Hepatic Mitochondrial Glutathione Depletion and Progression of Experimental Alcoholic Liver Disease in Rats

TAKESHI HIRANO,[1] NEIL KAPLOWITZ,[1] HIDEKAZU TSUKAMOTO,[2] SEIICHIRO KAMIMURA[2] AND JOSE C. FERNANDEZ-CHECA[1,3]

[1]*Division of Gastrointestinal and Liver Diseases, University of Southern California School of Medicine Department of Veterans Affairs Outpatient Clinic, Los Angeles, California 90033;* [2]*MetroHealth Medical Center and Department of Veterans Affairs Medical Center, Case Western Reserve University, Cleveland, Ohio 44109; and* [3]*Unidad de Higado, Hospital Clinic: Provincial Universidad de Barcelona, Barcelona 08036, Spain*

Long-term ethanol feeding has been shown to selectively reduce hepatic mitochondrial glutathione content by impairing mitochondrial uptake of this thiol. In this study, we assessed the role of this defect in evolution of alcoholic liver disease by examining the mitochondrial glutathione pool and lipid peroxidation during progression of experimental alcoholic liver disease to centrilobular liver necrosis and fibrosis. Male Wistar rats were intragastrically infused with a high-fat diet plus ethanol for 3, 6 or 16 wk (the duration that resulted in induction of liver steatosis, necrosis and fibrosis, respectively). During this feeding period, the cytosolic pool of glutathione remained unchanged in the ethanol-fed animals compared with that in pair-fed controls. In contrast, the mitochondrial pool of glutathione selectively and progressively decreased in rats infused with ethanol for 3, 6 or 16 wk, by 39%, 61% and 85%, respectively. Renal mitochondrial glutathione level remained unaffected throughout the experiment. Serum ALT levels increased significantly in the ethanol-fed rats at 6 wk and remained elevated at 16 wk. In the mitochondria with severely depleted glutathione levels at 16 wk, enhanced lipid peroxidation was evidenced by increased malondialdehyde levels. Thus a progressive and selective depletion of mitochondrial glutathione is demonstrated in the liver in this experimental model of alcoholic liver disease and associated with mitochondrial lipid peroxidation and progression of liver damage. (HEPATOLOGY 1992;16: 1423-1427.)

The pathophysiology of alcoholic liver disease (ALD) remains uncertain. Many theories with supporting data have been proposed. A variety of evidence suggests a role for lipid peroxidation in the pathogenesis of ALD in experimental models and in human beings (1-3). A critical factor in the effects of prooxidants is the capacity for defense against peroxides. Glutathione (GSH) as a substrate for GSH peroxidase may be especially important. It is compartmentalized in cytosol and mitochondria. GSH is not synthesized in mitochondria, and a transport system mediates its uptake from cytosol (4-6). In view of the injury observed in a variety of organs when the mitochondrial pool of GSH is depleted below critical levels and of the subsequent protection by a selective increase in this compartmentalized pool of GSH, it has been thought that the mitochondrial pool of GSH is an important cell defense in the maintenance of vital cell functions (7-10). In addition, mitochondria do not contain catalase to cope with hydrogen peroxide produced under normal aerobic respiration (11).

We have previously examined the influence of ethanol feeding—in the form of the Lieber-DeCarli diet—on the compartmentalization of hepatic GSH (12, 13). These studies revealed a selective 40% to 50% decrease in mitochondrial GSH level that appeared to reflect impaired mitochondrial uptake (6). This depletion rendered hepatocytes more susceptible to the lethal effects of exogenous peroxide, whereas normalization of mitochondrial GSH levels in hepatocytes from ethanol-fed rats reversed this increased susceptibility (6). To ascertain whether the defect we described previously could be seen in a different model of ethanol feeding and to relate changes to the evolution of liver disease, we performed the following experiments with an intragastric ethanol infusion model known to be associated with liver damage—including inflammation, necrosis and fibrosis—in the rat.

MATERIAL AND METHODS

Animals. Male Wistar rats weighing 350 to 400 gm were implanted with single gastrostomy cannulas as previously described. They were then isocalorically infused for 2 to 3, 5 to 6 or 16 wk with a high-fat diet or a high-fat diet containing ethanol. Detailed descriptions of these diets have been given elsewhere (14-16).

Preparation of Cytosolic and Mitochondrial Extracts. We collected the cytosol- and mitochondria-enriched fractions with a discontinuous Percoll gradient and checked them for

Received May 4, 1992; accepted July 14, 1992.

This work was supported by Department of Veterans Affairs medical research funds, by the Alcoholic Beverage Medical Research Foundation and by U.S. Public Health Service grant AA06603.

Address reprint requests to: Neil Kaplowitz, M.D., LAC 11-221, Department of Medicine, USC School of Medicine, 2025 Zonal Avenue, Los Angeles, CA 90033.

31/1/41324

TABLE 1. Percentage recovery of cytosolic and mitochondrial markers in fractionation of pair-fed and ethanol-fed rat livers

Time	Control rats		Ethanol-fed rats	
	SDH	LDH	SDH	LDH
3 wk				
Cytosol	2.8 ± 1.0[a]	87.6 ± 4.9	2.9 ± 0.4	90.6 ± 0.1
Mitochondria	52.9 ± 3.2	1.1 ± 0.1	51.7 ± 2.6	1.0 ± 0.3
6 wk				
Cytosol	2.8 ± 0.9	91.0 ± 2.9	2.6 ± 0.6	90.1 ± 1.9
Mitochondria	50.9 ± 2.5	1.1 ± 0.3	50.4 ± 3.5	1.1 ± 0.2
16 wk				
Cytosol	6.7 ± 1.4	88.7 ± 5.2	5.4 ± 1.1	89.1 ± 5.1
Mitochondria	50.6 ± 3.9	1.5 ± 0.6	49.8 ± 9.5	1.2 ± 0.3

Recovery is the percentage of the total activity from homogenate recovered in the corresponding fraction.
[a]Data expressed as mean ± S.D. (n = 3 per group).

TABLE 2. Liver weights, hepatic mitochondrial malonaldehyde levels and serum ALT levels

Time	Control rats			Ethanol-fed rats		
	Liver weight (gm)	Mitochondrial malonaldehyde (nmol/mg protein)	ALT (U/L)	Liver weight (gm)	Mitochondrial malonaldehyde (nmol/mg protein)	ALT (U/L)
3 wk	15.3 ± 1.1	ND	11 ± 4	19.5 ± 1.3[b]	ND	21 ± 8
6 wk	14.3 ± 1.0	0.08 ± 0.06	12 ± 6	23.8 ± 2.4[b]	0.09 ± 0.02	99 ± 12[b]
16 wk	15.8 ± 0.5	0.29 ± 0.09	23 ± 8	29.5 ± 3.3[b]	0.40 ± 0.07[b]	154 ± 11[b]

ND = not determined.
[a]Data expressed as mean ± S.D.
[b]$p < 0.05$ vs. control (n = 3 rats per group).

TABLE 3. GSH compartmentation in cytosolic and mitochondrial fractions of pair-fed and ethanol-fed livers

Time	Control rats		Ethanol-fed rats	
	Cytosol	Mitochondria	Cytosol	Mitochondria
3 wk	4.5 ± 0.057[a]	0.46 ± 0.019	3.2 ± 0.16 (28)[b]	0.21 ± 0.008 (55)
6 wk	4.7 ± 0.60	0.37 ± 0.10	2.9 ± 0.95 (39)	0.085 ± 0.014 (77)
16 wk	4.6 ± 0.91	0.58 ± 0.06	3.1 ± 0.47 (32)	0.054 ± 0.009 (91)

[a]Data expressed as μmol/gm liver; they represent the mean ± S.D. (n = 3 per group). All values in the ethanol-fed group are significantly decreased compared with the respective control values ($p < 0.05$ on unpaired Student's t test).
[b]Values in parentheses denote percentage decreases from corresponding control groups.

cross-contamination by measuring the activity of cytosolic lactate dehydrogenase (LDH) and mitochondrial succinic dehydrogenase (SDH) in fractions and crude homogenates, as described previously in detail (6). Protein concentration was determined with a commercial reagent from Bio-Rad Laboratories (Richmond, CA).

Determination of Cytosolic and Mitochondrial GSH Levels in Liver. Cytosolic and mitochondrial fractions were derived and analyzed on HPLC, according to the method of Reed et al. (17), with an Altex 110A double-pump gradient system with a model 420 controller/programmer and an ISCO V4 detector (Cole Scientific, Calabassas, CA) as described previously (6). Values were corrected according to recovery of marker enzymes and expressed as GSH in each compartment per gram of liver and per 100 gm body weight; the latter represents total liver values multiplied by 100/body weight.

Determination of Cytosolic and Mitochondrial GSH of Kidney. Cytosolic and mitochondrial total GSH levels of kidney were measured by determination of the total GSH equivalents (reduced GSH plus oxidized glutathione) with the GSH recycling assay of Tietze (18).

Measurement of Malonaldehyde Formation. Malonaldehyde formation was measured as production of thiobarbituric acid–reactant according to the method of Uchiyama (19) in mitochondrial fractions.

Serum ALT Activity. ALT concentration was measured in serum samples with the Sigma Diagnostic ALT 50 kit (Sigma Chemical Co., St. Louis, MO).

Histological Study. Sections were removed from the left lobe of the liver and fixed in 3% paraformaldehyde in phosphate buffer for light microscopy. In addition to routine hematoxylin and eosin staining, reticulin and Masson's trichrome stains were employed for identification of reticulin and collagens fibers.

Statistical Analysis. Data were expressed as mean ± S.D. Statistical comparisons between groups were made with Student's unpaired t test.

TABLE 4. Compartmentation of GSH into cytosolic and mitochondrial fractions in kidneys of control and ethanol-fed rats

Time	Control rats	Ethanol-fed rats
3 wk		
Cytosolic GSH	1.7 ± 0.16[a]	1.8 ± 0.09
Mitochondrial GSH	0.16 ± 0.02	0.16 ± 0.001
6 wk		
Cytosolic GSH	1.9 ± 0.20	2.0 ± 0.35
Mitochondrial GSH	0.18 ± 0.05	0.19 ± 0.04

[a]Data expressed as mean ± S.D. of total GSH equivalents. No significant differences were found between groups (n = 3 rats per group).

RESULTS

When portions of the livers from pair-fed and ethanol-fed rats were fractionated into cytosol and mitochondria, no difference in the recoveries of LDH and SDH was observed at any time point (Table 1). No difference in body weights was observed between the two groups at any time point, whereas liver weights (Table 2) were greater in the ethanol group by 27%, 42% and 87% at 3, 6 and 16 wk, respectively. Therefore the GSH results are presented per 100 gm body weight. Cytosolic GSH level per 100 gm body weight remained nearly constant throughout the 16-wk study and was nearly identical in the ethanol-fed and control groups (Fig. 1A). When these data were expressed per gram of liver, a modest decrease (ranging from 28% to 39%) in cytosolic GSH level was seen in the ethanol group on comparison with the control group (Table 3). Unlike the cytosolic GSH level, mitochondrial GSH level exhibited a significant and progressive decrease in the ethanol-fed group (39% decrease at 3 wk, 61% at 6 wk and 85% at 16 wk) (Fig. 1B), whereas no significant change occurred in mitochondrial GSH level in controls over the same time course. When expressed per gram of liver, the decreases in mitochondrial GSH level were even more pronounced, as expected (Table 3). Also, the HPLC results indicated that at all times more than 90% of GSH in cytosol and mitochondria in both groups was in the reduced form (not shown). Notably, content of total GSH equivalents of kidney cytosol and mitochondria was unchanged at 3 and 6 wk of ethanol treatment, indicating that the mitochondrial GSH defect was not generalized to extrahepatic organs (Table 4).

Malonaldehyde levels, reflecting lipid peroxidation, were significantly increased in hepatic mitochondria at 16 wk but not at 6 wk. Liver injury was assessed according to serum ALT levels and histological appearance. Serum ALT levels and liver histological appearance confirmed the pattern of progressive ALD previously shown in this model (16). ALT levels were elevated by several times in the ethanol-fed rats at 6 wk and 16 wk (Table 2). Histological study showed induction of centrilobular liver necrosis at 6 wk and more advanced lesions, including focal perivenular and bridging fibrosis, at 16 wk (data not shown), similar to findings of previous reports (14, 15).

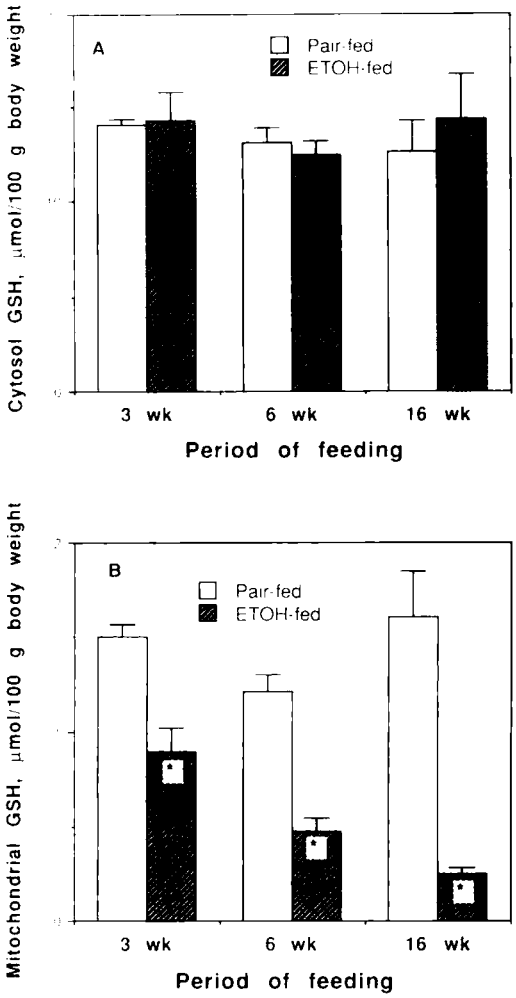

FIG. 1. Effect of chronic ethanol feeding on content of hepatic GSH in mitochondria and cytosol. Data expressed as mean ± S.D.; n = 3. *p < 0.01 vs. control by unpaired Student's *t* test. Body weights of the two groups were not significantly different, whereas liver weights were greater in ethanol-fed rats. To account for the latter difference, data are expressed per 100 gm body wt, thereby reflecting total liver GSH level.

DISCUSSION

These studies confirm our earlier results with the Lieber-DeCarli diet demonstrating a selective and significant decline in mitochondrial GSH level over 6 wk in a different model of ethanol feeding, namely, intragastric infusion. Furthermore, the defect appears to become progressively greater, leading to a rather profound deficiency of mitochondrial GSH level at 16 wk, a time when advanced liver injury is evidenced by enhanced mitochondrial lipid peroxidation and histological observation of liver necrosis and fibrosis.

The precise mechanism for mitochondrial GSH transport is not completely understood. Martensson, Lai

and Meister (5) provided evidence that GSH uptake by mitochondria is ATP dependent. The mechanism for the defect in mitochondrial uptake of GSH induced by ethanol is not understood and will require detailed investigation at the molecular level. Although ATP depletion has been observed with the feeding model used in this study (20, 21), maximum effects were seen at 3 wk, making it difficult to attribute the progressive decline in mitochondrial GSH level to decreased ATP needed for GSH uptake. We previously described an exchange mechanism for the cytosolic and mitochondrial pools of GSH with a half-time of about 18 min, and the transport of GSH from cytosol into mitochondria is impaired in the Lieber-Carli alcohol model (6). Although it is recognized that structural and functional changes in mitochondria are induced by ethanol (22, 23) and might "nonspecifically" lead to impaired GSH uptake, the striking decline in GSH in this study is far out of proportion to modest changes previously reported in ATP synthesis and respiration (24). Moreover, a significant decline in mitochondrial GSH levels occurs early (within 2 wk) and precedes histological liver injury. Thus, in comparing our newest findings with published work on the effects of ethanol on mitochondria, it has been shown that chronic ethanol feeding leads to a depression in state 3 respiration in all three centers of energy conservation of the electron transport chain with various periods of feeding. The magnitude of this depression ranged from 20% to 40% and was observed as early as after 3 wk of feeding, with little variation at longer feeding times (24, 25). The most dramatic ethanol-related alterations in mitochondria are the 50% to 60% decreases in the activity and heme content of the cytochrome oxidase (26-28). Electron transport and proton translocation through the NADH-ubiquinone reductase portion of the electron transport chain are decreased by 30% and 40%, respectively (26, 29). Thus all these changes in the electron transport chain and in the ATPase complex lead to slower respiration in the presence of ADP in the ethanol-fed animals, leading to a lower rate of ATP synthesis (24, 30) without changes in various transport mechanisms in the inner membrane of the mitochondria, including the ADP translocase, inorganic phosphate transport and various energy-rich substrates in the mitochondria and the shuttle NADH mechanisms (24, 29, 30).

Although we cannot determine with absolute certainty at this time whether GSH depletion leads to impaired mitochondrial function or vice versa, depletion of GSH may markedly increase the susceptibility to further mitochondrial dysfunction from oxidant stress produced by ethanol or simply from physiological consequences of aerobic respiration. Indeed, Meredith and Reed (31) showed that hepatocytes succumb to lethal oxidant stress when mitochondrial GSH level is profoundly depleted (31), with the source of oxidant stress being physiological respiration; 3% to 5% of O_2 consumed by hepatic mitochondria is incompletely reduced, leading to superoxide and hydrogen peroxide production (32). In fact, our results support the concept by demonstrating increased lipid peroxidation in mitochondria with the critically reduced level of GSH in the 16-wk ethanol-fed animals. Martensson and Meister (7) have demonstrated in GSH depletion models that relatively selective repletion of mitochondrial GSH with GSH monoesters prevents organ damage. Furthermore, in isolated hepatocytes we have been able to correct mitochondrial GSH depletion with GSH monoethyl ester. Therefore future studies will need to address the effect of correction of mitochondrial GSH depletion with GSH ester on the progression of liver disease in this experimental model.

In conclusion, we have confirmed that mitochondrial GSH is selectively depleted in livers of ethanol-fed rats and that the depletion becomes progressively more profound as histological liver disease progresses.

REFERENCES

1. Ishak KG, Zimmerman HJ, Ray MB. Alcoholic liver disease: pathologic, pathogenic and clinic aspects. Alcohol Clin Exp Res 1991;15:45-66.
2. Kaplowitz N, Fernandez-Checa JC, Ookhtens M. Glutathione, alcohol and hepatotoxicity. In: Halsted CH, Rucker RB, eds. Nutrition and the origins of disease. San Diego: Academic Press, Inc., 1989:267-281.
3. Kato S, Kawase T, Alderman J, Ivatomi N, Lieber CS. Role of xanthine oxidase in ethanol-induced lipid peroxidation in rats. Gastroenterology 1990;98:203-210.
4. Davis JS, Balinsky JB, Harington JS, Shepherd JB. Assay, purification and mechanism of action of γ-glutamyl-cysteine synthetase from the liver of the rat and xenopus laevis. Biochem J 1973;133:667-678.
5. Martensson J, Lai JCK, Meister A. High-affinity transport of glutathione is part of a multicomponent system essential for mitochondrial function. Proc Natl Acad Sci USA 1990;87:7185-7189.
6. Fernandez-Checa JC, Garcia-Ruiz MC, Ookhtens M, Kaplowitz N. Impaired uptake of glutathione by hepatic mitochondria from chronic ethanol-fed rats. J Clin Invest 1991;87:397-405.
7. Martensson J, Meister A. Mitochondrial damage in muscle occurs after marked depletion of glutathione and is prevented by giving glutathione monoester. Proc Natl Acad Sci USA 1989;86:471-475.
8. Martensson JA, Tain A, Frayer W, Meister A. Glutathione metabolism in the lung: inhibition of its synthesis leads to lamellar body and mitochondrial defects. Proc Natl Acad Sci USA 1989; 86:5296-5300.
9. Meredith M, Reed DJ. Depletion in vitro of mitochondrial glutathione in rat hepatocytes and enhancement of lipid peroxidation by adriamycin and 1,3-bis (2-chloroethyl)-1-nitrosourea (BCNU). Biochem Pharmacol 1983;32:1383-1388.
10. Olafsdottir K, Pascoe GA, Reed DJ. Mitochondrial glutathione status during Ca^{2+} ionophore-induced injury to isolated hepatocytes. Arch Biochem Biophys 1988;263:226-235.
11. Neubert D, Woitszak AB, Lehninger AL. Purification and enzymatic identity of mitochondrial contraction factors I and II. Proc Natl Acad Sci USA 1962;48:1651-1658.
12. Fernandez-Checa JC, Ookhtens M, Kaplowitz N. Effects of chronic ethanol-feeding on rat hepatocytic glutathione: compartmentation, efflux and response to incubation with ethanol. J Clin Invest 1987;80:57-62.
13. Fernandez-Checa JC, Ookhtens M, Kaplowitz N. Effects of chronic ethanol feeding on rat hepatocytic glutathione: relationship of cytosolic glutathione to efflux and mitochondrial sequestration. J Clin Invest 1989;83:1247-1252.
14. Tsukamoto H, French SW, Benson N, Delgado G, Rao GA, Larkin EC, Largman C. Severe and progressive steatosis and focal necrosis in rat liver induced by continuous intragastric infusion of ethanol and low-fat diet. HEPATOLOGY 1985;5:224-232.
15. Tsukamoto H, Towner SJ, Ciofalo LM, French SW. Ethanol-

induced liver fibrosis in rats fed high fat diet. HEPATOLOGY 1986;6:814-822.
16. Tsukamoto H, Gaal K, French SW. Insights into the pathogenesis of alcoholic liver necrosis and fibrosis: status report. HEPATOLOGY 1990;12:599-608.
17. Reed DJ, Babson JR, Beatty PW, Brodie AE, Ellis WW, Potter DW. High-performance liquid chromatography analysis of nanomole levels of glutathione, glutathione disulfide and related thiols and disulfides. Anal Biochem 1980;106:55-62.
18. Tietze F. Enzymatic method for quantitative determination of nanogram amounts of total and oxidized glutathione: applications to mammalian blood and other tissues. Anal Biochem 1969;27: 502-522.
19. Uchiyama M, Mihara M. Determination of malonaldehyde precursor in tissues by thiobarbituric acid test. Anal Biochem 1978;86:271-298.
20. Mujamoto K, French SW. Hepatic adenine nucleotide metabolism measured *in vivo* in rats fed ethanol and high fat-low protein diet. HEPATOLOGY 1988;8:53-60.
21. Takahashi H, Geoffrion Y, Butler KW, French SW. *In vivo* hepatic energy metabolism during the progression of alcoholic liver disease: a noninvasive ^{31}P nuclear magnetic resonance study in rats. HEPATOLOGY 1990;11:65-73.
22. Rottenberg H, Robertson DE, Rubin E. The effect of ethanol on the temperature dependence of respiration and ATPase activities of rat liver mitochondria. Lab Invest 1980;42:318-326.
23. Thayer WS, Rubin E. Effect of chronic ethanol intoxication on oxidative phosphorylation in rat liver submitochondrial particles. J Biol Chem 1979;254:7717-7723.
24. Cunningham CC, Coleman WB, Spach PI. The effects of chronic ethanol consumption on hepatic mitochondrial energy metabolism. Alcoholism 1990;25:127-136.
25. Cunningham CC, Kouri DL, Beeker RK, Spach PI. Comparison of effects of long-term ethanol consumption on the heart and liver of the rat. Alcohol Clin Exp Res 1989;13:58-65.
26. Bernstein JD, Penniall R. Effects of chronic ethanol treatment upon rat liver mitochondria. Biochem Pharmacol 1978;27:2337-2342.
27. Rubin E, Beattie DS, Toth A, Lieber CS. Structural and functional effects of ethanol on hepatic mitochondria. Fed Proc 1972;31: 131-140.
28. Thayer WS, Rubin E. Molecular alterations in the respiratory chain of rat liver after chronic ethanol consumption. J Biol Chem 1981;256:6090-6097.
29. Bottenus RE, Spach PI, Filus S, Cunningham CC. Effects of chronic ethanol consumption on energy-linked processes associated with oxidative phosphorylation: proton translocation and ATP-Pi exchange. Biochem Biophys Res Commun 1982;105:1368-1373.
30. Spach PI, Bottenus RE, Cunningham CC. Control of adenine nucleotide metabolism in hepatic mitochondria from rats with ethanol-induced fatty liver. Biochem J 1982;202:445-452.
31. Meredith MJ, Reed DJ. Status of the mitochondrial pool of glutathione in the isolated hepatocyte. J Biol Chem 1982;257: 3747-3753.
32. Jones DP. Intracellular catalase function: analysis of the catalytic activity by product formation in isolated liver cells. Arch Biochem Biophys 1982;214:806-814.