RESPONSE OF THE EXOCRINE PANCREAS TO THE CCK ON OFFSPRING RATS OF ETHANOL DAMS. EFFECTS OF FOLIC ACID

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Abstract — Aims: The aim of this study was to study the reverse effect of folic acid administered during gestation and lactation to ethanol-treated dams, on cholecystokinin (CCK) stimulus-secretion coupling in pancreatic exocrine secretion in offspring rats. Methods: Animals were randomized into three groups: Control group (C) received water and basic diet during pregnancy and lactation period; ethanol-treated rats (E) received ethanol and basic diet; the ethanol + folic acid group (EF) received folic acid supplement concomitantly with ethanol administration. Results: Body and pancreatic weight was lower in offspring after ethanol treatment. Folic acid supplementation increased these parameters with respect to ethanol rats. After CCK stimulation, a significant decrease in amylase, lipase and chymotrypsin activities in the duodenal juice were detected in ethanol, this trend was partially corrected with folate supplementation. Conclusion: Ethanol exerts its action on exocrine pancreatic secretion by two pathways: 'per se' and diminishing the folic acid content, because a folic acid supplement in rats during pregnancy and lactation periods produces an advantageous effect on amylase, lipase and chymotrypsin secretion in their offspring. Although extrapolation from animal studies may be tenuous, the present findings may explain the use of folic acid in the prevention of ethanol-induced damage by increasing the enzyme levels to adequate physiological concentrations.

INTRODUCTION

It has been postulated that ethanol-induced pancreatic damage may be mediated by the oxidation of ethanol within the pancreas producing secondary toxic metabolic changes. Haber et al. (1998), showed that pancreatic acinar cells oxidize significant amounts of ethanol. At intoxicating concentrations of ethanol, pancreatic acinar cell ethanol oxidation, may have the potential of contributing to pancreatic cellular injury. Matsumura et al. (2001), showed that chronic oxidative stress, induced by DDC (Diethyl Dithiocarbamate), causes pancreatic fibrosis.

Effects of ethanol administration on pancreatic function in the rat remain controversial. Factors such as strain of rats, mode of ethanol administration or route taken, may contribute to the differences reported in several studies (Singh et al., 2003). We previously reported that under basal conditions, ethanol treatment during gestation and lactation negatively affects the digestive function in offspring. The fact that ethanol causes a wide spectrum of health problems, as a consequence of altering several metabolic pathways in every organ of the body, has been well documented (Thakker, 1998; Lieber, 2005). Oxidative stress is considered a key step in the pathogenesis of ethanol-induced damage (Alevy et al., 1999). In previous works, we studied the pro-oxidant effect of ethanol in the progeny, which is supported by the increase of thiobarbituric reactive substances (TBARS), and carbonyl groups in the liver and pancreas of the offspring. Results also showed that the pancreas seems to be more sensitive to the effect of ethanol and its metabolism (Cano et al., 2001). In fact, the metabolic pathways of ethanol in pancreas differ from those found in liver (Laposata and Lange, 1986). Pregnant women are prone to folate deficiency since there is an increased folate catabolism and a significant increase in folate requirement during pregnancy (Higgins et al., 2000). Moreover, alcohol abuse produces folate deficiency because ethanol interferes with the absorption and metabolism of folic acid (Carreras et al., 1990; Tavares et al., 1999). Balaghi and Wagner (1995), suggested that folate deficiency might affect the production of zymogen granules as well as their release. Therefore, severe folate deficiency impairs pancreatic exocrine function.

The major function of pancreatic acinar cells is to synthesize and secrete a variety of digestive enzymes. Amylase, chymotrypsin, and lipase (representative enzymes) secretion can be physiologically activated by a variety of hormonal and neural stimuli, including CCK, secretin and Ach. In a previous article (Cano et al., 2003), we reported that under basal conditions, ethanol treatment during gestation and lactation negatively affects the digestive function in offspring. The effect of ethanol was slightly attenuated in rats supplemented with folic acid for amylase activities. In fact, we determined the effect of folic acid supplementation on CCK stimulus-secretion coupling in pancreatic exocrine secretion in offspring rats exposed to ethanol during pregnancy and lactation.

METHODS

Animals

Male and female Wistar rats weighing about 150–200 g were randomized into three groups (10 rats/group): Control group...
(C): basic diet and water; ethanol group (E): basic diet and ethanol; and Ethanol Folic Group (EF): supplemented folic acid diet and ethanol.

Ethanol was administered mixed with tap water by a previously described method (Cano et al., 2001). Ethanol-treated rats received increasing amounts of ethanol (5, 10, 15% V/V each week) in the drinking fluid for 3 weeks (5.5 ± 0.2, 7.8 ± 0.4 and 8.9 ± 0.4 g ethanol/Kg/day) ad libitum. A consumption of 20% ethanol (16.6 ± 2.1 g ethanol/Kg/day) was given for 4 weeks and maintained during the entire gestation and suckling periods (21 days). Ethanol folic acid groups received the same ethanol treatment and were fed a folic acid supplement diet during the gestation and lactation periods. Drinking water with or without ethanol and a normal diet or supplemented diet was given ad libitum.

Male and female rats were mated to obtain the first offspring. Pregnant rats were housed individually in plastic cages and were fed with rat diet ad libitum. E and EF dams were kept on 20% ethanol in the drinking water during the entire gestation and suckling periods.

The day of parturition was designated as day 1 of lactation, and the day 21 after parturition as the end of the lactation period. In all the groups, experiments were performed at the 21st day postpartum. The amount of milk consumed was estimated by subtracting the body weight of the pups before and the day 21 after parturition as the end of the lactation period. In all the groups, experiments were performed at the 21st day postpartum. The amount of milk consumed was estimated by subtracting the body weight of the pups before and after 30 min of suckling (Subramanian, 1997).

The rats were maintained under an automatically controlled temperature (22–23°C) and a 12-h light-dark cycle. Animal care complied with the Guide for the Care and Use of Laboratory Animals (National Academy Press, Washington, D.C., 1996).

Diets

Diets were prepared according to The Council of the Institute of Laboratory Animal Resources (ILAR, 1979), which details the nutrient requirements for most of the common laboratory animals (g/Kg of diet): casein: 200; glass sucrose: 510; cornstarch: 140; fibre, cellulose: 50; corn oil: 50; AIN-76 mineral mix: 35; AIN-76 vitamin mix: 10; folic acid 0.2; choline bitartrate: 2; DL-methionine: 3. Diet ingredients were mixed and homogenized in a double-cone blender (Rest, Haan, Germany).

Control and ethanol-treated groups received an average of 0.2 g of folic acid/Kg of diet (60µg/day) during pregnancy and the lactation period. In the Ethanol Folic Acid Group (EF) the amount of folic acid introduced in the solid diet was 0.8 g/Kg of diet (152µg/day) for the same period.

Folic acid supplement was started 6 weeks before the reproduction period.

Collection of pancreatic secretion

After 21 days postpartum, animals from each group were anaesthetized with a subcutaneous injection of 125 mg urethane/g body wt after an overnight fast. The collection of pancreatic secretion was realized by a previously described method (Carreras et al., 1990). Briefly, the abdominal cavity was opened and the bile duct ligated. The inflow cannula was tied at the beginning of the duodenum and the perfused was collected beside the Treitz ligament. After cannulation, the loops were replaced inside the body wall. The duodenum was irrigated with 37°C saline at a constant rate of 0.7 ml/min. The duodenum was washed with saline before the start of the first collection to remove any residual secretion to obtain the basal secretion. After CCK solution, (Sigma n° P-2000) was injected into the femoral vein (1 U/Kg body wt), while perfusion continued. This dose of CCK was chosen on the basis of our previous studies (Carreras et al., 1990). The effluents were obtained 10, 20, 30, 40, 50, 60, 70, 80, and 90 min after CCK administration. The volume of perfusate was determined by weight. Volume, pH, protein content, Na+ and K+ levels and pancreatic enzymatic activities were assayed in the duodenal juice. The gathered volume is the sum of saline solution perfused and pancreatic duodenal secretion.

Blood samples

Blood samples were taken from starved and anaesthetized rats by cardiac puncture through the thorax and collected into tubes. Serum was prepared using low-speed centrifugation. Amylase levels were obtained by the method described in the pancreatic enzyme assay section. Serum ethanol was determined by a commercial enzymatic colorimetric test (Sigma) according to the technique described by Bucher and Redetzki (1951) with Alcohol Dehydrogenase (ADH), at a wavelength of 340 nm. Serum folic acid levels were obtained by means of a radioassay kit (Waxman and Schreiber, 1980).

Pancreatic enzyme assay

Amylase content was assayed by an enzymatic colorimetric test using p-nitrophenyl-α-D-maltotetraoside as substrate (Kruse-Jarres et al., 1989). Chymotrypsin was determined by a method described by Delmar et al., 1979. Lipase activities were determined by the colorimetric method described by Neumann and Ziegenhorn, 1978. Protein concentration was measured according to the method of Lowry et al., 1951 using bovine serum albumin as the standard.

Sodium and potassium duodenal concentrations

Na+ and K+ duodenal concentrations were measured by a flame photometer (Digiflame, 2000, GDV) in the duodenal juice.

Statistical methods

Data were expressed as X ± SEM and were analysed by the Analysis of Variance (ANOVA). If ANOVA indicated significant treatment effects, the significance of differences between individual means was determined using the Tukey-Kramer parametric test or Kruskal-Wallis nonparametric test followed by Dunn’s multiple comparison tests. A P-value <0.05 was considered statistically significant.

RESULTS

General parameters

Table 1 shows that ethanol-treated offsprings (E, EF) consumed significantly less milk than control rats; they also
showed a decrease in body weight at 21 days postpartum. E body weights during the whole experiment were significantly lesser than C and EF. Similar results were observed in pancreas weight. The pancreatic protein concentration in E or EF did not differ from that of the control rats. Serum ethanol was similar in both ethanol groups. Serum folic acid was significantly decreased in ethanol group; however, a significant increase was found in ethanol folic acid group.

**Duodenal content flow**

As illustrated in Figure 1, Panel A, the duodenal juice volume at the beginning of the experiment (basal conditions, B) did not differ among the three offsprings at 21 days postpartum groups. However, at 20 and 80 min after CCK stimulation, this parameter slightly increases in C with respect to E. Ethanol alters the biphasic pattern of volume activation in monophasic patterns at 30 min. In EF also, there is a biphasic pattern, but this time is at 30 and 80 min.

Under basal conditions, a significant pH reduction in duodenal juice was observed in ethanol-treated groups (E and EF) as opposed to C (Fig. 1, Panel B). CCK administration did not significantly alter the duodenal pH in control group and in folic acid supplemented group. However, ethanol groups showed increased values at 30 min after CCK stimulation, which were higher than that for EF and similar to that for C. Folic acid group showed the lowest duodenal pH values which were higher than that for EF and similar to that for C. In the duodenal juice was observed in ethanol-treated groups (E and EF) no significant difference in protein concentration of rats at 40 min, which lasted for 30 min. In ethanol-treated rats (E, EF) no significant difference in protein concentration of the duodenal juice was found during the whole experimental time.

**Na\(^+\) and K\(^+\) duodenal concentration**

Under basal conditions, Na\(^+\) duodenal concentration was similar among the three groups of offsprings after 21 days postpartum. At 10 min, Na\(^+\) duodenal concentration increase was observed in response to CCK in all groups. There was a significant second and third enhancement at 40 and 70 min in EF with respect to C and E (Fig. 2, Panel A). In E group, there was a second slight enhancement at 40 min; the enhancement happened in C group at 50 min, but this increase was significantly higher than that for E or EF.

Under basal conditions and in response to CCK, K\(^+\) duodenal concentration was similar in E and C groups; these values were significantly lower than that of EF. CCK administration did not significantly alter the K\(^+\) duodenal concentration in C and E, but a significant increase in K\(^+\) duodenal concentration was observed in supplemented rats, with a biphasic response at 10 and 40 min (Fig. 2, Panel B).

**Enzymatic activity**

Amylase, Chymotrypsin and Lipase activities were determined in order to examine the time-dependent zymogen activation by CCK stimulation. The enzymatic activities, expressed as U/mg protein, in the offspring at 21 days, obtained under basal conditions and in response to CCK are shown in Fig. 3. Panel A, Panel B and Panel C.

Under basal conditions, amylase activity was significantly lower in E group, and similar in C and EF. CCK produced an expected increase in amylase activity in control group; this trend was also found to a lesser extent in EF; however, E rats did not change their amylase activity after CCK administration (Fig. 3, Panel A). CCK stimulation induced amylase activity in a time-dependent manner; it increased over basal levels for 90 min in control group and EF. During this 90 min two peaks appeared at 20 and 60 min in control group and at 30 and 60 min in EF.

### Table 1. Ethanol effects on offspring rats during pregnancy and lactation period.

<table>
<thead>
<tr>
<th></th>
<th>C (N = 10)</th>
<th>E (N = 10)</th>
<th>EF (N = 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Milk consumption (g/30 min) at 21 days after birth</td>
<td>0.541 ± 0.047</td>
<td>0.300 ± 0.030(^b)</td>
<td>0.313 ± 0.028(^a)</td>
</tr>
<tr>
<td>Body weight at birth</td>
<td>6.7 ± 0.11</td>
<td>5.89 ± 0.28</td>
<td>6.53 ± 0.14</td>
</tr>
<tr>
<td>Body weight at 21 days after birth</td>
<td>35.27 ± 0.5</td>
<td>21.65 ± 0.3</td>
<td>29.2 ± 0.7(^f)</td>
</tr>
<tr>
<td>Pancreatic weight (g)</td>
<td>0.111 ± 0.007</td>
<td>0.059 ± 0.004(^b)</td>
<td>0.134 ± 0.013(^f)</td>
</tr>
<tr>
<td>Pancreatic protein concentration (mg/g pancreas)</td>
<td>100.76 ± 4.26</td>
<td>102.45 ± 4.98</td>
<td>98.80 ± 2.83</td>
</tr>
<tr>
<td>Serum Ethanol (mg/dl)</td>
<td>—</td>
<td>4.59 ± 0.44</td>
<td>5 ± 0.5</td>
</tr>
<tr>
<td>Serum Folic acid (ng/ml)</td>
<td>13.3 ± 0.8</td>
<td>9.18 ± 0.6</td>
<td>15 ± 0.7(^f)</td>
</tr>
<tr>
<td>Solid diet of dams on the 3rd week of the gestation (g/day)</td>
<td>21.93 ± 2.13</td>
<td>15.20 ± 0.94(^a)</td>
<td>14.02 ± 0.45</td>
</tr>
<tr>
<td>Solid diet of dams on the 3rd week of the lactation (g/day)</td>
<td>38.70 ± 2.87</td>
<td>26.10 ± 2.46(^d)</td>
<td>27.12 ± 1.77</td>
</tr>
</tbody>
</table>

The results are expressed as mean ± SEM. Numbers in parentheses indicate the number of animals in each group. E versus C: \(^a\) p < 0.01, \(^b\) p < 0.001; EF versus C: \(^c\) p < 0.01, \(^d\) p < 0.001; EF versus E: \(^e\) p < 0.05; \(^f\) p < 0.001; \(^g\) p < 0.001.
Under basal conditions, chymotrypsin activity was significantly higher in C group; E group presented the least values. CCK produced an expected marked progressive increase in chymotrypsin activity in control group for the first 40 min, this increment lasted 30 min in EF and E; these values were lesser than that in C. After this progressive increase, chymotrypsin activity greatly decreased in the three groups. Chymotrypsin activity also increased in the control group at 80 min (Fig. 3, Panel B).

Under basal conditions, lipase activity was similar in C and EF, but was significantly low in E. After CCK administration, C and EF increased lipase activity gently up to 80 min, with a peak at 50 min (Fig. 3, Panel C). These increases were similar in both groups during the whole experiment, but at 50 min...
Fig. 2. Na\(^+\) (Panel A) and K\(^+\) (Panel B) duodenal concentration (mM) under basal conditions (B) and in response to CCK. Values are expressed as mean (12 values) ± SEM. E versus C \(^*\) \(p<0.01\); EF versus C \(^*\) \(p<0.001\); EF versus E \(^\bullet\) \(p<0.01\), \(^{3}\) \(p<0.001\).

after CCK stimulation, the increase in C was significantly higher than that of EF. Ethanol rats only showed a slight increase of lipase activity at 10 min after CCK administration; their values were always lesser.

**DISCUSSION**

In this study, we determined the effect of folic acid supplementation on CCK stimulus-secretion coupling in pancreatic exocrine secretion in offspring rats exposed to ethanol during pregnancy and lactation. Folic acid supplemented group showed similar serum folic acid values compared to the control group; this explained the decrease in serum folate during ethanol situations and the importance of its supplementation.

In a previous article (Cano et al., 2003), we found that under basal conditions, ethanol treatment during gestation and lactation affects negatively the digestive function in offspring. We showed that ethanol has a negative effect during the suckling period, provokes a retardation of body and pancreatic weight in the offspring, and produces a decrease in amylase activity, which is partially prevented by administering folic acid (Cano et al., 2003), despite this milk consumption was similar in both E and EF groups, and significantly lower than in C. Presently, we have also found that the effect of ethanol was slightly attenuated in rats supplemented for lipase activity.

Under our basal conditions, pH was more alkaline in control group with respect to the two ethanol groups. In the folic acid group, pH values were lower than in ethanol group, although these differences were not significant. These results are similar to those found by us in a previous study (Cano et al., 2003). Curiously, the supplementation with folate under basal condition also provoked a significant increase in K\(^+\) duodenal concentration compared to rest of the groups.

CCK stimulation increased the duodenal pH, but not the volume in ethanol offsprings. This could be explained because of an increase in Na\(^+\) output, indicating an increase in bicarbonate secretion. However, folic acid supplementation did not alter duodenal pH, because it increases both K\(^+\) and Na\(^+\) concentrations and duodenal volume. CCK induces a significant increase in Na\(^+\) and K\(^+\) duodenal concentration at 10, 40, 90 min, showing that pancreas of EF offsprings are rich in Na\(^+\) and K\(^+\). Folic acid increases K\(^+\) output after CCK administration and under basal conditions, so
we concluded that folic acid *per se* increase K⁺ concentration. Several studies in pancreatic acinar cells showed that ethanol decreased amylase secretion after CCK administration because of an alteration hormone-receptor coupling (Nakamura *et al.*, 1991). Tachibana *et al.*, 1996 investigated the effect of 600 mM ethanol on CCK-8-stimulated amylase release, in isolated rat pancreatic acini. They found that ethanol concentration inhibits CCK-8-stimulated amylase release by inhibiting Ca-pump activity on the plasma membrane. Recently, Gonzalez *et al.* (2006) have incubated pancreatic cells with 50 mM ethanol reducing amylase stimulated by CCK-8. The inhibitory effect of ethanol on CCK-8 induced amylase secretion was abolished by dithiothreitol. Ethanol induces generation of Reactive oxygen species (ROS) by Ca²⁺-dependent mechanism and reduces CCK-8-evoked amylase secretion in exocrine pancreatic cells. Siegmund
C (PKC) activation. López et al. (1996), showed that nutritional factors, especially the protein and fat content of the diet, may change pancreatic morphology after ethanol induced injury. These authors found that amylase, lipase, and cholesterol esterase content were reduced in malnourished rats; but long term ethanol ingestion, regardless of the nutritional state, increased lipase content and decreased amylase. The low amylase activities in ethanol offspring rats represent an adaptation to the low carbohydrate content of an alcoholic diet. In our case, ethanol consumption was accompanied by a decrease in milk uptake because the litters nurtured by the ethanol-treated dams (Murillo-Fuentes et al., 2001). Thus, total milk nutrient levels decreased, which could reduce enzymatic secretion.

CCK provokes a release of Chymotrypsin secretion was decreased in ethanol groups. Folic acid supplementation increased the chymotrypsin output. However, the maximum response was in control rats. The slight first increase in lipase activity of alcoholic rats could represent a metabolic adaptation to long-term ethanol ingestion, because pancreatic lipase is able to metabolize ethanol through a non-oxidative pathway (Riley et al., 1990). Alcohol may first stimulate protein synthesis, but later protein concentration diminishes, perhaps due to acinar cell injury (Grönroos et al., 1988). This could explain that the ethanol treatment, during gestation and lactation, decreases the lipase release.

Chronic ethanol abuse often leads to the development of vitamin deficiencies, one of the most common being folate deficiency (Fernández-Borrachero et al., 1996). Although the poor diet of the chronic alcoholic plays a major role in the etiology of folate deficiency, ethanol ingestion interferes with the absorption and metabolism of folates. In our studies, folic acid supplemented group presented an increase in amylase, chymotrypsin, and lipase activities, significantly higher than for ethanol group. The decrease in enzymatic activities in ethanol offspring could, in part, be due to a folate deficiency, because a folic acid supplementation during gestation and lactation periods leads to an advantageous effect on pancreatic enzyme secretion. However, Saluja and Bhagat (2003) found that ethanol administration results in a transient increase in pancreatic amylase output and plasma CCK levels which are mediated by a trypsin-sensitive CCK-releasing factor of the duodenal lumen.

Folate is an essential co-factor for many biochemical reactions; since de novo synthesis of methyl groups requires the participation of folate co-enzymes, Balaghi and Wagner (1992) investigated the effect of folate deficiency on pancreatic exocrine function. They reported that pancreatic secretion was significantly reduced in the folate deficient group. Later, Balaghi and Wagner (1992) suggested that folate deficiency might affect production of zymogen granules as well as their release. In similar experimental animals, we reported that folic acid was an antioxidant agent (Cano et al., 2001). This property could be responsible for the increased amylase, chymotrypsin, and lipase secretion in response to CCK in folate supplemented group.

Dietary folate deficiency and several other abnormalities of folate metabolism are common in alcoholic patients. Our research group (Fernández-Borrachero et al., 1996) described that the intake and the serum folic acid levels are significantly reduced in ethanol-fed adult rats; the same happened this time in the offsprings. Folate deficiency may contribute to pancreatic injury in malnutrition and alcoholism (Elseweidy and Singh, 1984).

In conclusion, our findings indicate that, after CCK stimulus-secretion coupling, ethanol intake by mothers negatively affects the digestive function in offspring during basal conditions and CCK stimulation. The effects of ethanol on the enzymatic activities were attenuated by folic acid. Thus, ethanol exerts its action over exocrine pancreatic secretion by two pathways: ‘per se’ and diminishing the folic acid content. Ethanol could alter the physiological process of secretion, creating a status that could generate pancreatic pathologies. When we supplemented rats with folic acid during pregnancy and lactation, we obtained an advantageous effect on amylase, lipase, and chymotrypsin release in their offspring. Although extrapolation from animal studies may be tenuous, the present findings may explain the use of folic acid in the prevention of damage induced by ethanol to increase the enzyme levels to adequate physiological concentrations.

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