Pancreatic polypeptide cells of rat pancreas after chronic ethanol feeding

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Male Wistar rats, (2 months old) were randomly divided into two groups according to the diet offered (C-control and E-ethanol treated rats). Final body weight was significantly increased but pancreatic weight as a percentage of body weight was decreased in ethanol treated rats. Volume density, number of pancreatic poly peptide (PP)-cells per islet and per um² of islet were significantly increased. PP-cells were abundant and occupied the whole periphery of islets in the splenic part of the pancreas. Those cells showed strong immunopositivity. At the ultrastructural level PP granules had predominantly less electron density. The mean diameter of PP granules was significantly increased and the number of granules of larger diameter was greater in the E group of rats, than in the controls.

Pancreatic polypeptide (PP) is a 36 amino acid hormone belonging to a family of structurally related peptides including peptide YY and neuropeptide Y. PP is synthesized in and released from endocrine PP cells situated within the pancreatic islets and scattered throughout the exocrine tissue.

The relative proportions of different pancreatic islet cells show regional and species differences. In both the human and rat pancreas, two distinct types of islets have been described, differing from each other in their cellular content: a PP-rich, glucagon-poor islet (PP islet) characteristic of the ventral lobe (derived from the ventral primordium) and glucagon-rich, PP-poor islet (glucagon islet) characteristic of the dorsal primordium.

Under physiological circumstances, PP is mainly secreted in response to ingestion of a meal. The physiological function of the peptide is poorly understood, although it has been demonstrated that under experimental conditions, the peptide inhibits the secretion of insulin in rats, dogs and mice, and inhibits exocrine pancreatic secretion in man and in dogs.

The secretion of PP is predominantly regulated by the parasympathetic nervous system. The basal level of serum PP has become a field of interest mainly due to the role of PP as a tumour marker, and as a marker of pancreatic endocrine function.

In comparison with general mucosal alteration of the gastrointestinal tract, relatively little is known about the effect of alcohol on the diffuse neuroendocrine system (DNES) and its neurohormonal response. Alcohol-associated effects are either due to direct local action or mediated by numerous other substances. Although controversy regarding the definition of different histological types of alcoholic chronic pancreatitis in humans and animal models prevails in the literature, the association between ethanol and chronic pancreatitis is well established. Functional studies have shown that exocrine deficiency has a significant effect on islet cell function, and there are exocrine pancreas-insulin, glucagon, pancreatic polypeptide and somatostatin interactions, as well as enteropancreatic interaction in chronic pancreatitis.

To the best of our knowledge, complex quantitative analysis of the light microscopic and fine structure of islet endocrine cells has not been carried out in chronic alcoholism, especially on the PP-cells. The aim of this study is to examine PP-cells at the light and ultrastructural levels in rats after long term (4 months) alcohol ingestion.
Materials and Methods

Animals and feeding protocol — Male Wistar rats (2 months old), weighing approximately 240 g were randomly allocated to two groups (C-control; E-ethanol treated animals). The control group of 15 rats was fed a commercial diet. The E group of 14 rats was given free access to the diet and a solution of 25% sucrose-32% ethanol as recommended by Hartroft. The quantity of food offered to the control animals was adjusted to the energy intake of animals receiving alcohol (i.e. control rats were pair fed). The amount of tap water given to the ethanol-treated rats was 25 ml, and the mean consumption per rat per day of the alcohol solution was 12 ml per animal. Ethanol treated rats therefore consumed 23% of the total calories as alcohol (mean 8.42 g/kg/day).

Blood ethanol determination — Samples for blood ethanol determination were obtained from the tail vein, and blood ethanol concentration was determined using the Sigma diagnostic alcohol procedure (No. 322-UV, Sigma Chemical Co., St. Louis, Mo). Mean blood ethanol concentration was calculated from multiple blood determinations (at 1000 hrs on day 1, 15, 30, 45, 60, 75, 90, 105 and 120) throughout the exposure period of 120 days.

Blood glucose and plasma insulin determination — Fasting blood glucose level was measured by the glucose oxidase method. Plasma insulin was measured by radioimmunoassay using commercial kits in accordance with the instructions (INEP-Diagnostics, Zemun) and rat insulin standard (Novo Industry, Bagsvaerd, Denmark).

Preparation of histological samples — After 4 months, rats were starved for 24 hr and given water ad libitum before being killed in the morning (0700 hrs) by a blow on the neck. The pancreas was removed, weighed in air, and the splenic part of the pancreas was cut and used for light and electron microscopic investigation. Samples were immersed for 24 hr in Bouin fixative and embedded in paraffin according to the standard procedure. Immunohistochemistry was performed in 5 μm serial sections using polyclonal antibodies against somatostatin, insulin, glucagon and pancreatic polypeptide. Sections were immunostained employing the streptavidin-biotin or PAP technique. Conventional morphometry and standard stereological equations were used to calculate the volume density and frequency of pancreatic endocrine cells.

Electron microscopy — Several pieces of the splenic part of the pancreas of each animal were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer and postfixed in 2% osmium tetroxide in the same buffer. After dehydration the specimens were cut on an LKB ultratome, double stained with uranyl acetate and lead citrate and examined under Philips CM 12 electron microscope.

Statistics — Data were subjected to Student's t-test and the results expressed as mean ± SE.

Results

There was no significant difference between the ethanol-treated and control rats with regard to daily energy intake and energy from protein and fat (Fig. 1). The average ethanol intake of group E was 3.09 g/day which was 23% of the daily energy intake.

Mean blood ethanol concentration was 125 ± 13 mg/ml and ranged from 107 to 138 mg/100 ml.

Animals in the examined groups had similar weight gains (data not shown) and body mass increased during the experiment continually. Total pancreatic

Fig. 1—Daily energy intake and percentage of particular nutritional ingredients in the rats of groups C and E.
weight and volume were similar in both groups, but relative pancreatic weight in rats of group E was decreased (Table 1). The results for fasting blood glucose and insulin level were similar in the two groups of animals. There was significant a difference between the ethanol-treated rats and controls with regard to final body weight.

Volume density, number of PP-cells per islet and per μm² of islet were significantly increased (Table 2). Mean profile area of the PP-cell was similar in both groups of rats. The number of PP-cells in the exocrine part of the pancreas was reduced, but statistically significant differences were not found.

Glucagon-producing A-cells, somatostatin-producing D-cells and pancreatic polypeptide-producing PP-cells were increased in number, while insulin-producing B-cells were decreased (Table 3). Results obtained for individual endocrine cells per islet showed changes in the percentage of those cells in the E group of rats (Fig. 2). The percentage of A- and PP-cells was increased and that of B-decreased in ethanol-treated rats. The percentage of D-cells was unchanged. In comparison with the control, the increase in number of PP-cells amounted to about 42% per islet and 83% per μm² of islet.

It was observed that PP-cells were situated peripherally on the islets singly or in small clusters in the C group of rats while in some islets of Langerhans there were no PP cells. In the exocrine part of the pancreas, pancreatic polypeptide-producing-PP-cells were found both in the acini and in the small ducts.

In ethanol-treated rats PP-cells were abundant and occupied the whole periphery of the islets (Fig. 3c). There were no islets without PP-cells unlike in the control (Fig. 3b). In the exocrine tissue PP-cells were found in the acini and in the small ducts (Figs 4a,b). A great number of PP-cells had very strong immunoreactivity suggesting accumulation of pancreatic polypeptide granules in the cytoplasm.

Electron microscopic examination showed that PP-cells in alcohol-treated rats contained granules in the whole cytoplasmic area. PP granules had less dense cores and closely fitting limiting membranes.

Table 2—Volume density, number or PP cells and mean profile areas of PP cells

<table>
<thead>
<tr>
<th></th>
<th>Group C</th>
<th>Group E</th>
</tr>
</thead>
<tbody>
<tr>
<td>Volume density</td>
<td>0.13 ± 0.015</td>
<td>0.24 ± 0.030**</td>
</tr>
<tr>
<td>Npp/μm² of islet</td>
<td>11.4 ± 1.64</td>
<td>16.7 ± 1.76</td>
</tr>
<tr>
<td>Npp/μm² islet</td>
<td>0.0012 ± 0.0001</td>
<td>0.0022 ± 0.0002**</td>
</tr>
<tr>
<td>Npp/m² of pancreas</td>
<td>25.6 ± 4.91</td>
<td>19.8 ± 2.92**</td>
</tr>
<tr>
<td>Mean profile area</td>
<td>1.36 ± 0.24</td>
<td>1.15 ± 0.18**</td>
</tr>
<tr>
<td>88.96 ± 3.67</td>
<td>87.13 ± 4.95*</td>
<td></td>
</tr>
</tbody>
</table>

P values: ** <0.001; * not significant

Table 3—Number of particular cells/islet

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Group C</th>
<th>Group E</th>
</tr>
</thead>
<tbody>
<tr>
<td>α</td>
<td>21.2 ± 1.95</td>
<td>27.1 ± 3.75**</td>
</tr>
<tr>
<td>β</td>
<td>30.8 ± 1.48</td>
<td>25.5 ± 2.73**</td>
</tr>
<tr>
<td>D</td>
<td>3.7 ± 0.38</td>
<td>4.2 ± 0.55**</td>
</tr>
<tr>
<td>PP</td>
<td>11.0 ± 1.52</td>
<td>16.7 ± 1.75*</td>
</tr>
</tbody>
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P values: * < 0.05; ** not significant

Table 1—Body and pancreatic weight, total pancreatic volume and fasting blood glucose and insulin level in the rats

<table>
<thead>
<tr>
<th></th>
<th>Group C</th>
<th>Group E</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial body weight (g)</td>
<td>232±1.27</td>
<td>240±2.37**</td>
</tr>
<tr>
<td>Final body weight (g)</td>
<td>359±5.61</td>
<td>426±18.00**</td>
</tr>
<tr>
<td>Pancreatic weight (g)</td>
<td>1.30±0.039</td>
<td>1.33±0.073**</td>
</tr>
<tr>
<td>Pancreatic weight (% of body weight)</td>
<td>0.36±0.012</td>
<td>0.32±0.014*</td>
</tr>
<tr>
<td>Total pancreatic volume (cm³)</td>
<td>1.24±0.037</td>
<td>1.26±0.070**</td>
</tr>
<tr>
<td>Glucose (mmol/L)</td>
<td>5.28±0.26</td>
<td>5.36±0.13**</td>
</tr>
<tr>
<td>Insulin (mIU/L)</td>
<td>22.0±1.34</td>
<td>21.5±3.06**</td>
</tr>
</tbody>
</table>

P values: ** <0.001; * <0.02; * not significant
The mean diameter of PP granules was significantly increased (209 ± 3.8 in the control and 224 ± 4.0 in the E group of rats). Although, the largest number of PP granules was found in the same diameter group in both groups of rats (222 nm), in the ethanol treated group the number of granules of larger diameter was greater (Fig. 6).

**Discussion**

In a series of experiments, started in 1989, we have been investigating the diffuse neuroendocrine system (DNES) in the upper part of the digestive tract and pancreas after long-term (4 or 6 months) ethanol consumption with or without adequate protein in the diet. Our study mainly consisted of morphofunctional and stereological investigations at the light and electron microscopic level because there are few data about this problem. In the present study, as well as in previous investigations we applied a nutritional schedule that made it possible to obtain similar mean daily energy intakes in both groups of rats. Rats given a chronic dose of ethanol (about 23% of total calories) consumed an adequate amount of other nutrients and had a normal growth rate when compared to pair fed controls. In this respect, the ethanol diet was nutritionally adequate, and possible effects of alcohol were not confounded with the effects of malnutrition.

There are no data in the available literature about the detailed morphometric parameters of the PP-cells after long-term of ethanol ingestion in rats and our results are the first in this area of investigation. Owiang et al. showed that the number of PP-cells...
appear to increase in chronic alcoholic pancreatitis. Islets were composed largely of PP-cells, and a large number of isolated PP-cells were frequently found in close contact with duct epithelium. This observation suggests that the increase in PP-cells may represent a regenerative phenomenon after pancreatic injury.

There was morphological evidence for an alcohol-induced selective intrapancreatic nerve degeneration in mice after 4 months of alcohol consumption. This affected mainly the nerve fibers that are inhibitory to the exocrine pancreas, and may represent the morphological background of the hypersecretory state of the pancreas in chronic alcoholism. A slight decrease was found in the intensity of VIP and SP immunoreactivity and the PP fibers almost disappeared. Majority of periacinar nerve terminals showed degenerative changes whereas the perivascular nerve fibers were relatively well preserved.

The present results showed a significant increase in number of PP-cells in the islets of Langerhans after 4 months consumption of ethanol. It is not clear what is the functional significance of this increase. Other results obtained in the present experiment showed that the number of A-and D-cells was slightly increased while that of B-cells decreased. In addition, some morphological abnormality of A-and B-cells was recorded. The profile area of A-cells was smaller, as well as the profile area and volume density of their nuclei. The B-cells appear more affected and sensitive than the other endocrine cells in the islets. Both B-and A-cells showed degenerative processes suggestive of apoptotic death. The D-and PP-cells in the islets of Langerhans did not express similar morphological changes.

Previous results for other endocrine cells belonging to DNES also showed changes in their number. Thus all endocrine cells in the fundic and pyloric part of the stomach were significantly decreased in number and some cytological abnormality was also observed.

In the rat pancreas glucagon and pancreatic polypeptide are present in distinct cell types, but they also coexist in one population of islet cells termed glucagon/PP-cells. The glucagon/PP-cells were found in significant numbers in all pancreatic regions, being more numerous than the other two types of PP-and A-cells.

Many of the PP granules in ethanol treated rats were electron pale. It seems that these PP-cells produced predominantly pancreatic polypeptide. In this context present results showed that there was proliferation of these cells, although no mitoses was observed in the PP-cells. A possible explanation may be that the population of cells with coexistence of both peptides changed their genetic programme and began to produce predominantly pancreatic polypeptide. The increased diameter of PP granules in the E group of rats probably results from accumulation of pancreatic polypeptide.

Functional studies using various tests for insulin secretion have been performed both in patients and animals in chronic pancreatitis. Fasting insulin levels in chronic pancreatitis were found to be normal or moderately raised or diminished and the response to oral glucose was variable. Fölich et al. reported that blood glucose concentrations were higher in patients with pancreatitis when compared to controls. On the other hand, serum immunoreactive...
Fig. 5—(a) PP granules in control rat; uranyl acetate; lead citrate; bar 1 μm; (b) PP granules with less dense cores in alcoholized rat; uranyl acetate; lead citrate; bar = 1 μm.
insulin (IRI) concentrations were diminished in chronic human alcoholics, while ethanol had no effect on IRI in ethanol-treated rats. Infusion of ethanol at increasing concentrations caused progressive inhibition of insulin and glucagon secretion. However, Jauhonen and Hassinen observed that ethanol itself produced no marked changes in blood glucose, IRI and glucagon after intravenous infusion. The present results showed that there was no hypoglycaemia which is a strong stimulus for production of pancreatic polypeptide.

The plasma level of PP increased significantly after intravenous ethanol administration. Ingestion of alcohol by healthy volunteers did not stimulate the release of cholecystokinin (CCK), which is the chief hormonal stimulant of pancreatic enzyme secretion, nor did it significantly alter fasting levels of pancreatic polypeptide. These data suggest that, in patients who do not have chronic pancreatitis, alcohol does not induce acute pancreatitis, either by stimulating CCK release or by stimulating enzyme secretion directly. On the contrary, in chronic alcoholic pancreatitis, fasting and postprandial levels of pancreatic polypeptide were significantly increased and the link between CCK and pancreatic polypeptide was disrupted. In addition, Hajnal et al. tested the effect of a 12% (v/v) alcohol solution added to a meal on basal and postprandial levels of PP in both nonalcoholic volunteers and chronic alcoholics. Basal plasma PP levels were similar in both groups, but the postprandial increments in PP levels observed in nonalcoholics were not observed in alcoholics. However, basal and postprandial levels of gastrin and CCK were similar in alcoholics and nonalcoholics. They concluded that postprandial hypersecretion of pancreatic enzymes in alcoholics is not related to increased plasma levels of CCK or gastrin but it is possible that the impaired release of PP may participate in the mechanism for increased pancreatic enzyme secretion in chronic alcoholics.

Hyperplasia of PP-cells in the PP-rich region of the pancreas (ventral embryonic origin) was observed in patients previously treated surgically for functioning duodenal or pancreatic gastrinoma not associated with multiple endocrine neoplasia syndrome. PP hyperplasia was observed in the pancreas of juvenile diabetes. Although hyperplasia of PP-cells took place predominantly in altered exocrine pancreases, in the present study all rats (with or without histological disturbances of the exocrine pancreas) showed hyperplasia of PP-cells only in the islets of Langerhans. Proliferation of PP-cells was also observed in ethanol treated rats which were fed with a low protein diet (data not shown). It may be concluded that ethanol had a direct effect on this proliferation, although the exact mechanisms of this phenomenon cannot be explained at present.

Acknowledgement
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