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ENHANCEMENT OF ETHANOL-INDUCED LIPID PEROXIDATION IN RAT LIVER BY LOWERED CARBOHYDRATE INTAKE

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Abstract—In order to investigate the effect of carbohydrate intake on ethanol-induced lipid peroxidation and cytotoxicity, rats were maintained on four different test diets, a medium-carbohydrate (carbohydrate intake, 8.4 g/day/rat on average), a low-carbohydrate (carbohydrate intake, 2.8 g/day/rat on average), an ethanol-containing medium-carbohydrate (carbohydrate and an ethanol intake, 8.4 and 2.9 g/day/rat on average, respectively), and an ethanol-containing low-carbohydrate diet (2.8 and 2.9 g/day/rat on average, respectively). Ethanol and the low-carbohydrate diet each increased the liver malondialdehyde content, but the combined effect of both (ethanol-containing low-carbohydrate diet) was much more prominent than either alone. The degree of increase in malondialdehyde content almost paralleled the activity of the microsomal ethanol oxidizing system. Both the low-carbohydrate and the ethanol-containing low-carbohydrate diets decreased the liver glutathione content, but ethanol combined with the medium-carbohydrate diet had no effect on the content. Ethanol treatment increased the liver triglyceride content only when combined with the low-carbohydrate diet. The rate of NADPH-dependent microsomal malondialdehyde formation was much higher in microsomes from rats maintained on the ethanol-containing low-carbohydrate diet than in those from rats on the ethanol-containing medium-carbohydrate diet, indicating that lowered carbohydrate intake augments ethanol-induced malondialdehyde accumulation in the liver by enhancing the rate of lipid peroxidation. In addition, when incubated with red blood cells in the presence of NADPH, microsomes from rats fed the ethanol-containing low-carbohydrate diet caused marked hemolysis, which was prevented by the addition of 5 mM glutathione to the incubation system. Furthermore, addition of 50 mM ethanol to the reaction system greatly accentuated the hemolysis. These results suggest that lowered carbohydrate intake at the time of ethanol consumption potentiates ethanol cytotoxicity by enhancing ethanol-induced lipid peroxidation.

A considerable amount of evidence has accumulated regarding the important role of lipid peroxidation in the causation of alcoholic liver injury [1–3]. Two mechanistic explanations—an increase in the rate of lipid peroxide production and a decrease in the defense mechanism against the peroxidation—have been proposed for the ethanol-induced lipid peroxidation [4]. Both explanations are possible since chronic ethanol treatment induces the production of free radicals [5] and malondialdehyde (MDA§) [6–9] in the liver microsomal membrane as well as causing depletion of hepatic reduced glutathione (GSH) content in the liver [6, 9–11].

However, ethanol-induced lipid peroxidation is still controversial: some investigators have reported an increase in MDA production in the liver [6–9], the presence of conjugated dienes in liver lipid extracts [11, 12], and ethane and pentane exhalation [13, 14], while others have failed to confirm the occurrence of lipid peroxidation in ethanol-treated rats [15, 16].

It has been suggested that this discrepancy derives from the experimental conditions including the nutritional state of the animals during ethanol treatment [1]. The ethanol-containing liquid diet which has frequently been employed in experimental studies includes ethanol in the replacement of isocaloric carbohydrate (CHO) [17]. This means that the ethanol diet is a low-CHO diet supplemented with ethanol. The question therefore arises whether the ethanol-induced lipid peroxidation is attributable to the effect of ethanol only or to the effect of ethanol combined with low-CHO intake.

We have found that 1-day food deprivation enhances hepatic cytochrome P450-related metabolism of a variety of foreign chemicals 2–3-fold [18]. A CHO-free diet containing protein, fat and other nutrients in adequate amounts also enhances the activity almost to the same degree as that caused by complete food deprivation [19]. In addition, lowered CHO intake at the time of ethanol treatment markedly augmented the ethanol-induced enhancement of the drug-metabolizing enzyme activity [20]. All these findings suggest that dietary CHO plays a very important part in the activity of cytochrome P450-related enzymes.

We have recently reported that the dietary CHO intake level at the time of ethanol ingestion modifies the development of alcoholic fatty liver in rats [21].

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§ Abbreviations: MDA, malondialdehyde; GSH, glutathione; CHO, carbohydrate; TG, triglyceride; TBA, thiobarbituric acid; RBC, red blood cell; MEOS, microsomal ethanol oxidizing system.

Since cytochrome P450-mediated ethanol oxidation is responsible for ethanol-induced lipid peroxidation [2] and since the lipid peroxidation is proposed as a mechanism for ethanol-induced fatty liver [22], it is possible that dietary CHO intake is associated with the liver damage through the effect on lipid peroxidation.

The present study was undertaken to assess the effect of dietary CHO on ethanol-induced lipid peroxidation and cytotoxicity in the rat liver.

MATERIALS AND METHODS

Animals. The experiment was performed in accordance with Guidelines for Animal Experiments of the Shinshu University School of Medicine. Male Wistar rats were purchased from Nippon SLC (Shizuoka, Japan). They were housed individually in stainless steel wire-bottom cages in an air-conditioned room (18–22°) with a 12-hr light (6 a.m.–6 p.m.) and a 12-hr dark (6 p.m.–6 a.m.) cycle. All rats were maintained on pellet food (Nippon Clea CE-2) and water *ad lib.* until they reached 8 weeks of age. Animals were divided into four groups each consisting of five rats. Each group was allotted one of the four liquid dietary regimens which were prepared essentially as described by Lieber *et al.* [17]. A diet (1 kcal/mL) containing casein sodium salt as protein (15% of total calories), a mixture of olive and corn oils as fat (25%) and sucrose as CHO (60%), vitamins, minerals, fibers, and other essential nutrients (L-cystine, DL-methionine and ethyl linoleate) was designated as the medium-CHO diet (control diet). The low-CHO diet was prepared by removing the sucrose (amounting to 36.4% of total calories) from the medium-CHO diet. Thus, the diet was hypocaloric in comparison to the control diet. Supplementation of 5.1 g ethanol to 100 mL of the medium-CHO) or the low-CHO diet provided two ethanol-containing diets, one the ethanol containing medium-CHO diet (hypercaloric in comparison to the control diet) and the other the ethanol-containing low-CHO diet (isocaloric to the control diet), respectively.

Animals received one of these test diets daily as the only source of food and water until killing. Rats assigned to the ethanol-containing low-CHO diet usually had the lowest spontaneous food consumption. They received their diet *ad lib.*, and the daily food consumption was recorded. Animals of the other groups were given the same volume of their particular diets as the rate-limiting group had consumed on the previous day. Daily observation revealed that the average CHO intake was 8.4 g/rat/day in groups kept on the control and the ethanol-containing medium-CHO diets and 2.8 g/rat/day in groups on the low and the ethanol-containing low-CHO diets. The average ethanol intake was 2.9 g/rat/day in both ethanol-fed groups.

Subcellular fraction. After being maintained on each test liquid diet for not less than 3 weeks, rats were decapitated, and the livers removed. Part of the liver was fixed in 10% buffered formalin (pH 7.4) for histological examination. After perfusion with an ice-cold 1.15% KCl solution, the remaining portion was made into a 25% liver homogenate with the same solution. A portion of the homogenate was processed for the assay of lipid peroxides, triglycerides (TG) and reduced glutathione (GSH). The other was centrifuged at 10,000 *g* for 60 min. The supernatant was further centrifuged at 105,000 *g* for 60 min. The pellet was suspended in an aliquot of the salt solution and again centrifuged at 105,000 *g* for 60 min. The washed microsomal pellet was resuspended with distilled water to a concentration of 15 mg protein/mL, aerated with N_2 and stored frozen at −85° until use.

Biochemical analysis. Microsomal protein and cytochrome P450 contents were determined spectrophotometrically according to the methods of Lowry *et al.* [23] and Omura and Sato [24], respectively. The activity of the microsomal ethanol oxidizing system (MEOS) was determined by the head space method of Petersen *et al.* [25]. The liver GSH content was assayed according to the method of Boyne and Ellman [26]. TG in the liver was extracted with chloroform according to the method of Bligh and Dyer [27] and the content was determined using a TG Kit (Wako Pure Chemical, Osaka, Japan). Lipid peroxide content in the liver was determined by measuring thiobarbituric acid (TBA)-positive reactants according to the methods of Uchiyama and Mihara [28] and the content in the serum according to the method of Jose and Slater [29]. The TBA reactant content was expressed in terms of the MDA content by using 1,1,3,3-tetraethoxypropane as a standard.

Microsomal NADPH-dependent lipid peroxidation was assessed using a reaction mixture containing 0.75 mg microsomal protein, 50 mM Tris–HCl buffer (pH 7.4), 1 mM NADP, 20 mM glucose 6-phosphate, 50 mM $MgCl_2$ and 2 IU glucose 6-phosphate dehydrogenase in the final volume of 0.5 mL. The mixture also contained 0 or 50 mM ethanol. The reaction was initiated by the addition of an NADPH-generation system and stopped by 0.2 mL 15% trichloroacetic acid. After various incubation periods at 37°, the rate of MDA production was determined by measuring TBA-positive reactants by the method of Uchiyama and Mihara [28].

The cytotoxicity of ethanol was assessed according to the method of Benedetti *et al.* [30] using red blood cells (RBC) as the target. Stored microsomal solution centrifuged at 105,000 *g* for 60 min and the pellet was resuspended in a 0.09 M NaCl–50 mM phosphate buffer to a final microsomal protein concentration of 15 mg/mL. Rat RBC was prepared as follows: blood was taken from the abdominal aorta using heparin as an anticoagulant and centrifuged at 1600 *g* for 5 min at 4° to obtain packed RBC, which was washed five times with a 0.9% NaCl solution. A final 20% (v/v) RBC solution in 0.9% NaCl was stored at 4° until use. RBC was incubated with microsomes plus an NADPH-generating system at 37° with and without ethanol for predetermined periods of time. The incubation mixture contained 0.75 mg microsomal protein, 0.09 M NaCl–50 mM sodium phosphate buffer (pH 6.6), 0.2% RBC, 0.083 mM NADP, 1.67 mM glucose 6-phosphate, 4.17 mM $MgCl_2$ and 2 IU glucose 6-phosphate dehydrogenase in a volume of 6 mL with or without 50 mM ethanol. After incubation at 37°, the reaction mixture was centrifuged

Table 1. Microsomal protein and cytochrome P450 contents

Diet	Body weight (g)	Liver weight (g)	Microsomal protein (mg/g liver)	Cytochrome P450 (nmol/mg protein)	Microsomal ethanol oxidation (nmol/mg protein/min)
Medium-CHO	213 ± 3*	8.2 ± 0.3	21.8 ± 2.2	0.55 ± 0.04	9.1 ± 1.0
Medium-CHO + ethanol	232 ± 8†	8.6 ± 0.3	21.2 ± 1.5	0.86 ± 0.07†	13.4 ± 1.2†
Low-CHO	156 ± 3†	5.0 ± 0.1†	23.0 ± 1.6	0.70 ± 0.03	12.1 ± 0.7†
Low-CHO + ethanol	171 ± 8†‡	6.6 ± 0.5†‡	27.7 ± 0.6†‡	1.44 ± 0.09†‡	20.3 ± 2.21†‡

* The values represent the mean ± SD for five rats.
† Significantly different from rats fed a medium-CHO diet ($P < 0.05$).
‡ Significantly different from rats fed a low-CHO diet ($P < 0.05$).

Table 2. Malondialdehyde (MDA), glutathione (GSH) and triglyceride (TG) levels after chronic ethanol consumption

Diet	MDA Liver (nmol/g)	MDA Plasma (nmol/mL)	GSH Liver (mg/g)	TG Liver (mg/g)
Medium-CHO	139 ± 12*	5.7 ± 0.3	1.81 ± 0.09	24.4 ± 2.4
Medium-CHO + ethanol	240 ± 66†	5.7 ± 0.2	1.67 ± 0.05	27.7 ± 4.3
Low-CHO	203 ± 21†	5.6 ± 0.3	1.46 ± 0.10†	13.7 ± 1.0†
Low-CHO + ethanol	779 ± 47†‡	5.9 ± 0.5	1.44 ± 0.10†	40.6 ± 10.2†‡

* The values represent the mean ± SD for five rats.
† Significantly different from rats fed a medium-CHO diet ($P < 0.05$).
‡ Significantly different from rats fed a low-CHO diet ($P < 0.05$).

at 10,000 *g* for 10 min. The optical density of the supernatant was then measured at 542 nm and the percentage of hemolysis was calculated by comparison with the optical density of an equal concentration of erythrocytes completely hemolysed in distilled water.

Statistics. Statistical analysis was performed by analysis of variance. When there was significant difference among groups means were tested by Student's *t*-test. The 0.05 level of probability was used as the criterion of significance.

RESULTS

Microsomal protein and cytochrome P450

Ethanol combined with the low-CHO diet had a much greater enhancing effect on the hepatic microsomal protein and cytochrome P450 contents than did ethanol with the medium-CHO diet (Table 1). Ethanol and the low-CHO diet each enhanced MEOS activity in the liver, but their combined effect was much greater than either alone, indicating that lowered CHO intake at the time of ethanol consumption augments the MEOS-inducing effect of ethanol.

MDA, GSH and TG

The low-CHO diet, the ethanol-containing low-CHO diet and the ethanol-containing medium-CHO diet all increased MDA content in the liver in comparison with the control diet, although neither of them had an effect on the serum level (Table 2). The hepatic content was almost doubled by the reduction of CHO from, or addition of ethanol to, the medium-CHO diet (control diet), while it was quadrupled by the combination of the low-CHO diet with ethanol. These results indicate that a low-CHO diet not only induces lipid peroxidation by itself but also potentiates ethanol-induced lipid peroxidation in the liver (Table 2).

The low-CHO diet decreased the liver GSH content. Addition of ethanol to the hypocaloric diet did not further decrease the content, suggesting that the decrease in liver GSH content caused by the ethanol-containing low-CHO diet may be due to the reduction of CHO rather than to the addition of ethanol.

Although lowered CHO intake itself markedly decreased the liver TG content, the low-CHO diet combined with ethanol increased it in comparison with those diets which contained CHO in moderate amount. In accordance with this, fat accumulation was notable around the centrilobular area, but not around the portal area, in the liver of rats treated with ethanol in combination with the low-CHO diet. In contrast, only a few fat droplets were seen in the centrilobular region, though many droplets were observed around the portal area, in the liver of rats fed ethanol with a moderate amount of CHO. These findings were also observed in the liver of rats fed a moderate-CHO diet without ethanol. No significant histological change was noticed in the liver of rats fed the low-CHO diet alone (data not shown). These results indicate that concomitant administration of a low-CHO diet potentiates ethanol-induced fat accumulation in the rat liver.

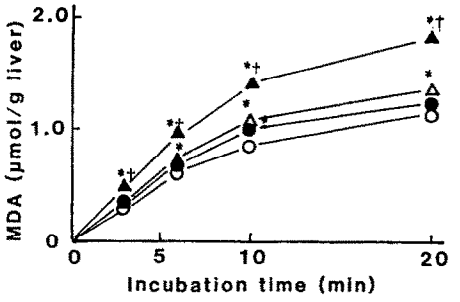

Fig. 1. Effects of carbohydrate and ethanol intake on the microsomal production of malondialdehyde *in vitro*. Each symbol represents the mean for five rats fed: (○) medium-carbohydrate diet; (●) ethanol-containing medium-carbohydrate diet; (△) low-carbohydrate diet; (▲) ethanol-containing low-carbohydrate diet. * Significantly different from rats fed a medium-carbohydrate diet ($P < 0.05$). † Significantly different from rats fed a low-carbohydrate diet ($P < 0.05$).

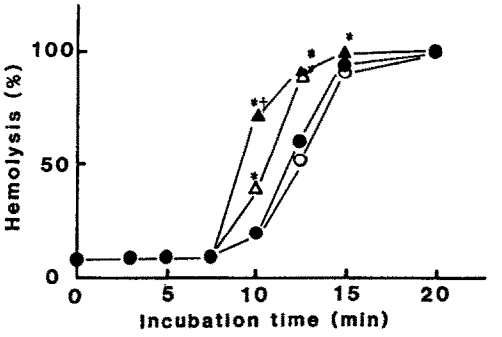

Fig. 2. Effects of carbohydrate and ethanol intake on hemolysis in the microsome–NADPH–RBC system. Each symbol represents the mean for five rats fed: (○) medium-carbohydrate diet; (●) ethanol-containing medium-carbohydrate diet; (△) low-carbohydrate diet; (▲) ethanol-containing low-carbohydrate diet. * Significantly different from rats fed a medium-carbohydrate diet ($P < 0.05$). † Significantly different from rats fed a low-carbohydrate diet ($P < 0.05$).

MDA formation in vitro

As shown in Fig. 1, significantly higher amounts of MDA were generated in microsomes from the liver of rats maintained on the low-CHO diet than in those from rats on the medium-CHO diet. MDA formation was also enhanced in microsomes from the liver of ethanol-treated rat, and this enhancement was much more pronounced when ethanol was administered in combination with the low-CHO than with the medium-CHO diet, indicating that lowered CHO intake may augment the ethanol-induced enhancement of microsomal MDA formation. However, direct addition of ethanol to the incubation mixture had no augmentative effect on the MDA formation in microsomes from any group of rats (data not shown).

RBC hemolysis

RBC was incubated with microsomes of various sources and the rate of RBC hemolysis was measured with incubation time (Fig. 2). Ten minutes after the start of incubation, a marked difference in the rate of hemolysis was noted among the microsomes used. The degree of hemolysis was in decreasing order as follows: microsomes from rats on ethanol-containing low-CHO diet > low-CHO diet > ethanol-containing medium-CHO diet = medium-CHO diet (control diet). Thus, ethanol treatment increased the rate of hemolysis only when it was administered in combination with a low-CHO diet.

Fifty millimolar ethanol directly added to the incubation system affected the rate of hemolysis differently according to the source of the microsomes. Hemolysis was much more prominent when the ethanol was incubated with microsomes from rats maintained on either the low-CHO or the ethanol-containing low-CHO diet than with microsomes from rats assigned to the other dietary regimens. The degree of hemolysis was in the same decreasing order as observed in the incubation without ethanol: microsomes from rats on ethanol-containing low-CHO diet > low-CHO diet > ethanol-containing medium-CHO diet = medium-CHO diet (control diet). However, when 50 mM ethanol directly added to the incubation system without NADPH-generating system, no significant hemolysis was found with any source of microsomes (data not shown). These results indicate that the enhanced MEOS activity may play a role in the augmentative effect of lowered CHO intake on the blood cell injury (Table 3).

Addition of 5 mM GSH to the incubation system completely inhibited the NADPH oxidation- and ethanol metabolism-dependent hemolysis by microsomes from the liver of rats fed the low-CHO diet or the low-CHO diet containing ethanol.

DISCUSSION

Lipid peroxidation has been associated with the development of ethanol-induced liver injuries in animals [1–4]. In the present study, CHO intake at the time of ethanol consumption greatly affected ethanol-induced lipid peroxidation in the liver: lowered CHO intake accentuated lipid peroxidation as manifested by an increase in MDA contents *in vivo* and by an enhanced rate of MDA formation *in vitro*.

Hepatic GSH has been implicated in the development of lipid peroxidation because of its ability to scavenge free radicals that may initiate a chain of peroxidation processes as well as to remove hydrogen peroxides formed in cells [31, 32]. In the present study, hepatic GSH content decreased in rats fed either a low-CHO or an ethanol-containing low-CHO diet. Since there was no significant difference in the content between both groups and since an ethanol-containing medium-CHO had no effect, it can be said that lowered CHO intake rather than ethanol treatment was more closely related to the decrease in the GSH content. However, it is uncertain whether the decrease in hepatic GSH content by lowered CHO intake is due to a decrease in the GSH synthesis, or to an increase in the GSH

Table 3. Hemolysis in the microsome–NADPH–RBC system

Diet	Hemolysis (%)	
	Ethanol (−), GSH (−)	Ethanol (+), GSH (−)
Medium-CHO	3.7 ± 1.7*	6.5 ± 3.3
Medium-CHO + ethanol	5.2 ± 2.1	7.0 ± 2.4
Low-CHO	21.2 ± 7.5†	42.2 ± 10.4†§
Low-CHO + ethanol	47.6 ± 17.1†‡	72.1 ± 9.8†‡§
	Ethanol (−), GSH (+)	Ethanol (+), GSH (+)
Medium-CHO	3.9 ± 0.2	3.0 ± 1.0
Medium-CHO + ethanol	3.4 ± 1.1	3.6 ± 0.3
Low-CHO	3.6 ± 0.9	3.7 ± 0.4
Low CHO + ethanol	4.2 ± 0.5	5.2 ± 2.8

* The values represent the mean ± SD for five rats.
† Significantly different from rats fed a medium-CHO diet ($P < 0.05$).
‡ Significantly different from rats fed a low-CHO diet ($P < 0.05$).
§ Significantly different from the incubation system without ethanol ($P < 0.05$).

synthesis, or to an increase in the GSH depletion related to the enhanced lipid peroxidation in the liver.

Vitamin E is also recognized as one of the major antioxidants, and its deficiency has been shown to enhance the toxicity of some chemicals [33, 34]. In our additional experiments, no difference was noticed in the level of hepatic vitamin E between control (7.5 ± 2.0 μg/g liver) and ethanol-treated rats (6.8 ± 2.2 μg/g liver), suggesting that vitamin E does not concern itself with ethanol-induced lipid peroxidation.

The extent of hemolysis in mixtures of RBC with microsomes and NADPH is an indicator of cellular injury caused by some free radical species [30, 35, 36]. In our experiment, liver microsomes from rats fed a low-CHO diet had a higher capacity to cause the hemolysis than those from rats fed a medium-CHO diet. Microsomes from ethanol-treated rats also caused hemolysis to a greater degree, but the effect of ethanol was noticed only when ethanol was administered in combination with a low-CHO diet (Table 3). This finding is in good agreement with the results of MDA formation *in vitro* where the lipid peroxide formation was most prominent in the incubation system using microsomes from rats kept on ethanol combined with low CHO diet (Fig. 1).

Addition of 50 mM ethanol to the microsome–NADPH system amplified the hemolysis when microsomes from rats fed either a low-CHO or an ethanol-containing low-CHO diet were used (Table 3), but the addition of ethanol produced no influence on MDA formation in the *in vitro* system, a finding consistent with the report by Shaw *et al.* [37]. These findings suggest that cytotoxic substances other than lipid peroxides as manifested by MDA are generated via ethanol oxidation. This may be enhanced by feeding rats ethanol in combination with a low-CHO diet.

Addition of a small amount of GSH to the incubation mixture completely prevented the hemolysis in microsomes from rats fed a low-CHO or an ethanol-containing low-CHO diet (Table 3). This suggests that lowered CHO intake may potentiate ethanol-induced cytotoxicity as a consequence of the decrease in liver defensive ability (GSH) against hepatotoxic lipid peroxidation as well as the increase in generation of microsomal toxic substances such as oxygen radicals or acetaldehyde.

Lowered CHO intake not only induces P450IIE1* by itself but also augments the induction of this isozyme due to ethanol (unpublished data). The isozyme is highly associated with the microsomal H_2O_2- or O_2^--production and lipid peroxide generation in the liver [38]. The enhanced induction of P450IIE1 by a combination of ethanol with a low-CHO diet may be responsible, at least in part, for the increased generation of lipid peroxides and ethanol cytotoxicity. Enzymes other than cytochrome P450, e.g. cytochrome P450 reductase, is also accepted as a mechanism of ethanol-induced microsomal lipid peroxidation [39]. Whether the low-CHO diet affect this enzyme remains to be studied.

In the present study, lowered CHO intake potentiated not only ethanol-induced lipid peroxidation but also TG accumulation in the liver. Whatever the underlying mechanism, the experimental evidence presented here seems to be in favor of the proposition that lipid peroxidation is involved in the development of alcoholic fatty liver [22].

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* The nomenclature used is described by Nebert *et al.*, *DNA* **8**: 1–13, 1989).

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