INCREASED BILIARY GLUTATHIONE DISULFIDE RELEASE IN CHRONICALLY ETHANOL-TREATED RATS

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1. Introduction

Glutathione disulfide (GSSG) release from liver [1] has been evaluated as an indicator of oxidative stress, including lipid peroxidation (cf. hydroperoxide metabolism review in [2]). At present, the occurrence of enhanced rates of lipid peroxidation or of H_2O_2 production in the liver of rats chronically treated with ethanol is controversial (cf. review in [3] and references therein) but appears to be a central concept in experimental pathology. Therefore, we have applied a non-invasive approach and have examined the release of both oxidized and reduced glutathione from the liver of rats chronically treated, i.e., for 6 weeks with ethanol, essentially as in [4]. According to the finding that GSSG release occurs into bile [5], measurements were carried out in bile samples obtained from anesthetized rats.

2. Materials and methods

2.1. *Animals and diets*

Male Wistar rats (95–115g initial body weight) were either treated with ethanol for 6 weeks (chronic ethanol) or pair-fed isocalorically with sucrose substitution (controls) as in [4]. The absence of fatty liver is typical of this model of chronic alcoholism [4]. The composition of the basal diet was: laboratory chow (Cargill), 93%; corn oil, 4%; choline chloride, 1%; vitamin diet fortification mixture (ICN Life Sciences Group, Cleveland), 1%; salt mixture Hegsted (ICN Life Sciences Group), 1%. The caloric distribution was: carbohydrate, 54%; lipid, 19%; and protein 27%.

The calory percentages of the ingredients in the final regimen, i.e., basal diet plus ethanol solution, consumed by the animals of the alcohol group were as follows: ethanol, 41.5%; carbohydrate, 42.6%; lipid, 6.6% and protein, 9.3%. The composition of the diet of the control group was the same except that the ethanol-derived calories were replaced by sucrose. The animals in the ethanol group were kept without ethanol for 18 h prior to the experiment but were allowed access to the basal diet and to drinking water.

2.2. *Experiments and assays*

The animals were anesthetized with sodium pentobarbital (50 mg/kg body wt, i.p.) and after opening of the peritoneal cavity, the bile duct was cannulated with a stainless steel cannula. Bile obtained for the first 10 min was discarded and then samples were collected at 15 min intervals for at least 60 min. Care was taken to transfer samples directly to 0°C, and glutathione assays were run immediately. Measurements were carried out with a thermostatted (30°C) Gilford model 2000 spectrophotometer. GSSG was assayed by following NADPH utilization in 50 mM K-phosphate (pH 7.0), 1 mM EDTA after addition of 0.1 U/ml yeast glutathione reductase (Sigma). Total glutathione, i.e., GSH plus 2 GSSG, was determined in the catalytic assay using yeast glutathione reductase and 5,5′-dithiobis-(nitrobenzoic acid) [6,7]. Results are given for GSH, obtained after subtraction of the GSSG value measured in the GSSG assay. We have shown [8] that this procedure gives GSH values

Table 1
Intake of calories and growth in control and chronically (6 week) ethanol-treated male Wistar rats

Group	Calory intake kcal/day per 100 g body wt	Ethanol intake g/day per kg body wt	Growth rate g/day	Liver weight g/100 g body wt
Control	38.9 ± 1.2 (6)	none	2.4 ± 0.2 (10)	4.3 ± 0.4 (5)
Chronic ethanol	41.0 ± 0.9 (6)	24.4 ± 1.9 (6)	2.0 ± 0.1 (10)	4.0 ± 0.2 (5)

Animals were treated as indicated in section 2. Data are means ± SEM, with the number of different animals in parentheses

similar to those obtained by separate GSH measurements.

Activity contents of GSSG reductase and GSH peroxidase were determined at 30°C in extracts obtained from homogenates made in 50 mM K-phosphate, 1 mM EDTA (pH 7.0), 1% Triton X-100 at a ratio of 10 ml/g liver, after centrifugation at 105 000 × g for 30 min. Assays were performed in the same buffer. For GSSG reductase, NADPH oxidation was followed at 340 nm, in the presence of 0.5–1.0 mM GSSG; for GSH peroxidase, NADPH oxidation was followed in the presence of 1 mM GSH, 0.1 U/ml yeast glutathione peroxidase (Sigma) and 0.5 mM *tert*-butyl hydroperoxide (Peroxid-Chemie, München).

3. Results

3.1. *Calory and alcohol intake and growth*

The relevant data on calory and alcohol intake and growth rate are collected in table 1. It is seen that calory intake in the two pair-fed groups was maintained at a similar level of about 40 kcal/day per 100 g body wt (167 kJ/day per 100 g body wt) and that growth rates were also similar. The animals were 200–250 g after 6 weeks of dietary treatment when experiments were performed.

3.2. *Biliary glutathione concentration and bile flow*

The biliary glutathione concentration was measured in samples taken at 15 min intervals in each experiment and they were found to be practically constant. The data for each animal were averaged and the results obtained with 8 different animals of the chronic alcohol and the control groups are presented in table 2. The control value of 0.38 mM GSSG is similar to that obtained [9] in bile samples from perfused livers. In bile samples from ethanol-treated rats, the GSSG concentration was significantly higher, 0.90 mM. On the contrary, GSH concentration was similar in both groups.

Bile flow was also determined at 15 min intervals. The data shown in table 2 again refer to mean values calculated for each experiment; bile flow, however, was not constant throughout the experiment. On an average, bile flow was ~10% higher at the beginning of the experiment than at the end. Clearly, there is no significant difference in bile flow in the 2 groups. Multiplication of the glutathione concentration by

Table 2
Biliary glutathione concentration and bile flow in control and chronically (6 week) ethanol-treated male Wistar rats

Group	Glutathione in Bile		Bile flow
	GSSG (mM)	GSH (mM)	$\mu l \cdot min^{-1} \cdot g\ liver^{-1}$
Control	0.38 ± 0.09 (8)	1.43 ± 0.40 (8)	1.47 ± 0.15 (5)
Chronic ethanol	0.90 ± 0.18 (8)	1.46 ± 0.53 (8)	1.57 ± 0.24 (5)

GSH was calculated by difference between the catalytic assay (GSH + 2 GSSG) and the GSSG assay as indicated in section 2. Data are expressed as means ± SEM, with the number of different animals in parentheses

the average bile flow gives the average rate of glutathione release into bile (nmol.min^{-1}.g liver^{-1}): GSSG release was 0.6 in controls and 1.4 in ethanol-treated animals, whereas GSH release was 2.1 and 2.3, respectively.

3.3. *Activity content of GSSG reductase and GSH peroxidase*

The activity of GSSG reductase was 12.0 ± 1.8 U/g liver (n = 4) in controls compared to 16.8 ± 2.1 U/g liver (n = 3) in the ethanol-treated group. The activity of glutathione peroxidase, assayed with 0.5 mM *tert*-butyl hydroperoxide, was 23.4 ± 3.7 U/g liver (n = 4) in controls and 25.5 ± 0.5 U/g liver (n = 3) in the ethanol-treated group. These preliminary data demonstrate that the activity content of these glutathione-related enzymes is practically unchanged in ethanol treatment, in agreement with a very slight increase – given without the absolute values of the activity content – of these enzymes observed in an earlier study [10].

4. Discussion

The experimental finding of a substantial increase in biliary glutathione disulfide (GSSG) concentration in chronically ethanol-treated rats as compared to isocalorically pair-fed controls may be of relevance in the discussion of liver damage related to chronic alcohol intake. As mentioned above, the long-standing dispute in the literature regarding the occurrence of lipid peroxidation in alcoholism (cf. [3]) may become resolved when experimental approaches of non-invasive and non-destructive nature become increasingly employed. In this respect, GSSG release into bile may prove to be a particularly useful tool (H. S., A. Wahlländer, in preparation). Enhanced rates of GSSG release have been observed during oxidative transitions associated with drug oxidation and during the metabolism of external and endogenous hydroperoxides [11–13]. It is of interest to note that increased rates of glutathione release have been associated with increased rates of lipid peroxidation occurring under hyperbaric oxygen in perfused rat liver and lung, particularly in tocopherol deficiency [14].

Although the mechanism of biliary glutathione release is only poorly understood, as is the mechanism of bile formation in general [15,16], the following arguments may be taken to indicate an enhanced occurrence of hydroperoxides in the liver of chronically ethanol-treated rats:

(i) The measured activity content of GSSG reductase does not indicate a limitation in the ethanol-treated state, so that an increased GSSG release due to an impaired reducing capacity appears unlikely.

(ii) It has been shown in perfusions with model hydroperoxides, e.g., *tert*-butyl hydroperoxide, that there is an approximately linear relationship between extra GSSG release and the rate of hydroperoxide supply to the liver [11].

(iii) The increased rates of GSSG release in ethanol-treated versus control animals are due to increased biliary concentration and not to increased bile flow.

(iv) They are maintained over an extended experimental period of 60 min.

Since the selenium-dependent glutathione peroxidase reacts both with H_2O_2 and with organic hydroperoxides, the present experiments do not provide information on whether H_2O_2 or organic hydroperoxides are involved. Such distinction is possible, however, in similar experiments in progress carried out in selenium-deficiency. Microsomes isolated from the liver of alcohol-treated rats show increased rates of H_2O_2 production [18], and lipid peroxidation was increased in isolated hepatocytes from ethanol-treated rats [19]. The whole liver homogenate of alcohol-treated rats exhibits an enhanced chemiluminescence that may also indicate an increased rate of lipid peroxidation [2,19]. Using the non-invasive and non-destructive approach of measuring alkanes [20] in the expired air, significantly higher amounts of ethane were observed in the rats treated with alcohol [21]. Increased lipid peroxidation may play an important role in the development of cellular injury and death in alcoholic hepatitis.

We have shown [8,12] that the GSSG release is selectively enhanced during oxidative transitions while GSH release remains essentially unchanged; this situation is also found in the present experiments on biliary glutathione release. While intrahepatic glutathione concentration was not measured in these experiments, it should be pointed out that the pools of hepatic amino acids in rats after long-term ethanol feeding remain practically unaltered [22].

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