Does chronic ethanol administration have influence on pancreatic regeneration in the course of caerulein induced acute pancreatitis in rats

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This study was undertaken in order to determine the influence of chronic ethanol administration on pancreatic regeneration during acute pancreatitis (AP). Rats were pair fed with isocaloric diet containing or not ethanol. After 8 weeks of such feeding AP was induced by sc injection of caerulein (Cae). 6 h, 24 h and 5 days after first Cae dose pancreatic weight, amylase, chymotrypsin, protein, RNA, DNA contents were determined and phosphatidic acid (PA) production in isolated pancreatic acini was measured. Proliferating cells were quantified by immunochemical staining of cells incorporating bromodeoxyuridine (BrdU).

Results:
Pancreatic weight was significantly higher at 6 h after first Cae injection in both, ethanol fed (EF) and control groups (C), however at 24 h pancreatic weight did not differ from prior to AP induction in EF rats. Ethanol feeding (EF) did not influence significantly protein, chymotrypsin and amylase content in pancreatic tissue in groups with AP. In EF rats RNA content after 5 days of AP was higher than in control animals. Total DNA content in EF rats with AP was lower 6 h after AP induction, earlier than in control animals with AP. Immunohistochemistry showed higher labelling index for BrdU after 6 h, 24 h and 5 days of AP in EF rats. In contrast to this findings, in EF animals, AP induction was not able to stimulate further PA accumulation.

Conclusion: We conclude that chronic ethanol feeding, while inhibiting PA accumulation in comparison to control group, does not impair pancreatic tissue regeneration during the early phase of Cae-induced AP. Stimulation of regenerative/reparative processes in EF rats during Cae-induced AP seems to be even more pronounced than in the control group.

Key words: ethanol feeding, acute pancreatitis, phosphatidic acid, pancreatic regeneration

Introduction

Long-standing alcohol abuse is associated with the development of chronic pancreatitis (1). Although the mechanisms by which ethanol leads to pancreatic damage remain unknown, there is growing evidence that ethanol exerts direct toxic effects on the pancreatic acinar cells (2—6). Certain toxic effects of ethanol have been attributed to oxidative metabolite of alcohol, acetaldehyde.
However pancreas shows only minimal oxidative ethanol metabolism, therefore acetaldehyde induced pancreatic damage is considered unlikely (7). Recently it was shown that pancreatic injury may be induced by fatty acid ethyl ester, a nonoxidative metabolite of alcohol (5, 6). On the other hand it is known that ethanol exerts its pharmacological effects on the lipids of the cell membrane and can cause alteration in the function of integral membrane proteins (8—10). Recent evidence suggests that chronic alcoholic pancreatitis may develop as a result of repeated necro-inflammatory episodes of acute pancreatitis (11, 12).

It is known that the injury to the pancreatic tissue during acute pancreatitis or other destructive processes is followed by a spontaneous reparative/regenerative process (13—15), however the mechanisms and agents involved in the tissue recovery are still poorly clarified. The data concerning pancreatic regeneration after chronic ethanol feeding are few and controversial (16, 17).

The activation of phospholipase D (PLD) in the exocrine pancreas has been described (18, 19). This enzyme stimulates phosphatidic acid (PA) production from phosphatidylcholine (PC), but in the presence of ethanol phosphatidylethanol (PEt) is generated as a result of transphosphatidylation. The functional significance of transphosphatidylation is unknown. PA has been recognized in some cell systems as a mitogenic factor, acting by calcium mobilization. Regarding the pancreas, an increase of PA production was observed after caerulein (Cae) infusion or in response to growth—associated processes stimulated by pancreatic juice diversion, subtotal pancreatectomy or in the regenerative period after acute pancreatitis (20, 21). The inhibition of PA production in the pancreas after acute alcohol ingestion was shown in vitro and in vivo (22). It was suggested, that the reduction of PA accumulation contributes to ethanol-induced impairment of pancreatic regeneration.

Recently we have shown that chronic ethanol feeding can increase the basal PA production in pancreatic acinar cells, however we observed also the lack of PA accumulation after growth stimulated processes such as Cae infusion or subtotal pancreatectomy (23). On the other hand we were not able to notice any significant changes in other parameters of pancreatic regeneration (pancreatic weight, protein, chymotrypsin, DNA and RNA content) after chronic ethanol treatment. The tissue degeneration in acute pancreatitis and a great reparative ability of the pancreas has been described mainly after caerulein-induced acute pancreatitis. Since increase of phosphatidic acid was usually associated with pancreatic regeneration, one can suspect that chronic ethanol treatment could impair pancreatic recovery after acute pancreatitis by depletion of phosphatidic acid and its replacement with PEt. This could be one of the mechanisms considered in the pathogenesis of chronic alcoholic pancreatitis, which may develop as a result of repeated acute episodes. In view of all those findings and hypotheses the aim of the present study was to
determine the influence of chronic ethanol administration on pancreatic regeneration after caerulein-induced acute pancreatitis in rats.

MATERIALS AND METHODS

Materials

Bovine serum albumine (BSA; fraction V and fatty acid free), soybean trypsin inhibitor type 2-S (SBTI), N-2-hydroxylethyl piperazine-N\(^{-1}\)-2-ethane sulfonic acid (HEPES), standards and solvents for thin-layer chromatography (TLC) and for enzymes and nucleic acid determination were purchased from Sigma (ST. Louis, MO). Purified collagenase was from Worthington Biochemical’s (Freehold, NJ). Silica gel TLC plates were obtained from Merck (Warsaw, Poland). \(^{3}\)H myristic acid (56 Ci/mmol) was from Du Pont (Wilmington, DE). Caerulein was from Sigma (ST. Louis, MO). Mouse monoclonal antibodies against 5-bromo-2’-deoxyuridine (BrdU) and ABC kit for immunolocalization of BrdU were purchased from Dako (Glostrup, Denmark).

Animals

Male Wistar rats weighing 200—220 g (n = 48) were housed in separated cages in rooms maintained at 20\(^{\circ}\) ± 2\(^{\circ}\)C using a 12-hour dark cycle. Care was provided in accordance with the procedures outlined in the National Institute of Health Guide for the Care and Use of Laboratory Animals (Bethesda, MD).

Experimental design

Animals were pair fed for a period of 8 weeks, isocaloric amounts of one of two diets: (i) a nutritionally adequate control diet (control rats C) or (ii) a nutritionally adequate diet containing ethanol as 36% of energy (ethanol rats EF), according to the formulation of Lieber and DeCarli (24). In this model of ethanol feeding daily ethanol intake is usually 10—12 g. Every week the animals were weighted. After 8 weeks of ethanol feeding the rats were divided into 8 groups (1 group without pancreatitis and 3 groups of pancreatitis each in control and ethanol fed rats). Acute pancreatitis (AP) was induced by sc. injections of caerulein 12 µg · kg body weight\(^{-1}\) every 8 h for 2 days. This analogue of cholecystokinin (CCK) was dissolved in gelatin (16% w/v) to prolong its absorption. The control animals were injected with saline in gelatine sc. in equivalent volumes. 1 hour before sacrificing rats were injected intraperitoneally with BrdU 50 mg/kg body weight freshly dissolved in PBS.
The experiment was finished respectively at 6 and 24 h and 5 days after first caerulein injection, rats were decapitated, pancreas was dissected as quickly as possible, free of connective tissue, lymph nodes and weighted.

The representative specimens of pancreatic tissue from 5 rats from each group were fixed in 10% buffered formaline and embedded in paraffin. The proliferating cells (S-phase cells), which nuclei incorporated BrdU (a thymidine analogue), were detected using mouse anti-BrdU monoclonal antibody and LSAB-HRP kit (secondary biotinitated antibody in streptavidin-peroxidase complex) and visualised with DAB. Random sections were scored at 40-x magnification. Labelling indices (LI) of acinar cells were calculated as the ratios (expressed as percentage) of BrdU-labelled cells to the total numbers of cells counted.

Separate sections were stained with haematoxylin and eosin (H&E). The histological grading of necrosis (degranulation, fragmentation) and vacuolisation refers to the approximate percentage of cells involved: 0 – absent, (±) <5%, (+)- 5—15%, (++)- 15-30%, (+++)- > 30%. The grading of oedema and inflammatory alterations and tubular fibrosis refers to a scale ranging from (±) as minimal to (++++) as maximal alteration.

Preparation of pancreatic acini

Acini were prepared as reported by Peikin et al. (25) from 300 mg pieces of pancreatic tissue. Acini from each pancreas were resuspended in 4 ml of an enriched HEPES buffered solution ([in mM] 24.1 HEPES, 98 NaCl, 6 KCl, 2.1 KH2PO4, 0.5 CaCl2, 1.2 MgCl2, 5 sodium pyruvate, 5 sodium fumarate, 5 sodium glutamate, 11.4 glucose, and 0.01% [w/v] SBTI, 0.5% [w/v] BSA fatty acid free, adjusted to pH 7.4).

Assays

The pieces of pancreas were homogenized using a motor-driven ground-glass homogeniser either in 0.6 mol/L perchloric acid for nucleic acids determination or in ice-cold Tris-HCl buffer (pH = 8.0) for protein, α-amylase and chymotrypsin assays. Protein was determined as described by Lowry et al. (26), with BSA as a standard. RNA and DNA were extracted as described by Mainz et al. (27). DNA was determined according to Volkin and Cohn using calf thymus DNA as the standard (28). RNA was hydrolysed overnight in 0.3 N KOH and measured by determining the absorption at 260 nm of the final 0.1 N PCA extract, as described by Munro and Fleck (29). Tissue α-amylase and chymotrypsin activities were determined according to Bernfeld and Hummel respectively (30, 31).
PA measurement

After 1 h of incubation with 5 µCi/ml of \( {}^3 \)H myristic acid, the 4 ml of acini in each flask was washed twice and resuspended in freshly oxygenated medium containing 200 µM propranolol, to favour PA accumulation. PA was measured after 20 min of incubation (18). At the end of each time period, 1 ml of acini was removed and quickly centrifuged at 10,000 g in a microcentrifuge for 15 s. The supernatant was removed, the pellet washed with incubation medium, then 2 ml of methanol: 10 mM glycine (5:2 v/v) was added and cells were detached mechanically with a spatula. Lipid phase was extracted with chloroform (32). Radioactivity present in the chloroform phase was determined. PA was separated in a solvent system containing chloroform: acetone: methanol: acetic acid: water (50:20:15:10:5, vol/vol) (18, 33). After separation and exposure to iodine vapour, the area containing PA was scraped, and radioactivity was counted. Radioactivity in PA was expressed as a percentage of total radioactivity in the chloroform phase.

![Graph](image-url)

*Fig. 1. Effect of acute pancreatitis and 3 days of rest on pancreatic weight in control and ethanol treated rats. Values are the means ± SD of six rats per group. Pancreatitis induction is described in methods section. * significantly different from control values prior to pancreatitis induction ^ significantly different from control treated animals at 6 h ** significantly different from ethanol treated animals prior to pancreatitis induction
**Statistical analysis**

Data are expressed as mean ± SD. Statistical analysis was performed using Student’s t test. P values < 0.05 were considered significant.

**RESULTS**

**Body weights**

All animals were pair fed and weighted every week. Body weight of the rats during 8 weeks of ethanol feeding did not significantly differ. The body weights of rats 5 days after pancreatitis induction decreased significantly in comparison to the time before induction by 9.5%, because of reduction of their food consumption during pancreatitis by 27%. However their pair fed controls received at this time reduced amount of liquid diet and the body weight of rats after acute pancreatitis induction was comparable in both groups at the same time point (control/ethanol- 302 ± 8.5 g / 300 ± 9.4 g; 6 h after first caerulein injection — 299.5 ± 10.8 / 287 ± 10.5 g; 24 h after first caerulein injection — 288.4 ± 12.3 / 268 ± 8.9 g, and 5 days after first caerulein injection — 273 ± 11.3 / 269 ± 11.3 g)

**Pancreatic weight and total protein, enzyme and nucleic acid content during acute pancreatitis**

*Pancreatic weight.* In animals fed with control diet pancreatic weight significantly increased at 6 and 24 h after first caerulein injection and significantly decreased at 5 days after first Cae injection, in comparison to the time before pancreatitis induction. This effect was not as marked in ethanol fed groups, since 24h after first Cae injection pancreatic weight did not significantly differ from ethanol fed animals receiving saline injection. After 5 days of pancreatitis, pancreatic weight in both, control and ethanol fed group was significantly lower than before pancreatitis induction (*Fig. 1*).

*Protein, chymotrypsin and amylase content.* After 24 h and 5 days of acute pancreatitis the significant decrease of protein content (by 52 and 57% respectively) in pancreas of control groups (fed without ethanol) was found. Chronic ethanol treatment did not influence this effect; in ethanol fed groups significant (45 and 54% respectively) decrease of protein content was observed after 24 h and 5 days of pancreatitis. (*Fig. 2*). Pancreatic chymotrypsin content decrease significantly after 24 h and 5 days in control groups by 47 and 32%, and in ethanol treated animals by 35 and 47% respectively (*Fig. 2*). Acute pancreatitis caused significant decreases of total amylase content after 24 h and
5 days of pancreatitis by 82 and 73% respectively. Since total amylase content in ethanol treated animals prior to pancreatitis induction was significantly lower in comparison to control groups, thus in ethanol fed animals the decrease of amylase content observed during pancreatitis was not statistically significant (Fig. 2).

**Nucleic acid content.** Caerulein induced acute pancreatitis resulted in significant decreases in total RNA content in control fed animals after 24 h and 5 days of acute pancreatitis (52 and 45% respectively). In ethanol fed animals similar effect was also observed (by 49 and 32% respectively). However as was shown in Fig. 3, in ethanol treated group RNA content after 5 days of AP was significantly higher than in the control fed rats. Total DNA content was significantly lower in control treated animals 24 h and 5 days after pancreatitis induction (by 36 and 42% respectively), and ethanol treatment did not

### Table 1.

<table>
<thead>
<tr>
<th>Time after first caerulein injection</th>
<th>Control animals</th>
<th>Ethanol fed animals</th>
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<td>0 hours (control)</td>
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<td>V (-)</td>
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Light microscopic study: O – oedema; V – vacuolisation of acinar cells; II – inflammatory infiltration (mononuclear cells); N – necrosis; TF – peritubular fibrosis + < 5% + 5—10% ++ 10—30% +++ > 30%.
significantly affect this parameter (the observed values were comparable: by 53 and 41% respectively). However in ethanol treated group total DNA content was significantly lower (34%) 6 h after pancreatitis induction, earlier than in control group.

Fig. 2. Effect of acute pancreatitis and 3 days of rest on total contents of protein, amylase and chymotrypsin in pancreas. Values are the means ± SD of six rats per group. Pancreatitis induction is described in methods section. * significantly different from respective controls prior to pancreatitis induction o significantly different from control treated rats at the same time of observation
Light microscopic study

As shown in Table 1, 8 weeks of ethanol feeding did not significantly affect pancreatic histology. Six hours after first Cae injection the vacuolisation of acinar cells was more pronounced in ethanol treated group in comparison to control one. 24 h after the first Cae injection the histological changes were also very similar in control and ethanol treated groups, however after ethanol treatment, the lower degree of oedema, and higher of vacuolisation and necrosis was observed. 5 days after AP induction the acinar cells vacuolisation and tubular fibrosis were still more pronounced in EF group, suggesting the deeper degree of tissue destruction.

PA accumulation

As shown in figure 4 chronic ethanol feeding was not able to stimulate PA accumulation in EF rats in contrast to control group.
Immunochemistry

Pancreatic acinar cells prior to pancreatitis induction showed only a few BrdU-positive nuclei (0.95% in control treated and 0.65% in ethanol treated rats). In control animals labelling index (LI) measured by BrdU incorporation at 6 h, was similar to that before pancreatitis induction, but it increased significantly 24 h and 5 days after AP induction. However, in ethanol treated group, BrdU LI was significantly higher at 6, 24 h and 5 days after pancreatitis induction in comparison to control treated rats at respective time points and also in comparison to values prior to AP, thus confirming the earlier and stronger induction of the recovery process in ethanol treated animals (Fig. 5).

DISCUSSION

We present the data on the influence of chronic ethanol treatment on trophic parameters in pancreas during the early phase of pancreatic regeneration after acute pancreatitis. The capability of pancreas to regenerate is observed after injuring processes as acute pancreatitis and subtotal pancreatectomy. Pancreas growth could be stimulated by Cae infusion in the trophic doses. It is known

Fig. 4. Effect of acute pancreatitis and 3 days of rest on PA accumulation in pancreatic acini in control and ethanol treated rats. Acini were prepared as described in method section. PA was expressed as a percent of total radioactivity incorporated into chloroform fraction. Values are the means ± SD of six rats per group. Pancreatitis induction is described in methods section. * significantly different from respective controls prior to pancreatitis induction o significantly different from control treated rats at the same time of observation.
that ethanol-induced injury to the pancreas evokes a regenerative/reparative process, which is characterized by a series of morphological and biochemical adaptative responses in subcellular organelles and by an increase in protein content and DNA replication. An inadequate or excessive regenerative response could be of a key importance in perpetuating tissue injury in the alcoholics (16). Most studies describing the influence of ethanol on pancreatic tissue have focused on the analysis of chronic ethanol administration on various cellular events. It is known that ethanol can affect intracellular calcium homeostasis (34), modify protein synthesis (3, 35), cause premature zymogene activation or the alteration in membrane lipids (36) and selectively impair endocytosis in the rat pancreas (4).

Recently we have demonstrated a significant increase in basal phosphatidic acid accumulation after chronic ethanol feeding in comparison to the control group and the disability of Cae to increase this level (23). Furthermore, in the ethanol treated group, we have observed the lack of PA accumulation in the pancreas subsequently to the growth-associated processes as Cae infusion and subtotal pancreatectomy. However 6 weeks of chronic ethanol feeding did not cause any significant changes in other trophic parameters such as DNA, RNA, protein and chymotrypsin content (23). Since the involvement of PLD-derived PA in the early stages of pancreatic regeneration was postulated, we suggested that chronic ethanol feeding can affect this process, probably by the inhibition of hydrolytic PLD activity and production of PEt instead of PA. In some cell types PA acts as a mitogenic agent, involved in phagocytosis, respiratory burst,
proliferation etc. (37, 38). On the other hand Cae, the most potent trophic factor for rat pancreas, significantly stimulates PA accumulation in pancreatic acini. The increase of PA was also observed in growth-associated processes such as subtotal pancreatectomy, Cae infusion and acute pancreatitis (20, 21). Therefore it was intriguing to speculate that the reduction of PA accumulation contributes to ethanol-induced impairment of pancreatic regeneration after acute pancreatitis, especially since ethanol concentration achievable in vivo in the blood of humans (0.1-0.6%) provides sufficient acceptor to support transphosphatidylation (0.3—2%). Present data however do not support this concept. Total DNA, RNA, protein, amylase and chymotrypsin contents in control animals were significantly lower than before pancreatitis induction. In the control animals we have shown the significant increase of PA accumulation, especially 6 h after AP induction, followed by the significant increases of PCNA and BrdU LI, suggesting that parallely to the pancreatic tissue destruction caused by pancreatitis, the regenerative/reparative process is induced. This is in agreement with the other studies concerning the regeneration process after pancreatitis. Surprisingly, we were not able to observe any decrease in pancreatic regeneration parameters in the EF animals, except of significant inhibition of PA production during pancreatitis. It must be noted however, that in this group the significant increase of this parameter was observed before Cae injection. Pancreatic wet weight during pancreatitis was significantly higher at 24 h and lower at 5 days in control group. In ethanol treated rats we were not able to see any increase in pancreatic weight after 24 h. After 5 days of pancreatitis however, this parameter was similar to the control group, and lower than before AP induction. Protein and chymotrypsin contents were comparable in both ethanol treated and control animals, RNA content after 5 days of AP increased significantly in comparison to control treated rats, being still lower than before pancreatitis. Total DNA content in ethanol treated animals decreased significantly earlier than in control rats (6 h after first Cae injection vs. 24 h), what can suggest more pronounced loss of pancreatic tissue, what was confirmed by earlier necrosis shown in light microscopic study (table 1). LI for BrdU showed significant increase over the control treated groups at 6, 24 h and 5 days, indicating that stimulation of the regenerative/reparative processes in ethanol treated animals during caerulein-induced acute pancreatitis starts earlier and is slightly more pronounced than in control fed rats.

One can speculate that after chronic ethanol feeding, an over expression of PLD activity could be observed under basal conditions, similarly to the over expression of this enzyme activity in the lymphocytes of alcoholics (39). However this activity is not stimulated by Cae infusion, subtotal pancreatectomy (23) or by tissue destruction during acute pancreatitis (present data). Under experimental conditions we have used, it can be suspected that repeated
cellular impairment caused by chronic ethanol administration could be a source of pancreatic injury, and thus could stimulate an early phase of pancreas regeneration after AP independently of PLD activation and PA production. Light microscopic study, showed the higher degree of tissue destruction after pancreatitis induction in ethanol treated rats, thus confirming this explanation.

Recent data by Ponappa et al. (40) suggest that pancreata from rats fed with ethanol for 9—12 months were more susceptible to Cae-induced chymotrypsinogen activation compared to the pancreata from pair-fed controls. In contrast, up to 3 months of ethanol consumption, there was the resistance to Cae-induced changes. The relatively short time of observations after AP induction in our study, does not exclude the possibility that full pancreatic recovery could be delayed in ethanol fed rats. In this early phase however we observed the stimulation of some regenerative parameters, what suggest that recovery process under this condition is not necessary dependant on PLD activation and PA production, as it was shown are some other cells (37, 38). Chronic ethanol ingestion in rat model was also shown to increase cell regeneration of the upper gastrointestinal tract and of the rectum (41, 42). These alcohol-associated effects are supposed to be due to a direct local alcohol action, or mediated by neural stimuli and/or gastrointestinal hormones.

In summary we conclude that chronic ethanol feeding, while significantly inhibiting PA production, does not impair pancreatic tissue regeneration during the early phase of experimental, Cae-induced acute pancreatitis. This suggests that PLD and PA are not evidently involved in the regeneration after caerulein induced AP in ethanol fed rats. The influence of other factors such as polyamines synthesis, the increase of tyrosine kinase activity (21) or other unknown mechanism cannot be excluded.

Acknowledgment: the Grant 4 PO5B 073 10 offered by the Committee of Sciences in Poland has partly financially supported this study.

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Received: October, 18, 2001

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