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INCREASE IN HEPATIC GAMMA-GLUTAMYLTRANSFERASE (GGT) ACTIVITY FOLLOWING CHRONIC ETHANOL INTAKE IN COMBINATION WITH A HIGH FAT DIET*†

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Abstract—Increased hepatic gamma-glutamyltransferase (GGT) activity following chronic ethanol consumption has been attributed to enzyme induction, dietary carbohydrate imbalance, and/or to hepatic cell damage. In this study, hepatic GGT activity was increased in rats consuming ethanol (35% of kcals) in a high fat (35% of kcals) diet compared to pair fed and *ad lib.* fed high fat controls ($P \leq 0.01$), but no enhancement of activity was observed in those rats consuming ethanol on low fat (11% of kcals) diets. The high-fat-ethanol group also had increased hepatic lipid ($P \leq 0.01$) and decreased glutathione levels ($P \leq 0.05$) compared to their *ad lib.* fed control group. In rats that had ethanol removed from their diet for the final 4 weeks of the 12-week dietary treatment period, levels of GGT, lipid or glutathione were not different from control values. Histochemical evaluation of hepatic GGT activity showed increases associated with centrolobular lipid accumulation in ethanol-fed rats consuming a high fat diet. The cause of the increase in hepatic GGT activity could not be determined from this experiment. However, increased microsomal enzyme activity did not appear to be related to GGT activity. It is suggested that cellular damage following increased lipid accumulation, depletion of hepatic glutathione, or changes in biliary flow may be associated with the increased GGT activity.

Gamma-glutamyltransferase (GGT; EC 2.3.2.2) catalyzes the first step in the degradation of glutathione and other gamma-glutamyl compounds [1, 2]. In the normal adult animal, enzymatic activity is highest in the kidney, but measurable levels are also found in the pancreas, epididymis, seminal vesicle, jejunal epithelial cells, liver, and spleen [3]. In these tissues, GGT is located on the outer surface of the plasma membrane [4]. Plasma activity is low in the adult, but increases markedly in hepatobiliary disease including viral and chronic hepatitis, obstructive jaundice, liver tumors and liver metastases, Laënnec's and cardiac cirrhosis, and alcoholism [5–8]. Measurement of plasma enzyme activity has been used to follow the progression of various hepatobiliary diseases, [5, 6]. More recently, use of the enzyme in the diagnosis of alcoholism has been suggested. GGT is a sensitive indicator of both acute and chronic ethanol ingestion [9–11], and plasma levels are associated with the degree of hepatic histological damage [8, 12].

The liver has been identified as the source of the plasma enzyme [13–15]. Increases in hepatic enzyme activity have also been found in experimental animals following chronic ethanol consumption [16–19]. However, the cause of the increase in hepatic GGT activity is not known. Microsomal enzyme induction [13, 16, 18–22], dietary carbohydrate imbalance [17, 19, 23], and liver cell damage [6–8] have been suggested as possible factors involved in the increase.

In the studies reported herein, both biochemical and histochemical measurements of hepatic GGT activity, total liver lipids, and hepatic glutathione levels were assessed in rats consuming ethanol at 35% of total caloric intake in low fat (11% of kcals) and high fat (35% of kcals) liquid diets in order to further investigate the mechanism by which hepatic GGT activity is increased following ethanol consumption.

METHODS

Animal care. Seventy male weanling Sprague–Dawley (SD) rats were obtained from Charles River, Inc., Wilmington, MA. Rats were housed individually in wire-bottomed stainless steel cages with *ad lib.* access to water. Temperature (21–23°) and humidity (45–55%) were controlled. A 7:00 a.m.–7:00 p.m. light cycle was maintained. Rats were weighed twice a week. They were fed pelleted AIN-76 diet [24, 25] during the acclimation period.

Dietary composition and preparation. During the dietary treatment period, the rats were fed a liquid diet administered in calibrated feeding tubes which facilitated pair-feeding (Dyets, Inc., Bethlehem, PA). The diet was modeled on the DeCarli–Lieber diet [26], but was modified to contain the components of the AIN-76 diet [24, 25] (Dyets, Inc.) (Table 1). All diets contained dextrin-maltose as the carbohydrate source in place of sucrose. The forms of

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Table 1. Macronutrient composition of diets as percentages of total calories

	Ethanol low-fat (%)	Control low-fat (%)	Ethanol (high-fat (%)	Control high-fat (%)
Dextrin-Maltose	34	69	10	45
Casein	20	20	20	20
Corn oil	11	11	35	35
Ethanol	35		35	

some of the minerals and vitamins were changed to increase solubility but amounts were equivalent to those in the AIN-76 diet. Xanthan gum (4.2 g/liter of diet) was the suspending agent. The diets all provided 1 kcal/ml.

In the low fat diet, 11% of calories was supplied by fat, as in the AIN-76 formulation. Ethanol (35% of kcals) replaced dextrin-maltose in the ethanolic-low fat diet. In the control and ethanol-containing high fat diets, dextrin-maltose was replaced by additional corn oil (35% of total calories).

Diets were made fresh daily in a gallon-sized Waring blender. Rats were fed at 2:00–3:30 p.m. each day, and diet tubes were removed at 8:00–9:00 a.m. the following day. This feeding regime resulted in consumption of approximately one-third of the diet within 2 hr after feeding [27]. Diet tubes were washed daily in mild detergent.

Experimental design. When rats attained 100–120 g body weight, they were divided into ten weight classes. Within each weight class, rats were randomly assigned to one of seven dietary treatments (Table 2). Each of the seven dietary treatment groups contained ten rats. Group 1 rats received low fat diet containing ethanol. The rats in group 2 were individually pair-fed to those in group 1 and received control low fat diet. Group 3 animals were allowed *ad lib.* access to control low fat diet. Rats in groups 4–6 were the counterparts to those in groups 1–3 except these animals were fed a high fat diet. Group 7 animals received the high fat diet containing ethanol for the first 8 weeks of the dietary treatment period, then withdrawn from this diet and fed the control high fat diet for the final 4 weeks; this group was referred to as the regenerating group.

The experiment was terminated after 12 weeks of dietary treatment. Diet tubes were removed, and rats were killed between 9:00 and 11:00 a.m. They were killed while under CO_2 anesthesia and the livers were removed and weighed. A 0.25 to 0.5 cm thick cross-section of each lobe was frozen on solid CO_2 and stored at −70° for histochemical analysis. The remainder of the left median lobe was then frozen on solid CO_2 and stored at −20° for biochemical analysis of GGT activity and glutathione levels. The ventral lobe was frozen for analysis of total lipid content.

Table 2. Study design

Group	Diet
Low fat	
1	Ethanol-*ad lib.*
2	Control-pair fed to Group 1
3	Control-*ad lib.*
High fat	
4	Ethanol-*ad lib.*
5	Control-pair fed to Group 4
6	Control-*ad lib.*
7	Ethanol (8 weeks): Control (4 weeks)-*ad lib.*

GGT histochemistry. GGT was determined histochemically by a modification of the method of Rutenberg *et al.* [28]. Liver slices, embedded in O.C.T., a water soluble support medium (Fisher Scientific, NY) were sectioned at 10 μm in a Cryocut-11 cryostat (American Optical Co., Buffalo, NY) at a temperature between −15° and −18°. Sections were air dried and then defatted and fixed in ice-cold acetone for 24 hr. This step decreased diffusion of the fat soluble dye and increased the time before the dye crystallized. Air-dried sections were then incubated for 1 hr in the substrate medium that contained 4 mM gamma - glutamyl - 4 - methoxy - 2 - naphthylamide (Vega Biochemicals, Tucson, AR) and the dye fast blue BBN salt (Sigma Chemical Co., St. Louis, MO). Sections were rinsed in 0.9% saline, the dye was stabilized with 1 M cupric sulfate, and the sections were then rinsed again in saline. Sections were mounted in glycerin jelly. The dye was stable for 2–4 weeks before it crystallized and faded. Sections were evaluated for enzyme activity within 2 weeks.

Extent of GGT-positive staining was evaluated by projecting microscope slides onto a 50 × 40 grid of 2000 dots. The number of dots covered by stained tissue was counted and compared to the total number of dots within the outline of the liver section. That proportion was recorded as a percentage of the section that was positive for GGT.

GGT biochemistry. GGT activity in the liver membrane fraction was analyzed biochemically by a modification of the method of Cameron *et al.* [29]. Pieces (2 g) of the left median lobe of the liver were homogenized in 4 ml of 0.1 M Tris buffer (pH 7.6 at 4°) containing 0.25 M sucrose and 10 mM $MgCl_2$. Following centrifugation at 1000 *g* to remove cell debris, a membrane preparation was made by centrifuging the supernatant fraction at 105,000 *g* for 1 hr. The membrane preparation was resuspended in buffer containing 0.1 M Tris and 10 mM $MgCl_2$ (pH 8.0 at 37°), put into cuvettes (0.25 ml in each of two cuvettes), and allowed to preheat for 2–3 min. Prewarmed resuspension buffer (1.5 ml) was added to the reference cuvette, and prewarmed substrate solution (1.5 ml) was added to the sample cuvette. The substrate solution was comprised of resuspension buffer with 4 mM gamma-glutamyl-*p*-nitro-

aniline and 50 mM glycylglycine. The solution was made up immediately before use and heated to 50° to solubilize the substrate. The rate of production of *p*-nitroaniline was followed at 405 nm over a 10-min period in a Beckman spectrophotometer, model 25, with the heating jacket at 37°. Enzyme activity was calculated by use of the molar extinction coefficient of 10,050 mM^{-1} for *p*-nitroaniline and expressed as I.U. per milligram protein per minute. The protein content in a 1:10 dilution of the suspension in water was determined by the method of Lowry *et al.* [30].

Lipid analysis. Total lipid was extracted from 1 to 2 g wet weight of the ventral lobe of the liver by the Folch extraction procedure [31] with chloroform–methanol (2:1) containing 0.005% butylated hydroxyanisole and 0.005% butylated hydroxytoluene. After evaporation of the extracting solvent the dried residue was determined gravimetrically.

Glutathione. The hepatic glutathione content was determined in the crude homogenate prepared for the biochemical GGT analysis by the method of Ellman [32] as modified by Chen *et al.* [33]. Although this method measured soluble thiols in the liver, 90–95% of this fraction was glutathione [1].

Statistical analyses. Paired-groups were compared by the paired *t*-test, and the remaining comparisons were made by analysis of variance. Groups were blocked by weight, and two variables were compared: ethanol intake and dietary fat content. The Statistical Analysis System (SAS) computer package (SAS Institute, Raleigh, NC) was used for all analyses.

RESULTS

No difference in weight gain was observed between rats in pair fed groups 1, 2, 4, and 5 (Table 3). Weight gain was not related to either the level of fat or the presence of ethanol. The equivalent calorie consumption in these groups appeared to determine their equivalent weight gain. Conversely, the *ad lib.* fed animals (groups 3 and 6) gained about 25% more weight, which compared with the consumption of approximately 25% more food. Rats consuming ethanol diets drank 75–80 ml/day compared to 100–105 ml/day in the *ad lib.* groups. At the end of the experiment, ethanol intake was 8–9 g/kg body wt/day in groups 1 and 4. Rats in the regenerating group (group 7) consumed the ethanol high fat diet during the first 8 weeks and gained weight at the same rate as group 4; however, during the final 4 weeks when they consumed *ad lib.* the control high fat diet, weight gain increased and they weighed only 10% less than the *ad lib.* controls in group 6 at the termination of the experiment.

Liver weight was not different between rats consuming the ethanol low fat diet compared to their pair-fed low fat controls (Table 3). However, rats consuming the ethanol high fat diets had significantly greater liver weights than did their pair-fed high fat controls ($P \leq 0.01$). The liver weights of the regenerating rats were not different from the animals consuming the non-ethanol diets throughout the experiment.

Visual examination of the histochemical distribution of hepatic GGT showed greater activity in rats fed the high fat diet, especially in the livers of those animals consuming the ethanol-high fat diet (Fig. 1). The increased GGT activity appeared to be located centrolobularly in the areas of excessive lipid accumulation (Fig. 1). Determination of the percent area occupied for GGT activity confirmed this observation (Table 4). Biochemical measurement of hepatic membrane GGT activity also showed the same pattern (Fig. 2). Hepatic membrane GGT activity in rats consuming ethanol was significantly higher than activity in the pair-fed controls ($P \leq 0.01$). Rats on high fat diets had significantly greater activity than those on low fat diets ($P \leq 0.01$). Activities in the regenerating rats were not different from the *ad lib.* fed control animals, even though these animals had been fed the ethanol diets for the first 8 weeks of the experiment.

Hepatic total lipid levels were elevated significantly in rats consuming the ethanol high fat diet when compared to their pair-fed controls ($P \leq 0.01$) (Fig. 2). A similar effect of ethanol was not observed in animals fed the low fat diet. Rats fed the high fat diets (groups 4–6) had more hepatic lipid than animals fed the low fat diets (groups 1–3) ($P \leq 0.01$). Hepatic lipid levels of the regenerating animals switched to the high fat diet (group 7) were not different from animals fed the high fat diet throughout (group 6).

Rats consuming the ethanol high fat diet (group 4) had lower hepatic glutathione levels ($P \leq 0.01$) than those pair fed the control diet. In addition, rats on the high fat diets had lower levels than those on the low fat diets ($P \leq 0.01$).

Correlation coefficients between GGT activity, lipid levels, and glutathione levels were low when all animals in all groups were included in the analysis.

Table 3. Final body and liver weights

	Body weight (g)		Liver weight (g/100 g body wt)	
	Low fat	High fat	Low fat	High fat
Ethanol	(1) 484 ± 17	(4) 483 ± 11	(1) 3.0 ± 0.1	(4) 3.4 ± 0.1
Pair fed	(2) 492 ± 18	(5) 484 ± 10	(2) 2.9 ± 0.1	(5) 2.8 ± 0.1
Ad lib.	(3) 615 ± 27	(6) 597 ± 15	(3) 3.2 ± 0.1	(6) 3.2 ± 0.1
Regenerating		(7) 533 ± 15		(7) 3.0 ± 0.1

Data are expressed as means ± S.E.M. Numbers in parentheses are the respective group assignments (see Table 2).

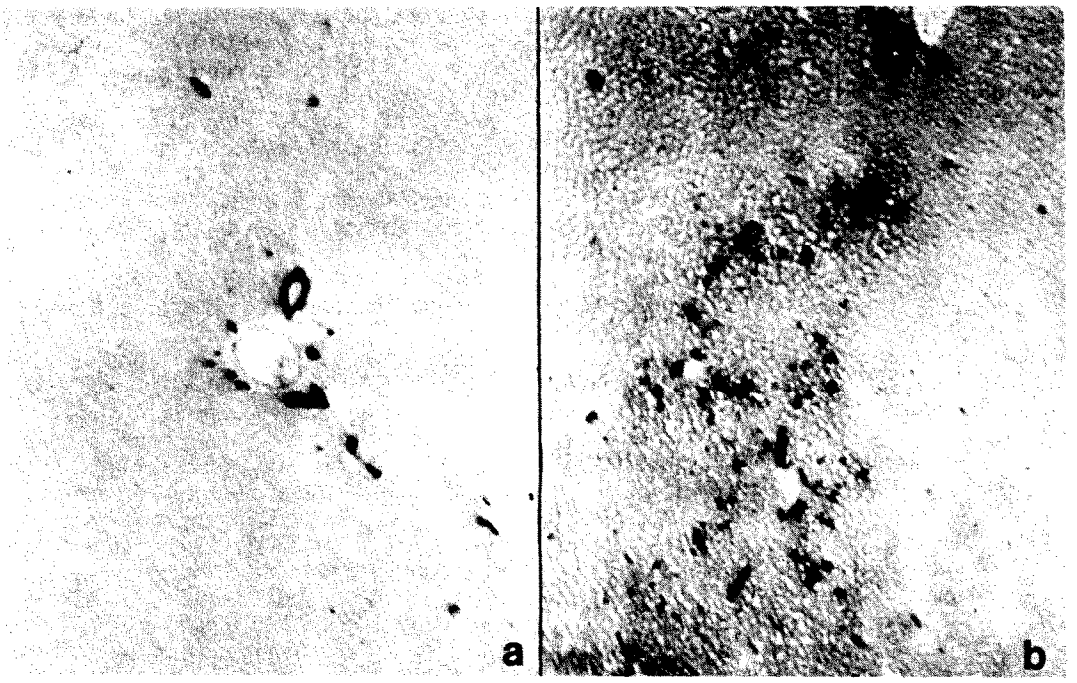

Fig. 1. (a) Hepatic GGT activity of a control rat consuming a low fat diet. Only the bile ducts are GGT-positive. (b) Hepatic GGT-positive activity of a rat consuming an ethanol-high fat diet. GGT activity is no longer limited to the bile ducts, but appears in hepatocytes in the centrolobular regions. The activity is highest in the areas where lipid has accumulated (represented by the empty circular spaces on the section). Magnification: × 100.

However, when only the ethanol diet animals were analyzed, correlations were significant for GGT activity and lipid levels ($r = 0.53$) and for lipid and glutathione levels ($r = -0.56$).

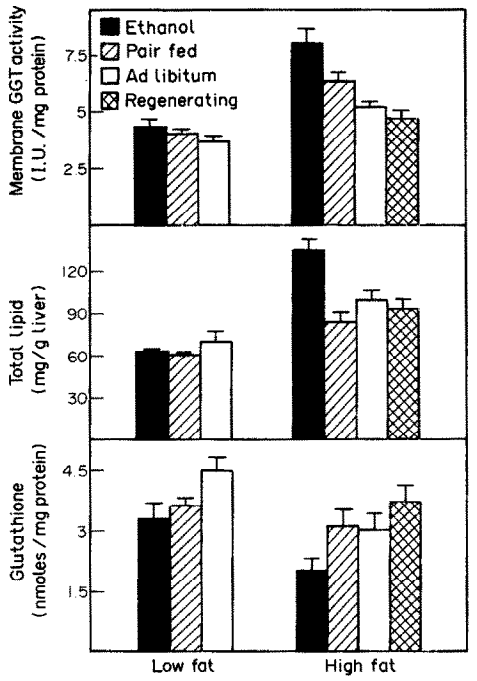

Fig. 2. Comparison of hepatic GGT activity, total lipid levels, and glutathione content in groups 1–7 (N = 10, mean ± S.E.M.).

DISCUSSION

Although elevated plasma GGT activity is used as a diagnostic tool to detect recent and/or prolonged ethanol consumption [7, 11], and the source of that enzyme is the liver [4], the mechanism for that increase is not yet understood.

Increases in plasma GGT activity were originally attributed to hepatic cellular damage [5–12]. That, however, has not, to these authors' knowledge, been shown to be the case with GGT.

Increases in GGT activity following ethanol consumption do not appear to be due to microsomal enzyme induction, as has been suggested by others [15, 16, 18, 20, 21]. These investigators have argued that, following ethanol consumption, GGT activity is enhanced in the microsomal fraction, along with other microsomal enzymes. However, GGT is localized on the plasma membrane [1], and plasma membrane fragments often contaminate microsomal

Table 4. Gamma-glutamyltransferase (GGT) activity of the ventral lobe

	GGT activity (% area occupied)	
	Low fat	High fat
Ethanol	8.8 ± 0.8	20.2 ± 3.1
Pair fed	7.6 ± 1.0	15.6 ± 2.6
Ad lib.	7.1 ± 1.0	12.3 ± 1.9
Regenerating		13.0 ± 2.0

Means ± S.E.M.

preparations [4]. Following the administration of certain inducing agents, isolated plasma membrane fractions show far greater enhancement of GGT activity than do microsomal fractions [4]. In addition, GGT co-sediments with 5′-nucleotidase, another plasma membrane enzyme that is also found in the microsomal fraction [34]. Moreover, this enzyme activity appears in the plasma in experimental animals consuming ethanol [15]. Thus, although enhanced GGT activity may be found in the microsomal fraction, this activity does not necessarily originate in the endoplasmic reticulum.

In previous studies from this laboratory, comparisons of microsomal enzyme induction showed no differences between rats on high or low fat diets following ethanol consumption, even though GGT activity was more elevated in rats on the high fat-ethanol diet [27].

Dietary carbohydrate imbalances have also been suggested as an apparent cause of increased GGT activity in rats consuming ethanol [17, 23]. This hypothesis is based on the observation that an excess intake of certain dietary carbohydrates, such as fructose, can repress enzyme activity [17]. In the ethanol diet in which ethanol isocalorically replaced carbohydrate, GGT activity was enhanced relative to the carbohydrate control diet. Recently, Teschke and Petrides [19] attempted to evaluate the effect of different levels of dietary carbohydrate on GGT activity. Their results are difficult to interpret because comparisons were made among non-isocaloric diets. By changing the levels of carbohydrate in their hypocaloric and hypercaloric diets, they changed the relative proportions of all the nutrients in each diet. The GGT activity changes they observed could have been due to changes in dietary carbohydrate and/or fat, both of which were simultaneously changed.

In the present study, changes in GGT activity were specifically localized in the areas of hepatic lipid accumulation that was observed only in rats on the high fat diet. Lieber and DeCarli [35] have also noted that hepatic lipid accumulation following ethanol consumption was dependent upon dietary fat level. Previous studies on GGT induction following ethanol consumption have been done in rats consuming the DeCarli–Lieber diet, in which fat provided 35% of the total caloric intake, a diet comparable to the high fat diet that was used in this study. Shaw and Lieber [18] found that steatosis was associated with increased plasma GGT activity, although no other evidence of cell damage was noted.

In a study on histochemical distribution of GGT activity in liver disease, Tanaka [36] noted an increased activity in alcoholic liver injury. The activity was specifically increased in the region surrounding cells showing fatty degeneration. He found that the activity was associated with proliferating connective tissue and attributed the increase to mechanical obstruction of the bile tract rather than to liver degeneration. Other changes in biliary flow have been observed in alcoholic liver disease. These changes may also become involved in the increased GGT activity following ethanol consumption.

Enhanced hepatic GGT activity could also be associated with depletion in hepatic glutathione levels. MacDonald *et al.* [37] found that hepatic glutathione was depleted following alcoholic liver injury, possibly as a consequence of increased lipid peroxidation. Since GGT is active in the metabolism of glutathione, it would have been interesting to have examined the histochemical distribution of glutathione in the livers of these rats. Morton and Mitchell [38] have shown that glutathione turnover increases in correlation with increased hepatic GGT activity following chronic ethanol administration. A reduction in glutathione levels in cells with high GGT activity would not be unexpected.

In conclusion, the micrographs from this experiment show for the first time that enhanced hepatic GGT activity following chronic ethanol consumption is localized solely in areas of the liver associated with lipid accumulation, perhaps in association with a depletion of glutathione levels. This increase in GGT activity could be associated with ethanol-induced damage to periportal hepatocytes. However, we do not believe there is sufficient evidence to conclude that cellular damage, microsomal enzyme induction, or dietary carbohydrate imbalance caused enhancement of GGT activity.

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