hydroxyproline content and pancreatic collagenase activity did not change, but pancreatic prolyl hydroxylase activity and collagenolytic cathepsin activity were significantly increased.

Ethanol given with pyrazole also resulted in a significant decrease in body weight up to 70% of the control pyrazole group ($P < 0.01$). In the ethanol-pyrazole group, the pancreatic hydroxyproline content did not change, but pancreatic amylase activity was decreased up to 11% of the control pyrazole group ($P < 0.01$). Pancreatic prolyl hydroxylase activity was significantly increased by about 1.6 times that of the control pyrazole group, and collagenolytic cathepsin was also slightly increased. However, pancreatic collagenase activity did not change. There were no differences in these parameters between the ethanol group and the ethanol-pyrazole group.

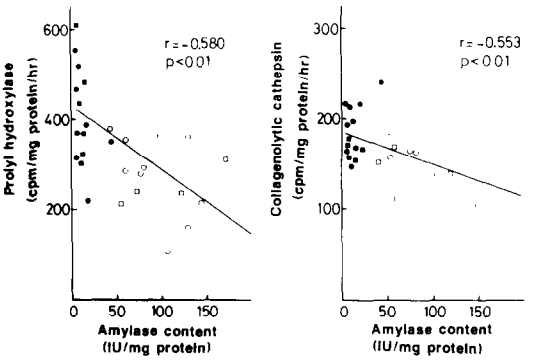

Fig. 1. Correlation of pancreatic amylase content to prolyl hydroxylase activity and collagenolytic cathepsin activity. (○: control, ●: ethanol, □: pyrazole, ■: ethanol-pyrazole).

As shown in Fig. 1, pancreatic prolyl hydroxylase activity and collagenolytic cathepsin activity were inversely related to pancreatic amylase activity, respectively. However, pancreatic prolyl hydroxylase activity was not correlated with pancreatic collagenolytic cathepsin activity.

DISCUSSION

Morphologically, chronic ethanol feeding to rats causes the accumulation of lipid droplets in pancreatic acinar and ductal cells, protein plugs in the pancreatic ducts, and fibrosis, associated with biochemical alteration of the pancreas such as a decrease in amylase and trypsin-inhibiting capacity [11–13].

Table 1. Effect of ethanol feeding with and without pyrazole on body weight, hydroxyproline content, amylase activity, prolyl hydroxylase activity, collagenase activity and collagenolytic cathepsin activity in rat pancreas

	Control	Ethanol	Pyrazole	Ethanol + pyrazole
No of rats	9	9	5	5
Body weight (g)	326 ± 10	229 ± 11**	268 ± 7	194 ± 10‡‡
Pancreas				
Protein (mg/g tissue)	98 ± 4	98 ± 6	96 ± 3	95 ± 4
Hydroxyproline (μg/mg protein)	6.04 ± 0.38	5.65 ± 0.33	5.15 ± 0.28	5.30 ± 0.37
Amylase (IU/mg protein)	97.5 ± 10.3	13.1 ± 4.5**	88.4 ± 20.6	9.80 ± 1.60‡
Prolyl hydroxylase (cpm/mg protein/hr)	271 ± 30	398 ± 33*	276 ± 26	429 ± 51†
Collagenase (cpm/mg protein/hr)	1.67 ± 0.57	1.29 ± 0.55	1.93 ± 0.40	1.59 ± 0.50
Collagenolytic cathepsin (cpm/mg protein/hr)	1.57 ± 0.07	1.94 ± 0.10**	1.31 ± 0.12	1.63 ± 0.05

Mean ± SEM. **$P < 0.01$, *$P < 0.05$ compared with control.
‡ $P < 0.01$, †$P < 0.05$ compared with pyrazole.

One of the earliest changes in pancreatic fibrosis seen after chronic ethanol consumption is periacinal fibrosis with infiltration of fibroblasts [14, 15]. However, little is known about the effect of ethanol consumption in pancreatic collagen metabolism.

We previously reported that the glycyl-prolyl dipeptidyl-aminopeptidase activity in the pancreas is significantly increased in rats after 4 weeks of ethanol feeding, correlating inversely with pancreatic amylase activity [4]. Glycyl-prolyl dipeptidyl-aminopeptidase is regarded as a kind of collagen peptidase, presumably participating in collagen degradation [16]. Therefore, collagen metabolism in the pancreas may be accelerated at the early stage of ethanol consumption. In the present study, the increased activity of prolyl hydroxylase and collagenolytic cathepsin correlated with pancreatic amylase depletion in ethanol and ethanol-pyrazole rats, suggesting that the ethanol-induced pancreas damage stimulates collagen synthesis, and also promotes the degradation of collagen in the pancreas.

Prolyl hydroxylase, located in the microsome, is the enzyme that hydroxylates peptide-bound proline in the process of collagen biosynthesis [17]. The activity is an index of collagen biosynthesis. Our present study demonstrated that prolyl hydroxylase activity in the rat pancreas was significantly increased by ethanol feeding. Although it is not clear what type of cell in the pancreas has prolyl hydroxylase activity, ethanol may accelerate collagen synthesis in pancreatic cells including acinar cells and ductal cells, which is induced by ethanol resulting in periacinar fibrosis. In the present study, the hydroxyproline content in the pancreas was not increased, indicating that collagenolysis in the pancreas is also accelerated at the early stage of ethanol ingestion.

Collagenase and collagenolytic cathepsin are known as the enzymatic systems for *in vivo* collagenolysis [18]. Pancreatic collagenase is known to be increased in a pathological condition such as acute necrotizing pancreatitis in rats [19]. Pancreatic cathepsin also has some pathophysiologic role in acute hemorrhagic pancreatitis [20]. The present study demonstrated the presence of collagenase activity and collagenolytic cathepsin activity in the pancreas. In the present study, following 7 weeks of ethanol feeding, pancreatic collagenase activity was not increased, but pancreatic collagenolytic cathepsin activity was significantly increased. We previously reported that at 4 weeks of ethanol feeding hepatic collagen synthesis was significantly increased, and collagenolytic cathepsin activity was significantly increased, though hepatic collagenase activity did not change [3]. Our findings on the liver and the pancreas show that ethanol feeding causes an increased synthesis of collagens, which could be efficiently removed intracellularly by the collagen peptidase and/or collagenolytic cathepsin, leading to no fibrosis.

An important factor responsible for hepatic injury by ethanol is considered to be the ethanol oxidative metabolites [21–24]. Ethanol itself does not stimulate collagen production in cultured liver and skin fibroblasts, lung fibroblasts or liver myofibroblasts [21, 25]. Our previous study indicated that hepatic collagen synthesis by ethanol ingestion is significantly related to hepatic GSH depletion, which is regulated by acetaldehyde generation [3]. However, the oxidative metabolism of ethanol is minimal in the pancreas, which is free of substantial acetaldehyde production [26]. Recently, many mammalian organs were found to metabolize ethanol through a nonoxidative pathway to form fatty acid ethyl esters, the esterification products of ethanol with various fatty acids. The pancreas manifests the highest concentration of fatty acid ethyl ester, which is markedly accelerated by ethanol ingestion, suggesting that fatty acid ethyl ester plays an important role in the development of pancreatic damage including fibrosis by ethanol feeding [27]. In fact, our study indicates that the increased collagen synthesis induced by ethanol is not inhibited by the administration of pyrazole, a specific inhibitor of ethanol, which inhibits the formation of acetaldehyde from ethanol *in vivo* [5]. Thus, these results suggest that unlike in the liver, collagen synthesis in the pancreas is mainly regulated by the nonoxidative ethanol metabolites rather than by its oxidative products.

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