

Chronic ethanol consumption increases the fragility of rat pancreatic zymogen granules

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Abstract

Intracellular activation of pancreatic digestive enzymes by lysosomal hydrolases is thought to be an early event in the pathogenesis of pancreatic injury. As ethanol excess is an important association of pancreatitis, experimental work has been directed towards exploring possible mechanisms whereby ethanol may facilitate contact between inactive digestive enzyme precursors and lysosomal enzymes. The aim of this study was to find out if chronic ethanol administration increases the fragility of rat pancreatic zymogen granules. Sixteen male Sprague-Dawley rats were pair fed ethanol and control liquid diets for four weeks. Zymogen granule fragility was then assessed in pancreatic homogenate by determination of (a) latency and (b) per cent supernatant enzyme after sedimentation of zymogen granules. Amylase was used as a zymogen granule marker enzyme. Latency was significantly reduced in pancreatic homogenates of ethanol fed animals suggesting increased zymogen granule fragility. In support of this finding, there was a trend towards increased supernatant enzyme after ethanol feeding. In conclusion, administration of ethanol increases the fragility of pancreatic zymogen granules in the absence of morphological evidence of pancreatic injury. It is proposed that zymogen granule fragility may play an early part in the pathogenesis of alcoholic pancreatitis by permitting contact between digestive and lysosomal enzymes.

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to find out if chronic ethanol consumption also increases the fragility of pancreatic zymogen granules.

Methods

ANIMALS AND EXPERIMENTAL DIETS

Sixteen male, littermate Sprague-Dawley rats (100-130 g) were pair fed nutritionally adequate liquid diets based on the formulation of Lieber and DeCarli⁵ containing either ethanol as 36% of energy or an isocaloric amount of carbohydrate for four weeks. At the end of the feeding period, the animals were killed by decapitation and the pancreas was quickly removed, debrided of fat and connective tissue in ice cold saline, and used for assessment of zymogen granule fragility.

ASSESSMENT OF ZYMOGEN GRANULE FRAGILITY

The principles for the assessment of zymogen granule fragility are similar to those used for studies of lysosomes.⁴ Zymogen granule fragility was assessed in two ways.

Determination of latency (Figs 1A and B) – latency is defined as the per cent increase in marker enzyme activity over initial enzyme activity in the sample after the addition of the detergent Triton X-100 (which disrupts all biological membranes). Latency was calculated as follows:

$$\frac{(\text{enzyme activity after Triton}) - (\text{enzyme activity before Triton})}{(\text{enzyme activity before Triton})} \times 100$$

Increased zymogen granule fragility is shown by a decrease in latency.

Determination of the proportion of total enzyme released into the supernatant after sedimentation of zymogen granules by centrifugation at 100 000 g for 30 minutes at 4°C (Beckman L8-70M Ultracentrifuge with TY65 rotor). Increased zymogen granule fragility is shown by an increase in supernatant enzyme.

PRELIMINARY EXPERIMENTS

To determine the optimum cellular fraction and in vitro conditions for assessment of zymogen granule fragility, preliminary experiments were conducted using pancreas from chow fed male Sprague-Dawley rats. Zymogen granule fragility was compared in pancreatic homogenate, a crude zymogen granule pellet, and a purified zymogen granule fraction.

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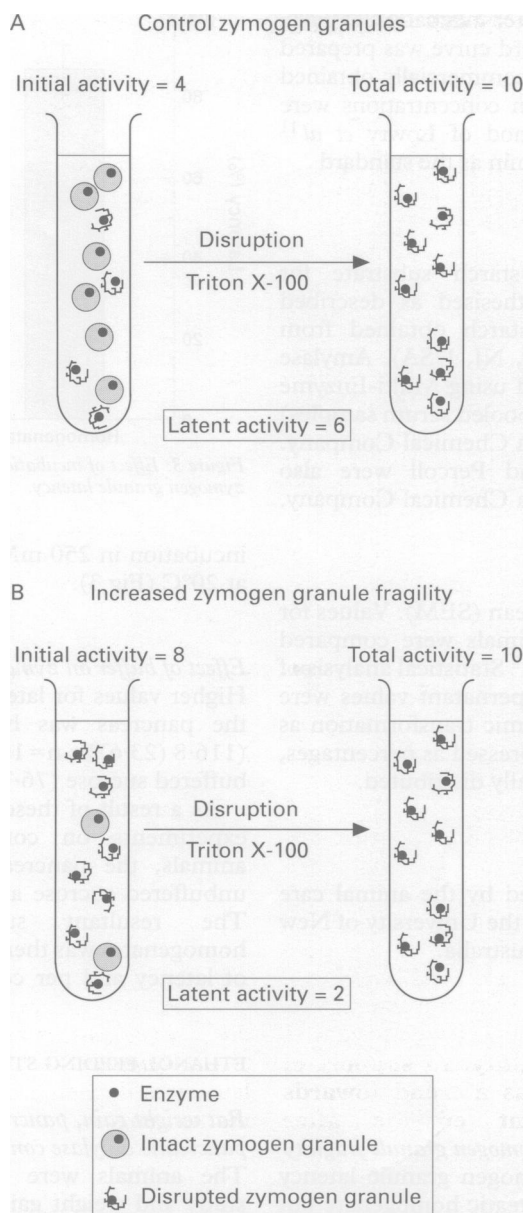
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Alcoholic pancreatitis is, at least in part, an autodigestive phenomenon.^{1,2} Recently, Steer *et al*³ have shown that colocalisation of digestive and lysosomal enzymes occurs in several forms of experimental pancreatic injury. This phenomenon may be a factor in the intracellular initiation of pancreatic autodigestion, as the lysosomal protease cathepsin B is capable of activating trypsinogen.

We have recently reported that chronic consumption of ethanol increases the fragility of pancreatic lysosomes⁴ – a change that may facilitate contact between lysosomal and digestive enzymes within the acinar cell. Digestive enzyme precursors are, however, normally segregated from the remainder of the acinar cell within membrane bound zymogen granules. Therefore, the aim of this study was



Figures 1A and 1B: Latency studies: in control samples, the available (pre-Triton) enzyme activity is low because enzyme within intact zymogen granules is not accessible to the assay substrate. Disruption of granules by Triton X-100 permits measurement of total enzyme activity in the sample. The difference between total and available enzyme activity is the latent enzyme activity of the sample. Under conditions of increased zymogen granule fragility, pre-Triton enzyme activity is high because the disrupted granules permit the substrate access to the enzyme. Total enzyme activity remains unchanged, however, and, as a result, latency of the sample is reduced. Thus, increased zymogen granule fragility is shown by a decrease in latency.

The pancreas was homogenised in 25 ml ice cold buffer pH 7.0 (250 mM sucrose, 5 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS), 0.1 mM MgSO₄, and 0.1 mM phenylmethylsulfonyl fluoride (PMSF)) using four up and down strokes of a Potter Elvehjem homogeniser (700 rpm) equipped with a 30 ml chamber and a Teflon plunger (B Braun, Germany). In later studies directed towards further improving the stability of the zymogen granule preparation, the pancreas was homogenised in unbuffered 250 mM sucrose because zymogen granules have previously been reported to be more stable in sucrose than in electrolyte solutions.^{6,7}

The crude homogenate was centrifuged at 150 *g* for 15 minutes at 4°C to remove unbroken cells and nuclei (Beckman J2-21 centrifuge, JS-7.5 rotor). An aliquot of the resultant supernatant (termed the pancreatic homogenate) was used to assess zymogen granule fragility. The remainder of the pancreatic homogenate was centrifuged at 1300 *g* for 15 minutes at 4°C. The resulting pellet was gently resuspended in fresh buffer and recentrifuged at 1300 *g* as before to yield the crude zymogen granule pellet.⁶ The crude granule pellet was resuspended gently and mixed with a buffer containing Percoll to yield final concentrations of 40% Percoll, 250 mM sucrose, 50 mM 2-(*N*-morpholino)-ethanesulfonic acid (MES) pH 5.5, 0.1 mM MgSO₄, 0.1 mM PMSF, and 2 mM ethyleneglycol-bis(β-aminoethylether)-*N,N'*-tetraacetic acid (EGTA). A density gradient was established by centrifugation at 100 000 *g* for 20 minutes at 4°C in a Beckman L8-70M ultracentrifuge using a Ti70 rotor (23° fixed angle). The zymogen granules were easily seen as a dense white band near the bottom of the tube and were removed using a peristaltic pump (Pharmacia model P-1, Sweden). The zymogen granule band did not form, however, when the Percoll density gradient was established with unbuffered sucrose in place of the buffer described above.

Preliminary experiments, using crude and purified zymogen granule fractions, showed that latency was absent from some preparations despite only small changes in the per cent supernatant enzyme, suggesting that latency was a more sensitive measure of the effect of variations in isolation methods on zymogen granule fragility. Therefore, comparisons between these methods were made by determination of zymogen granule latency. In a separate study, latency was also assessed after incubation of a sample of homogenate in 250 mM sucrose for 15 minutes at 20°C.

ASSAYS

Amylase was used as the zymogen granule marker enzyme. All measurements were made on fresh tissue without delay. Amylase activity was determined spectrophotometrically using the method of Jung.⁸ This method uses a chromogen coupled corn starch substrate (comprised predominantly of amylopectin) with a molecular weight ranging from 100 000 to several million Daltons. Amylase liberates the chromogen from the starch substrate by enzymatic hydrolysis. On centrifugation of the assay mixture at 5000 rpm for 10 minutes, the soluble chromogen remains in the supernatant and is available for measurement by spectrophotometry. The amylase assay buffer was modified to optimise latency values as follows: the buffer was made isotonic by the addition of 200 mM sucrose and the pH was adjusted to 5.5. Zymogen granules have previously been reported to be more stable under these conditions.^{6,7,9,10} The modified amylase assay remained linear with respect to time and protein concentration. Activity was determined from the

absorbance at 420 nm after incubation at 37°C for 10 minutes. A standard curve was prepared using serial dilutions of commercially obtained standards. Tissue protein concentrations were determined by the method of Lowry *et al*¹¹ using bovine serum albumin as the standard.

REAGENTS

The Procion Yellow starch substrate for amylase assay was synthesised as described by Jung⁸ using corn starch obtained from Argo (Englewood Cliffs, NJ, USA). Amylase activity was standardised using Multi-Enzyme Lin-Trol (derived from pooled serum samples) obtained from the Sigma Chemical Company. Reactive yellow 86 and Percoll were also obtained from the Sigma Chemical Company.

STATISTICAL ANALYSIS

Data are expressed as mean (SEM). Values for ethanol and control animals were compared using Student's *t* test.^{12 13} Statistical analysis of latency and per cent supernatant values were performed after logarithmic transformation as these parameters are expressed as percentages, which may not be normally distributed.

ETHICS

This study was approved by the animal care and ethics committee of the University of New South Wales, Sydney, Australia.

Results

PRELIMINARY STUDIES

Effect of purification on zymogen granule fragility

As Figure 2 shows, zymogen granule latency was present in the pancreatic homogenate but was progressively lost during further purification of zymogen granules.

Effect of incubation

Zymogen granule latency was present in the homogenate but latency was reduced after

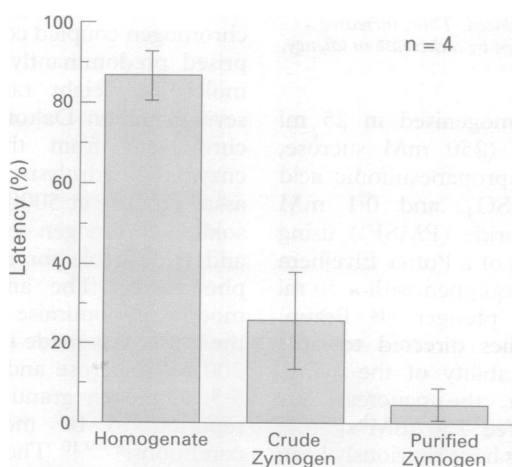


Figure 2: Effect of purification of zymogen granules on zymogen granule latency.

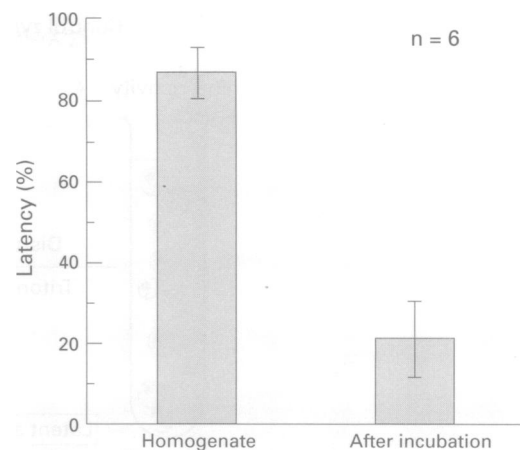


Figure 3: Effect of incubation of pancreatic homogenate on zymogen granule latency.

incubation in 250 mM sucrose for 15 minutes at 20°C (Fig 3).

Effect of buffer on zymogen granule fragility

Higher values for latency were obtained when the pancreas was homogenised in sucrose (116.8 (23.6)%; n=10) compared with MOPS buffered sucrose (76.7 (8.8)%; n=4).

As a result of these preliminary studies, for experiments on control and ethanol fed animals, the pancreas was homogenised in unbuffered sucrose and centrifuged at 150 *g*. The resultant supernatant (pancreatic homogenate) was then used for determinations of latency and per cent supernatant enzyme.

ETHANOL FEEDING STUDY

Rat weight gain, pancreatic weight, and pancreatic amylase content

The animals were healthy throughout the study and weight gain was similar in the two study groups (Table). Pancreatic weights and protein content were also similar in control and ethanol fed animals (Table).

Indices of zymogen granule fragility

There was evidence of increased zymogen granule fragility as shown by a significantly decreased zymogen granule latency in ethanol fed animals compared with controls (Fig 4). Per cent of supernatant enzyme was raised in the ethanol group compared with controls but the difference was not significant (control: 5.7 (0.8)%; ethanol: 9.9 (3.9)%; *p*=0.12).

Effect of chronic ethanol consumption on rat weight and pancreatic composition

	Control	Ethanol
Initial weight (g)	121.4 (2.1)	121.0 (2.3)
Final weight (g)	210.7 (10.1)	209.9 (9.1)
Weight gain (g)	89.7 (8.1)	88.5 (7.6)
Weight gain (g/day)	2.82 (0.23)	2.79 (0.23)
Pancreatic weight (g)	0.97 (0.06)	0.87 (0.03)
Pancreatic weight (g/100 g body weight)	0.46 (0.01)	0.42 (0.02)
Protein (mg/g pancreas)	117.2 (6.7)	114.4 (6.7)

Data are expressed as mean (SEM) and were compared by Student's *t* test as described in Methods.

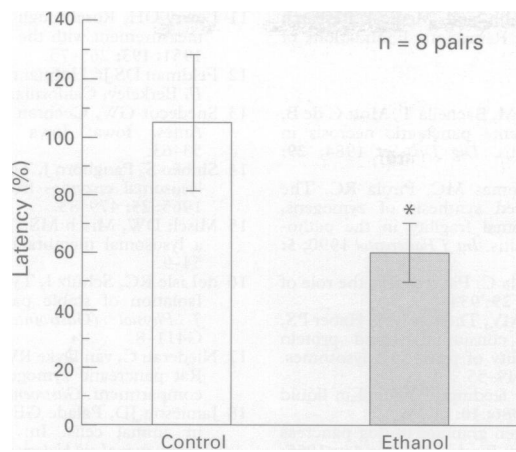


Figure 4: Zymogen granule latency for amylase in ethanol fed and control rats. * $p < 0.03$ compared with control.

Discussion

In these studies, chronic administration of ethanol was associated with increased fragility of rat pancreatic zymogen granules. The increased fragility was manifested by changes in both parameters of granule fragility. Latency for the zymogen granule marker enzyme amylase was significantly reduced in the ethanol fed animals. Per cent supernatant enzyme showed a trend towards an increase although the rise was not statistically significant.

The difference between the magnitude of the change in latency and the change in per cent supernatant enzyme may reflect differences in the nature of these two parameters. A similar phenomenon has been reported previously in a number of studies,^{4 14 15} with respect to lysosomes. The most probable explanation for this differential effect is that the marker enzyme remains bound to the membrane even when the degree of damage to the organelle is sufficient to permit the substrate access to intragranular enzyme. The available (pre-Triton) enzyme activity is increased and as total enzyme activity (measured after addition of Triton) is unchanged, latent activity (the difference between the available and total activities) is reduced. During centrifugation, the enzyme tends to remain bound to the membrane and therefore sediments with the organelle membranes. Consequently, the change in per cent supernatant enzyme is less noticeable than the change in latency. Thus, latency is generally regarded as a more sensitive indicator of damage as it is independent of whether or not enzyme is released from damaged granules.

These studies were performed using a pancreatic homogenate because multistep methods for purifying zymogen granules proved unsatisfactory. In preliminary studies using published methods for the isolation of zymogen granules,^{7 16} latency values were uniformly low. There is other evidence from published reports that such isolation procedures may damage zymogen granules. Intracellular zymogen granules are actively acidified in an energy dependent process.¹⁷ When these granules were purified using the above methods, however, acidification could no longer be shown.¹⁷ Thus this study and that

of Niederau *et al*¹⁷ suggest that the zymogen granule membrane is functionally changed during isolation.

Because a pancreatic homogenate was used for these studies, it is possible that the phenomenon described may result from fragility of amylase containing organelles other than zymogen granules such as rough endoplasmic reticulum, the Golgi complex or condensing vacuoles. Evidence from pulse-chase and immunolocalisation studies have shown, however, that the bulk of intracellular digestive enzymes are localised to zymogen granules.^{18 19} It is therefore most likely that the results reflect changes in the fragility of zymogen granules rather than other compartments. In any event, this is not an important distinction as a change in membrane stability of any digestive enzyme containing compartment would be of relevance to the pathogenesis of pancreatitis.

The mechanism by which chronic ethanol consumption results in reduced zymogen granule latency remains to be established. In this experimental model of ethanol administration for four weeks, histological evidence of pancreatitis is consistently absent.²⁰ Therefore, the changes in zymogen granule fragility probably do not result from the presence of pancreatitis. The effect may result from the presence of ethanol itself, or from other substances known to accumulate in the pancreas after ethanol consumption including acetaldehyde, fatty acid ethyl esters, or cholesteryl esters. The next logical step to determine the mechanism for this effect of chronic ethanol feeding would be in vitro incubation of pancreatic homogenate with each of the putative mediators and subsequent determination of latency. There are methodological problems entailed in this approach, however, in that zymogen granule latency diminishes rapidly after incubation (see Fig 3). Thus, further progress in this area awaits refinement of these methods.

Recent studies show that pancreatitis is an autodigestive process initiated intracellularly by activation of digestive enzymes by lysosomal enzymes.³ Colocalisation of digestive and lysosomal enzymes has been shown to be an early event in several dissimilar animal models of experimental pancreatitis.³ Colocalisation in itself, however, does not lead to pancreatitis. It is possible, therefore, that an additional factor such as a block in exocytosis is required to trigger autodigestion.²¹ Our hypothesis for the pathogenesis of alcoholic pancreatitis is that increased fragility of lysosomes and zymogen granules after ethanol consumption facilitates contact between lysosomal and digestive enzymes, thus establishing a 'primed' setting for the initiation of autodigestion in the acinar cell by some, as yet unknown, trigger factor.²

The study reported here has shown that chronic administration of ethanol increases the fragility of pancreatic zymogen granules. This effect combined with the previously reported increase in pancreatic lysosomal fragility⁴ may permit intracellular contact between digestive and lysosomal enzymes thus facilitating autodigestion.

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