Hyperlipemia and Early Pancreatic Injury Induced by Ethanol Intake in Rats

Chie YUASA,1 Osamu MIYOSHI,1 Kiyoshi FUKUI2 and Toshinori OKA1,*
1Pharmacology Research Laboratory, R & D Headquarters, Tokushima Research Center, Taiho Pharmaceutical Co., Ltd., 224–2, Ebisuno, Hiraishi, Kawanishi-cho, Tokushima 771–0194, Japan
2Institute for Enzyme Research, The University of Tokushima, 3–18–15, Kuramoto, Tokushima 770–8503, Japan (Received June 5, 2000)

Summary The pathogenesis of alcoholic pancreatitis is unknown, and even though hyperlipemia has been hypothesized to be a risk factor for alcoholic pancreatitis, no studies directly investigating whether there is a relationship between the two have ever been reported. Therefore, to determine if a relationship exists between hyperlipemia and alcoholic pancreatitis, especially the early stage of alcoholic pancreatic injury, we administered a regular liquid Lieber-DeCarli diet, with and without ethanol as 35% of total calories, to rats for 2 wk. Thereafter we measured their plasma lipid concentrations, pancreatic zymogen granule fragility, and plasma lipase activity and subsequently investigated the correlations between these parameters. Significant increases in plasma triglyceride, total cholesterol, phospholipid, nonesterified fatty acid, pancreatic zymogen granule fragility, and plasma lipase activity were observed in the ethanol liquid diet group, compared with the values of the control liquid diet group, and pancreatic zymogen granule fragility was correlated with plasma triglyceride (r=0.62), total cholesterol (r=0.77), phospholipid (r=0.76), nonesterified fatty acid concentrations (r=0.62), and lipase activity (r=0.63). These results show a possible relationship between hyperlipemia and the early stage of alcoholic pancreatic injury, and they may support the hypothesis that hyperlipemia contributes to the etiology of alcoholic pancreatitis.

Key Words early stage, ethanol intake, rat, hyperlipemia, pancreatic injury

Chronic alcohol intake induces the manifestations of alcoholic pancreatitis, but the pathogenetic mechanism has never been explained (1, 2). Lipids have often been demonstrated to be causally related to pancreatitis, and because alcohol intake promotes lipid synthesis in the liver and pancreas and induces the manifestations of fatty liver, hyperlipemia (3-5), and fatty pancreas (6-8), lipids have been attracting interest and have been investigated as a risk factor for alcoholic pancreatitis (1).

Haber and colleagues hypothesized the following pathogenetic mechanism for alcoholic pancreatitis: As a result of alcohol administration, lipids in the pancreas increase the fragility of the zymogen granules (9) and lysosomes (10, 11) in pancreatic acinar cells and induce the autoactivation of pancreatic zymogens such as trypsinogen. These activated enzymes destroy pancreatic tissue, thus triggering the initial stage of alcoholic pancreatic injury (12).

It has never been demonstrated, however, that hyperlipemia develops in the early stage of alcohol intake (3–5), and it is quite possible that increased lipids in the blood cause the early stage of alcoholic pancreatic injury. Surprisingly, however, there have been no reports of any direct experimental investigations of a relationship between hyperlipemia and the early stage of alcoholic pancreatic injury. Therefore, to investigate whether this relationship exists, we administered ethanol to rats in their diet for 2 wk. Thereafter we measured their plasma lipid concentrations, pancreatic zymogen granule fragility, and plasma lipase activity; then we examined the correlations between these parameters.

MATERIALS AND METHODS

Animals and experimental design. Four-wk-old male Sprague-Dawley rats (Clea Japan, Inc., Tokyo, Japan) were housed in a controlled environment, exposed to a 12-h light/12-h dark cycle, and provided laboratory rat food pellets and water ad libitum for a minimum of 5 d. The experiment was conducted under guidelines established by the Taiho Review Committee of Animal Experiments.

The animals (weighing 124–162 g) were randomly divided into three groups as detailed below, then housed in individual cages and match-fed isocaloric amounts of these diets ad libitum for 2 wk: (1) standard laboratory rat food, as the rat food powder group (n=8); (2) regular liquid Lieber-DeCarli diet (13), as the control liquid diet group (n=8); (3) Lieber-DeCarli diet with 35% of carbohydrate calories replaced with ethanol, as the

* To whom correspondence should be addressed.
E-mail: t-oka@taiho.co.jp
ethanol liquid diet group (n=8). The composition of the liquid diet for (3) was as described by Lieber and DeCarli (13). The diets for (2) and (3) had a total caloric content of 1,000 Kcal/L and were supplemented with regular amounts of vitamins and minerals. The dietary ingredients were purchased from Dyets (Bethlehem, PA, USA). The animals in the rat food powder group were allowed to consume water ad libitum, but not the animals in the liquid diet groups.

We measured daily food intake and body weight of each animal during the 2-wk feeding period. At the end of the period, the animals were anesthetized with intraperitoneal pentobarbital sodium (45 mg/kg), and blood was collected from the inferior vena cava; some was collected into chilled heparinized tubes. Immediately after blood collection, the animals were killed by exsanguination via abdominal aortic and vena cava puncture, and the pancreas of each was quickly removed and trimmed of other tissue. Part of each pancreas was sampled, frozen in liquid nitrogen, and stored at −80°C until analyzed for its tissue-protein concentration. One hundred milligrams was also sampled for immediate homogenizing in 1 mL of cold unbuffered 250 mM sucrose, pH 5.5. (250 mM sucrose) by four up-and-down strokes of a Handy Micro Homogenizer (Phycotron NS-310E, Microtec Co., Ltd., Chiba, Japan), and the crude homogenate was placed on ice until the dissection of all animals had been finished. The dissection was completed within 1 h, and the crude homogenate of all animals was immediately centrifuged at 150 g for 15 min at 4°C to remove unbroken cells, nuclear material, and debris. The resultant supernatant was used to assess pancreatic zymogen granule fragility.

Measurement of protein concentration in the pancreas. Frozen sections of each pancreas were homogenized in cold physiological saline, and 10% homogenates were prepared. The pancreatic homogenates were diluted 140-fold with 0.1 N NaOH, and the protein concentration in each diluted solution was measured by the method of Lowry et al. (14).

Plasma biochemistry. The blood collected into heparinized tubes was centrifuged at 2,500 g for 10 min. The plasma was removed and stored at −80°C until analyzed for plasma biochemical parameters. The plasma was removed and stored at −80°C until analyzed for plasma biochemistry. Plasma lipase was measured by the BALB-DTNB method (15) by using a Lipase Kit S (Dainippon Pharmaceutical Co., Ltd., Osaka, Japan). All other plasma biochemical parameters were analyzed with a Hitachi 7150 Automatic Analyzer (Hitachi, Ltd., Tokyo, Japan).

Assessment of pancreatic zymogen granule fragility. Pancreatic zymogen granule fragility was assessed by a modification of the method of Haber et al. (9). Briefly, amylase was used as the zymogen marker enzyme. The resultant supernatant from the pancreatic homogenate of the animals in the ethanol liquid diet group or in the control liquid diet group was diluted 100- or 750-fold with cold 250 mM sucrose. The diluted supernatant was then mixed with an equal volume of cold 250 mM sucrose with or without 0.2% Triton X-100, and the mixture was placed on ice and used as a sample for the measurement of amylase activity. Forty-five milligrams of blue starch polymers, which are a nonsoluble substrate of amylase (Neo· Amylase Test DAIICHI, Daiichi Pure Chemicals Co., Ltd., Tokyo, Japan), and 3.8 mL of 250 mM sucrose were well mixed and preincubated at 37°C for 10 min; 200 μL of the cold sample was then added to the mixture. After incubation with the sample at 37°C for 5 min, the reaction was stopped by adding 1 mL of 0.5 N NaOH. The reaction mixture was centrifuged at 1,500 g for 5 min, and the clear supernatant was measured at 620 nm with a DU® 7400 Spectrophotometer (Beckman Instruments, Inc., CA, USA). Amylase activity was quantified by using a Phadebas® Humylase Control (Amersham Pharmacia Biotech, Buckinghamshire, UK) as a standard. Latency was defined as the percent of increase in amylase activity over the initial amylase activity in the sample after the addition of the detergent Triton X-100, which disrupts all biological membranes. Latency was calculated as follows:

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\frac{\text{Amylase activity after Triton}}{-\text{Amylase activity before Triton}} \times 100
\]

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

Statistical analysis. Data were expressed as the mean±SD or ±SE. Comparisons between the control liquid diet group and the other groups were made by Dunnett’s method (SAS System Version 6.12, SAS Institute Inc.). Data regarding pancreatic zymogen granule fragility were analyzed by Student’s t-test (SAS System). Correlations between pancreatic zymogen granule latency and other parameters were examined by linear regression (SAS system). A probability value (p) of less than 0.05 was considered to be statistically significant.

RESULTS

The percent change in body weight, caloric intake, and pancreatic and plasma protein concentrations in the three groups of animals are shown in Table 1. The animals in all three groups were healthy throughout the study, and the general parameters of all three groups were similar.

Plasma lipid concentrations are shown in Table 2. Triglyceride, total cholesterol, phospholipid, and nonesterified fatty acid concentrations in the plasma of the animals in the ethanol liquid diet group were significantly increased compared with those of the animals in the control liquid diet group.

Amylase activity in the pancreas and pancreatic zymogen granule latency for amylase are shown in Fig. 1. These parameters were significantly decreased in the ethanol liquid diet group compared with those of the animals in the control liquid diet group. Decreased pancreatic zymogen granule latency, indicating increased fragility, occurred with ethanol administration.

Plasma lipase activity is shown in Fig. 2. Lipase activity in the plasma of the animals in the ethanol liquid
Table 1. General parameters in rats fed standard laboratory food, a control liquid diet, or an ethanol liquid diet, after 2 wk of consumption.

|                          | Rat food powder | Control liquid diet | Ethanol liquid diet |
|--------------------------|-----------------|---------------------|---------------------|
| Initial weight (g)       | 149 ± 8         | 142 ± 9             | 144 ± 8             |
| Final weight (g)         | 153 ± 7*        | 146 ± 4             | 145 ± 5             |
| Percent change in body weight | 2.98 ± 4.72    | 3.51 ± 5.49         | 0.70 ± 4.29         |
| Caloric intake (Kcal/d)  | 0.21 ± 0.01     | 0.22 ± 0.01         | 0.22 ± 0.01         |
| Protein concentration    |                 |                     |                     |
| Pancreas (mg/g tissue)   | 324 ± 107       | 297 ± 118           | 205 ± 10            |
| Plasma total protein (g/dL) | 4.8 ± 0.3      | 4.8 ± 0.2           | 5. ± 0.2            |
| Albumin (g/dL)           | 3.7 ± 0.2       | 3.8 ± 0.2           | 4.0 ± 0.2           |

Data are expressed as the means±SD for 8 rats per group. *p<0.05 compared with the control liquid diet group by Dunnett's test.

Table 2. Plasma lipid concentrations in the three groups of rats after 2 wk of diet consumption.

|                        | Rat food powder | Control liquid diet | Ethanol liquid diet |
|------------------------|-----------------|---------------------|---------------------|
| TG (mg/dL)             | 36 ± 10         | 44 ± 17             | 103 ± 39**          |
| T. Cho (mg/dL)         | 54 ± 9          | 65 ± 12             | 106 ± 9**           |
| PL (mg/dL)             | 106 ± 13*       | 126 ± 20            | 198 ± 14**          |
| NEFA (mEq/L)           | 0.24 ± 0.08     | 0.28 ± 0.06         | 0.64 ± 0.18**       |

Data are expressed as the means±SD for 8 rats per group. TG: triglyceride; T. Cho: total cholesterol; PL: phospholipid; NEFA: nonesterified fatty acid. *p<0.05 and **p<0.01 compared with the control liquid diet group by the Student’s t-test.

diet group was significantly increased compared with that of the animals in the control liquid diet group. Correlations between pancreatic zymogen granule latency and each of the plasma parameters in the control and ethanol liquid diet groups are shown in Fig. 3. Pancreatic zymogen granule latency was inversely correlated with triglyceride (r=−0.62, p<0.01), total cholesterol (r=−0.77, p<0.001), phospholipid (r=−0.76, p<0.001), nonesterified fatty acid concentrations (r=−0.62, p<0.001), and with lipase activity (r=−0.63, p<0.02).

**DISCUSSION**

To investigate whether there is a relationship between hyperlipemia and the early stage of alcoholic pancreatic injury, we administered ethanol to rats for 2 wk. We thereafter measured their plasma lipid concentrations, pancreatic zymogen granule fragility, and plasma lipase activity. We then examined the correlations between these parameters.

Because alcoholic pancreatic injury is well known to be greatly affected by nutritional status, such as hypernutrition and malnutrition (1), to investigate it accurately we conducted an experiment that used the Lieber-DeCarli diet (13, 16), and we standardized the nutritional conditions of the animals. The results 2 wk after the start of the ethanol administration essentially showed no differences between the rat food powder group, the control liquid diet group, and the ethanol liquid diet group in body weight change, caloric intake, pancreatic protein content, plasma protein and albumin content, or albumin-globulin ratio (data not shown). These results show that the general condition and the pancreas of the animals in all three groups
were normal, and thus we concluded that we could evaluate the injury induced by ethanol in our experiment free of the influence of nutrition.

We therefore first investigated whether there was any correlation between the plasma lipid concentrations and pancreatic zymogen granule fragility. Ethanol ad-
ministration for 2 wk increased the plasma triglyceride, total cholesterol, phospholipid, and nonesterified fatty acid concentrations (i.e., manifestations of hyperlipemia). It also increased pancreatic zymogen granule fragility. Furthermore, significant correlations were found between the increases in plasma lipid concentrations and the increase in pancreatic zymogen granule fragility. The mechanism by which plasma lipids increased pancreatic zymogen granule fragility is unknown. However, because the Lieber-DeCarli rat model showed no histological injurious changes in the pancreas 2 wk after the start of ethanol administration (8), and because our findings showed especially high correlations between the increase in pancreatic zymogen granule fragility and cholesterol and phospholipid, which are major structural components of cell membranes, we suspect that the plasma lipid components that had increased in response to ethanol administration had produced changes in the lipid components that form the cellular membrane of the pancreatic zymogen granules, and that the fragility of the pancreatic zymogen granules may have increased as a result. Haber and colleagues demonstrated that neither ethanol nor any of its metabolites—but rather cholesterol esters in the pancreas—have potent activity that increases the fragility of pancreatic lysosomes (11), and they reported that the same factors may also intensify pancreatic zymogen granule fragility (9). In the present study, however, we showed that there may be factors that increase pancreatic zymogen granule fragility among blood lipids as well as in the pancreas.

We next investigated whether any correlation exists between plasma lipase activity and pancreatic zymogen granule fragility. Ethanol administration for 2 wk increased plasma lipase activity, and a significant correlation was found between the increases in activity and the increases in pancreatic zymogen granule fragility. The mechanism of the process that leads from the increase in pancreatic zymogen granule fragility to the onset of the alcoholic pancreatic injury (i.e., the increase in plasma lipase activity) remains a puzzle. The following hypothesis is now being proposed: As a result of alcohol administration, ethanol and its metabolites increase the fragility of pancreatic acinar cell zymogen granules and lysosomes and induce the autoactivation of pancreatic zymogens, such as trypsinogen. The activated pancreatic enzymes destroy pancreatic tissue, allowing pancreatic enzymes (e.g., lipase) to leak into the blood, and alcoholic pancreatic injury is manifested (2, 12). Because correlations with the initial and final events of alcoholic pancreatic injury were confirmed when we referred to this hypothesis, we are certain that alcoholic pancreatic injury developed.

Therefore we have succeeded in experimentally demonstrating significant correlations between increases in plasma lipid concentrations and increases in pancreatic zymogen granule fragility, and between increases in pancreatic zymogen granule fragility and increases in plasma lipase activity. These findings show a possible relationship between hyperlipemia and the early stage of alcoholic pancreatic injury. We think that in the future it will be necessary to investigate the effect of increases in blood lipid components, especially cholesterol and phospholipid, in response to ethanol administration on the lipid structural components of the membranes of pancreatic zymogen granules and to elucidate the mechanism by which pancreatic zymogen granule fragility increases.

In this study we have demonstrated that a possible relationship exists between hyperlipemia and the early stage of alcoholic pancreatic injury. Our findings may support the hypothesis that hyperlipemia contributes to the etiology of alcoholic pancreatitis.

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