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EFFECTS OF CHRONIC ETHANOL FEEDING ON GLUTATHIONE TURNOVER IN THE RAT*,†

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Abstract—Glutathione (GSH) is important in protection of cells against electrophilic drug injury and against reactive oxygen species. Both steady-state concentrations and turnover of GSH are important determinants of susceptibility of the hepatocyte to injury. Chronic ethanol administration is known to enhance susceptibility to electrophilic drug injury. We have examined the effects of chronic ethanol feeding on GSH turnover and the hepatic activities of GSH peroxidase and enzymes of the γ-glutamyl cycle in the rat. Turnover of GSH was measured in individual animals by measuring the decrease in specific activity of GSH in bile over time after i.v. administration of [^{35}S]cysteine. Rats fed ethanol had significantly increased rates of GSH turnover, 0.287 ± 0.050 hr^{-1} vs 0.131 ± 0.041 hr^{-1} ($P < 0.001$), as well as steady-state GSH levels, 6.59 ± 1.55 vs 4.30 ± 1.28 μmoles/g liver ($P < 0.01$). The activities of gamma-glutamyltransferase (GGT) and GSH-synthesizing enzymes were correspondingly increased significantly. By contrast, GSH peroxidase activity was decreased in ethanol-fed rats, 194 ± 20.8 vs 311 ± 89.9 nmoles NADPH oxidized/min/mg protein ($P < 0.001$). Biliary output and concentrations of GSH and GSSG were similar in both groups. The increase in turnover of GSH was not due to an increase in oxidation of GSH. There was, however, an association between GSH turnover and the activity of hepatic GGT in ethanol-fed but not in control rats.

Glutathione (L - gamma - glutamyl - L - cysteinyl - glycine) is the most abundant thiol in all animal tissues and is contained in high (4–7 mM) concentrations within hepatocytes [1]. Glutathione is involved in a variety of intracellular functions, as recently reviewed [1, 2], and one important function is the protection of cells against damage from reactive electrophiles, free radicals and reactive oxygen intermediates formed during drug metabolism [2]. Steady-state concentrations of glutathione are important in the protection against electrophilic insults to the liver [3–5]. Reduction of intrahepatic glutathione stores by treatment with diethylmaleate potentiates injury from acetaminophen and a number of other drugs [3]. Previous work has suggested that the rate of *de novo* synthesis of glutathione as well as the steady-state concentration of this tripeptide are important in protection against electrophilic, drug-induced liver damage [6–8]. Thus, glutathione turnover rates may be equally important as, or more so than, steady-state levels of glutathione in determining susceptibility to electrophilic drug injury.

Chronic ethanol consumption increases the hepatotoxicity of a number of drugs in animals [9–11] and possibly in man [12, 13]. Since many hepatotoxins cause liver damage by free radical or electrophilic mechanisms, it is possible that some of the increased toxicity in animals chronically fed ethanol may be related to alterations in glutathione metabolism. Acute ethanol exposure decreases hepatic glutathione stores over several hours [14–16], whereas conflicting results have been reported on the effects of chronic ethanol consumption on steady-state hepatic glutathione content [15–17]. Chronic ethanol exposure increases the activity of gamma-glutamyltransferase (GGT) in serum and liver in both rodents [15, 18–20] and patients with alcohol-induced fatty liver [21, 22]. This increase is thought to be due to an induction of the enzyme by ethanol [23]. However, the role of hepatic GGT activity in glutathione turnover is unclear. Thus, we examined the effects of chronic ethanol feeding on the overall turnover rate of glutathione *in vivo* and on the *in vitro* activities of enzymes related to glutathione synthesis and degradation in the rat.

MATERIALS AND METHODS

Male Fischer 344 rats, initially weighing 200–225 g, were pair-fed Lieber-de Carli liquid diets (Bioserve, Rahway, NJ) containing 36% of calories as ethanol or an isocaloric mixture with maltose-dextrins substituted for ethanol [24]. The animals were housed in individual cages and fed for 8 weeks during which time body weights increased by 21.9 ± 6.3%. All rats were given equal amounts of control diet for 24 hr before experiments, to minimize short-term nutritional effects and to eliminate effects of short-term ethanol consumption superimposed on the chronic effects.

Glutathione turnover. At 8:00 a.m. rats were lightly anesthetized with ether, and a laparotomy was

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performed. Cannulae (PE-10 tubing) were placed in the common bile duct and femoral vein. The animals were housed in restraining cages and allowed to recover (45 min). Each animal then received 30 μCi of [^{35}S]cysteine (Amersham, Chicago, IL, sp. act. 34.8 mCi/mmole) intravenously over 1 min. Bile was collected into 5% metaphosphoric acid at hourly intervals for 5 hr. After centrifugation to remove precipitates, samples were chromatographed (Lab Data Control HPLC, Riviera Beach, FL) on Spherisorb ODS-5 μm (R. E. Gourley Co., Laurel, MD) using 1% acetic acid (mobile phase) at flow rate of 1 ml/min. Fractions (1.0 ml) were collected and assayed for radioactivity by liquid scintillation spectrometry and for glutathione as described below. The rate of glutathione turnover was calculated from the decrease in the nlog of specific activity of GSH over time using least squares linear regression. In all instances, the decrease followed a first order exponential decay.

GSH and GSSG assays. Hepatic glutathione (GSH) and glutathione disulfide (GSSG) were measured in livers of animals not used in turnover studies but fed in a similar fashion. GSH and GSSG were determined fluorometrically by the method of Hissin and Hilf [25] after separation of GSH and GSSG by high performance liquid chromatography (HPLC) as described above. Column recovery of synthetic standards of GSH and GSSG (Sigma Chemical Co., St. Louis, MO) was greater than 90% for both.

Gamma-glutamyltransferase. After turnover studies were completed, the liver of each rat was perfused with ice-cold saline and homogenized in 10 vol. of cold 50 mM Tris–HCl buffer, pH 8.2. Activity of gamma-glutamyltransferase was determined by release of *p*-nitroaniline from L-glutamyl-*p*-nitroanilide as described [26].

Glutathione-synthesizing enzymes. The activities of glutathione synthetase and gamma-glutamylcysteine synthetase were measured together using a modification of the assays described by Mooz and Meister [27]. A 105,000 *g* supernatant fraction was prepared from crude liver homogenates and incubated for 0–15 min at 37° in the presence of L-cysteine, L-glutamate and L-glycine with an ATP-generating system. The reaction was stopped by addition of 20% metaphosphoric acid, and activity was determined from the rate of formation of glutathione measured as described above.

Glutathione peroxidase. Activities of the selenium-dependent and selenium-independent peroxidases were assayed in the 105,000 *g* supernatant fraction of liver homogenates from unanesthetized rats by the method of Lawrence and Burk [28]. Activity of the selenium-dependent peroxidase was assayed using 0.25 mM H_2O_2 as a substrate, and combined activity of both peroxidases was determined using 1.5 mM cumene hydroperoxide.

Statistical analysis. All results are expressed as mean ± standard deviation unless otherwise specified. Student's *t*-test (two-tailed) was used to compare means of the ethanol and control groups. The relationship between hepatic GGT activity and GSH turnover rate was determined by comparison of the slopes of the regression lines of GGT versus turnover rate, using bivariate analysis of covariance. A value of $P < 0.05$ was considered the minimum level of statistical significance.

RESULTS

As shown in Fig. 1, chronic ethanol feeding more than doubled the fractional turnover rate of glutathione, from $0.131 \pm 0.041\ hr^{-1}$ (control) to $0.287 \pm 0.050\ hr^{-1}$ (ETOH) ($P < 0.001$). There was a corresponding increase in the activities of both GGT and the glutathione synthesizing enzymes measured *in vitro* (Table 1). The increases in turnover rate and rate of synthesis were paralleled by increased hepatic GSH concentrations in the chronic ethanol-fed rats, 6.59 ± 1.55 μmoles/g liver compared with control, 4.30 ± 1.28 μmoles/g ($P < 0.01$) (Table 1). Liver weight, expressed as a percentage of body weight, was increased significantly from 3.07 ± 0.52 to 3.89 ± 0.45 in ethanol-fed rats ($P < 0.0001$), whereas hepatic protein content was similar, 15.5 ± 6.26 (control) vs 16.2 ± 2.90 mg protein/100 mg liver (ethanol).

Biliary concentration and output of GSH and GSSG were similar in both groups (Table 2) as were the hepatic GSSG levels (Table 1). Glutathione peroxidase activity (Table 3) was decreased significantly in chronic ethanol-fed rats (for both H_2O_2 and cumene hydroperoxide).

The relationship between GGT activity and turnover rates for individual animals is shown in Fig. 2. As shown, there was little difference in GGT activity

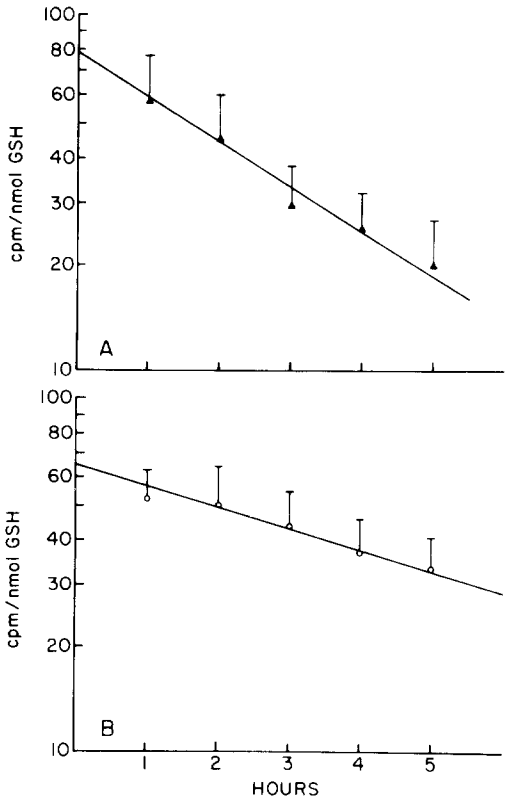

Fig. 1. Specific activity of biliary glutathione (GSH). Specific activity of GSH decreased exponentially with time in both ethanol-fed (▲) (A) and pair-fed control rats (○) (B). Each point represents the mean ± S.D. of six animals.

Table 1. Effects of chronic ethanol on hepatic GSH, GSSG and γ-glutamyl cycle enzymes*

	Hepatic GSH (μmoles/g liver)	Hepatic GSSG (μmoles/g liver)	GSH "Synthesis" (nmoles/min/mg protein)	GGT† (nmoles/min/mg protein)
Control	4.30 ± 1.28	0.38 ± 0.10	2.81 ± 1.15	2.31 ± 0.37
Ethanol	6.59 ± 1.55	0.49 ± 0.11	3.54 ± 1.20	9.9 ± 2.05
	P < 0.01	NS‡	P = 0.05	P < 0.001

* All results shown are the mean ± S.D.; N = 15 pairs except for the GGT values where N = 6 pairs.
† GGT activity was measured in animals used in turnover studies.
‡ Not significant.

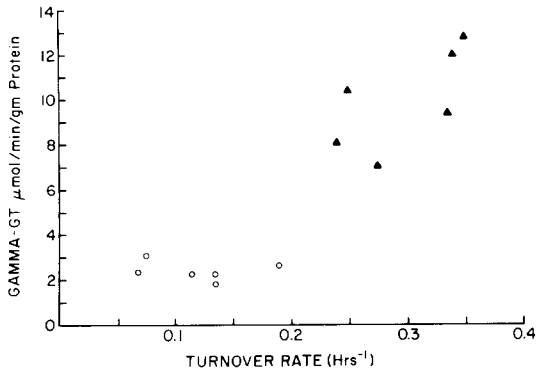

Fig. 2. Relationship between glutathione fractional turnover rate and gamma-glutamyltransferase activity (gamma-GT) in individual animals. Values for ethanol-fed rats are shown as closed triangles (▲) and those for control rats are shown as open circles (○). The slope of the regression line for ethanol-fed rats (y = 1.27 + 29.2 x) is significantly greater than that for control rats (y = 2.56 − 2.06 x) (P = 0.06).

over the range of turnover rate for control animals, whereas there was a close association in ethanol-fed animals. The slope of the regression line for ethanol-fed rats was greater than for controls (P = 0.06).

DISCUSSION

We have shown that chronic ethanol feeding significantly increased the turnover of hepatic glutathione in the rat. Our technique for measurement of glutathione turnover allows for measurement of turnover in individual animals and thus does not rely on pooled data for specific activity of hepatic GSH to calculate rate of turnover. The procedure uses awake animals although it is possible that even 15 min of light ether anesthesia may affect glutathione metabolism. We have assumed that the specific activity of biliary glutathione is similar to that in the liver and that decreases in the specific activity of GSH in livers are reflected by changes in the bile. Previous studies have shown that under most, but not all, circumstances there is a direct relationship between hepatic GSH concentration and biliary output of GSH [29]. One possibility which

Table 2. Effects of chronic ethanol on biliary output of GSH and GSSG*

	GSH (mM)	GSH output (nmoles/min/g liver)	GSSG (mM)	GSSG output (nmoles/min/g liver)
Control	1.30 ± 0.36	1.15 ± 0.37	0.15 ± 0.07	0.13 ± 0.05
Ethanol	1.01 ± 0.58	1.07 ± 0.67	0.10 ± 0.04	0.10 ± 0.04
	NS†	NS	NS	NS

* All results shown are the mean ± S.D.; N = 6 pairs.
† Not significant.

Table 3. Effects of chronic ethanol on glutathione peroxidase activity*

	Glutathione peroxidase activity (nmoles NADPH oxidized/min/mg protein)	
	Substrate	
	Cumene hydroperoxide	H_2O_2
Control	311 ± 89.9	290 ± 82.9
Ethanol	194 ± 20.8	135 ± 25.7
	P < 0.001	P < 0.001

* All results shown are the mean ± S.D.; N = 15 pairs for each of the substrates.

cannot be excluded is that the glutathione entering bile may be, in part, degraded by gamma-glutamyltransferase, an enzyme which is thought to be localized primarily to the bile canalicular membrane in the liver [30]. The increased turnover rate of glutathione observed in ethanol-fed rats is consistent with the observed increases in *in vitro* activities of both gamma-glutamyltransferase and the glutathione synthesizing enzymes measured in this study. The calculated initial specific activity of glutathione extrapolated to time zero was higher in ethanol-fed rats (78.25 ± 52.68) but not significantly different from control (64.29 ± 9.89). Such an increase would be consistent with an increased rate of synthesis *in vivo*.

We found a close association between hepatic GGT activity and the glutathione turnover rate in ethanol-fed, but not in control rats. Under normal circumstances the activity of hepatic GGT is low, particularly in comparison with the activity of renal GGT [31]. Thus, most of the hepatic GSH is exported from the liver into the plasma and degraded by GGT in the kidney. During fasting, there is increased activity of hepatic GGT and an increased turnover of hepatic glutathione [32]. Whether renal GGT activity modulates hepatic GSH turnover, perhaps through changes in plasma GSH levels, is unknown. Although a close association between hepatic GGT and hepatic GSH turnover rate was observed in ethanol-fed rats in the present study, we cannot be certain whether the increase in GGT accounts in part for the increased turnover rate or whether it is a consequence or independent of the turnover rate.

The increase in hepatic glutathione turnover does not appear to be related to increased oxidation of GSH to GSSG. Hepatic concentrations of GSSG were similar in both ethanol-fed and control animals. Furthermore, we did not observe any increase in the amount of GSSG exported from the liver into bile. Since GSSG is exported into bile preferentially [33], we would expect that any increase in oxidation of GSH to GSSG would be paralleled by an increase in biliary output of GSSG. Other investigators have found an increase in biliary output of GSSG from pentobarbital-anesthetized rats chronically fed ethanol [34]. However, our results do not confirm this observation. In the present study glutathione peroxidase activity was decreased in ethanol-fed rats. It is possible that the animals used in this study had a reduced capacity to generate GSSG from GSH in response to peroxide formation due to lower activities of the glutathione peroxidases.

Chronic ethanol consumption increases the hepatotoxicity of a number of drugs which are toxic because of formation of free radicals or electrophilic intermediates. The increased toxicity has been postulated to be due to an increased rate of formation of these intermediates by cytochrome P-450 [9–11]. Ethanol also increases the toxicity of hepatotoxins that do not require cytochrome P-450-mediated activation, such as allyl alcohol [35]. Allyl alcohol is, however, detoxified by glutathione [36]. Fasting and diethylmaleate treatment enhance the toxicity of a number of drugs, and both conditions not only lower hepatic glutathione levels but also increase glutathione turnover and *in vivo* synthesis of glutathione [8, 31]. Toxic doses of acetaminophen actually suppress the rate of synthesis of glutathione [32]. This effect is greater in fasted animals and may be related to an inability to compensate for a greater loss of glutathione through non-detoxifying pathways. Glutathione synthesis is dependent on ATP [27]. Thus, net synthesis may be limited by availability or capacity to generate ATP during electrophilic insult. Chronic ethanol feeding decreases ATP synthesis [37, 38] and may limit compensation (through increased glutathione synthesis) for such stresses.

In summary, we have shown that chronic ethanol consumption increases glutathione turnover. This increase does not appear to be related to increased oxidation of GSH to GSSG, but may result from increased degradation of GSH via the γ-glutamyl cycle. Further work will be necessary to determine the effects of this increased glutathione turnover on the susceptibility to injury from electrophilic drug metabolites.

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