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Effect of Diethylmaleate on Liver Extracellular Glutathione Levels Before and After Global Liver Ischemia in Anesthetized Rats

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ABSTRACT. Glutathione (GSH), present in a high concentration in the liver, serves important protective functions. We investigated the effect of lowered tissue GSH content, accomplished by diethylmaleate (DEM) administration, on liver extracellular GSH levels before and after global ischemia in anesthetized rats. Liver extracellular GSH levels were determined by microdialysis perfusion and an on-line high performance liquid chromatography system. Global liver ischemia was induced by ligation of the hepatic pedicles including the hepatic artery, portal vein, and bile duct. DEM (4 mmol/kg) significantly lowered both the liver tissue GSH levels (1.36 ± 0.26 μmol/g wet wt vs 9.50 ± 0.55 μmol/g wet wt for the untreated) and the liver extracellular GSH levels (4.3 ± 2.4 μM vs 25.2 ± 8.7 μM for the untreated). Global liver ischemia induced a dramatic increase in the liver extracellular GSH level. Although the liver tissue GSH level was lowered following DEM treatment, DEM administration did not affect significantly ischemia-induced elevation of extracellular GSH (when presented as fold increase relative to basal value). In conclusion, DEM showed a direct effect on liver extracellular GSH content in anesthetized rats. However, DEM treatment did not affect the relative release of GSH following global liver ischemia. *Copyright © 1997 Elsevier Science Inc.* BIOCHEM PHARMACOL 53;3:357–361, 1997.

KEY WORDS. liver ischemia/reperfusion; glutathione; diethylmaleate; microdialysis

GSH† is a tripeptide present in a particularly high concentration in the liver. GSH serves important protective functions including scavenging of oxygen-derived free radicals (or ROS), either directly or in association with GSH peroxidase. GSH may be involved in the detoxification of various drugs and chemicals such as acetaminophen, alcohol, and pesticides by forming a conjugate with these harmful substances followed by subsequent elimination via the biliary route. The detoxification results in a decrease in liver GSH content [1–4], which renders the liver more susceptible to pathophysiological damage [5]. DEM can react with GSH to form a stable conjugate [6, 7], leading to GSH depletion. Thus, DEM is commonly used in experiments involving the investigation of the physiological and biochemical effects of DEM-induced lowering of tissue GSH content.

Hepatic ischemia and reperfusion are unavoidable procedures in liver transplantation. Ischemia/reperfusion may result in the lowering of liver GSH content due to the reaction of GSH with ROS, increasingly formed in the procedure [8–10], or to ischemia-induced GSH release [11]. Lowered liver GSH content following hepatic ischemia may lead to exacerbation of ROS-induced damages [12, 13].

Although liver GSH is involved in the scavenging of ROS formed during liver ischemia/reperfusion, some reports have indicated that following liver ischemia/reperfusion, oxidative stress may occur in the extracellular space [14–16]. Monitoring of hepatic GSH export, usually accomplished by the measurement of GSH content in the sinusoidal efflux from an isolated perfused liver or the liver of an anesthetized animal [14, 17], can be performed by microdialysis perfusion and on-line HPLC [11].

Few reports have focused on the effects of lowered liver GSH content, as often encountered in chemical intoxication and abnormal physiological conditions, on ischemia-induced GSH release. Therefore, we investigated the effect of lowered liver tissue GSH, accomplished by diethylmaleate administration, on extracellular GSH concentration as determined by microdialysis perfusion and on-line HPLC analysis, before and after global liver ischemia/reperfusion in anesthetized rats.

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† *Abbreviations*: GSH, glutathione (γ-glutamyl cysteinyl glycine); DEM, diethylmaleate; and ROS, reactive oxygen species.

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MATERIALS AND METHODS

Chemicals

GSH, urethane, and diethylmaleate were purchased from the Sigma Chemical Co. (St. Louis, MO, U.S.A.). Monochloroacetic acid was obtained from Merck (Darmstadt, Germany). Heptane sulfonic acid was obtained from the Tokyo Kasei Kogyo Co. (Tokyo, Japan). Reagent-grade acetonitrile and methanol were obtained from J. T. Baker (Phillipsburg, NJ, U.S.A.). All other chemicals were reagent-grade, and deionized distilled water was used.

General Procedure for Microdialysis

The microdialysis system was obtained from Carnegie Medicine Associates (Stockholm, Sweden). Microdialysis probes (CMA/20) were purchased from CMA. The probe length was 24 mm. The probe membrane consisted of polycarbonate. Probe length and diameter were 4 and 0.5 mm, respectively. Molecular weight cut-off for the membrane was 20,000 Da. The probe was perfused (2 μL/min) with a CMA-100 perfusion pump in corresponding outer medium for 30–60 min with Ringer's solution before the start of measurement, to avoid changes in relative recovery over time. Microdialysates were collected in 10-min intervals with a 19.6 μL-loading-loop of a CMA-160 on-line injector. The time length for injection of the collected microdialysates onto the HPLC system was 8 sec.

Animal Preparation

Male Sprague-Dawley rats, weighing 400–450 g (aged 2.5 to 3 months old), were used in all experiments. The animals were starved for 12 hr prior to the start of the study, and were anesthetized with an i.p. injection of urethane (1200 mg/kg). A tracheal tube was inserted to facilitate breathing. Throughout the experiments, body temperature was maintained between 36 and 38° with a heating pad. Polyethylene cannulas (PE-50) were inserted into the femoral vein for administration of drugs and saline. All animals were heparinized (400 IU/kg of body wt). A midline laparotomy was performed, and the liver hilum was exposed. In animals undergoing hepatic ischemia, the entire hepatic pedicle (hepatic artery, portal vein, and common bile duct) was clamped for 30 min using a vascular clamp [12, 18]. During the ischemic period, 1.5 mL of saline was administered i.v., at 60-min intervals, to maintain hemodynamic stability and to replace losses due to portal stasis [12]. Reperfusion was established by removal of the clamp. Thirty minutes after removal of the vascular clamp, biopsies were taken from the median lobe of the liver for determination of liver tissue GSH concentration. When DEM was used, it was administered 1 hr prior the start of laparotomy by i.p. injection [7].

Determination of Extracellular GSH Content

The liver extracellular GSH analysis was based on a published procedure [11]. Briefly, a microdialysis probe was implanted and perfused with Ringer's solution at a flow rate of 2 μL/min. The microdialysis probe was placed in the median lobe 1 cm from the right edge and bottom edge of the lobe. A total length of 1 cm was inside the liver. The microdialysates, collected with a CMA-160 on-line injector containing a 20-μL loading loop, were injected onto an HPLC system consisting of a BAS (Bioanalytical System, Lafayette, IN, U.S.A.) PM-80 isocratic pump and a BAS LC-4C electrochemical detector with Au/Hg electrodes. Separation was achieved by Alltech 5 μm Econosphere C-18 reversed phase cartridge columns (4.6 × 150 mm).

Determination of Tissue GSH Content

A slice of liver tissue, removed from where the probe was implanted, was homogenized in 10% metaphosphoric acid solution (0.3 g/5 mL). The deproteinized homogenates were centrifuged at 13,000 *g* for 10 min. Supernatants were diluted with mobile phase (1:24) prior to injection onto the HPLC system. The HPLC system and mobile phase were identical to those used in the analysis of extracellular GSH content.

RESULTS

Following administration of DEM, liver tissue GSH concentration decreased significantly (1.36 ± 0.26 μmol/g wet wt), when compared with untreated rats (9.50 ± 0.55 μmol/g wet wt, $P < 0.01$) (Table 1). Liver extracellular GSH levels were examined, following probe implantation, in control rats and in rats treated with DEM. The results are shown in Figs. 1 and 2, respectively. The extracellular GSH levels for the first 20 min following probe insertion are not shown because the extracellular GSH levels in this period were usually overscaled on the chromatogram due to GSH leaking as a result of the cell rupture. The extracellular GSH levels reached equilibrium at 70 min after probe implantation in both the DEM-treated and untreated rats. It was obvious that, when compared at the same point in time following probe implantation, liver extracellular GSH levels were much lower in rats treated with DEM. When compared with the untreated rats, basal liver extracellular GSH levels were significantly lower in rats treated with DEM (4.3 ± 2.4 μM vs 25.2 ± 8.7 μM for the untreated). Both values are much higher than the detection limit in

TABLE 1. Comparisons of GSH concentration in DEM-treated and untreated rats

	GSH concentrations	
	Extracellular (μM)	**Tissue (μmol/g wet wt)**
Untreated	$25.2 \pm 8.7^{a,b}$	$9.50 \pm 0.55^{a,c}$
DEM-treated	$4.3 \pm 2.4^{b,d}$	$1.36 \pm 0.26^{c,d}$

Values with the same letters (a, b, c, d) were statistically different ($P < 0.01$, Student's *t*-test). Values are means ± SEM.

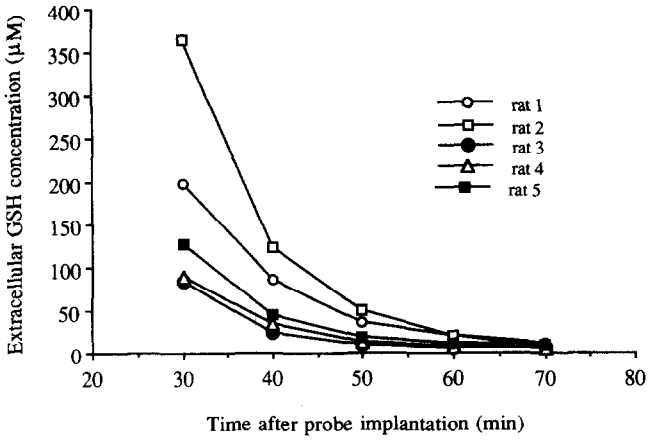

FIG. 1. Extracellular GSH concentration after probe implantation into the livers of anesthetized rats.

extracellular GSH levels (0.2 μM based on a signal to noise ratio of 3). These results indicate that liver extracellular GSH levels are directly related to tissue GSH reserves.

The effect of global liver ischemia on liver extracellular GSH content also was investigated. In untreated rats, extracellular GSH concentration increased significantly after the onset of global liver ischemia. GSH concentration remained elevated for the entire 30-min ischemic period and did not decrease toward the basal values until reperfusion. Following DEM treatment, extracellular GSH concentration also increased significantly. This increase, presented as the fold increase relative to basal value, was slightly less than in the untreated rats (Fig. 3). There was no significant difference in GSH increase between DEM-treated and untreated rats. Reperfusion gradually reduced the ischemia-induced liver extracellular GSH elevation in both DEM-treated and untreated rats. However, even at 30 min after reperfusion, GSH levels had not returned to basal values (Fig. 3).

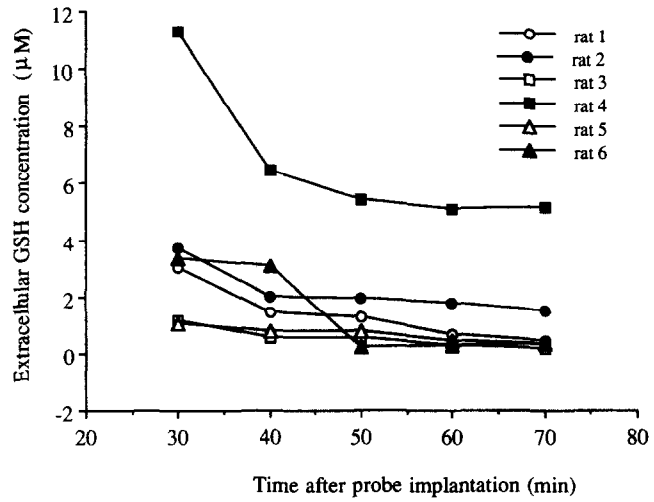

FIG. 2. Extracellular GSH concentration after probe implantation into the livers of anesthetized rats treated with DEM. DEM was administered (4 mmol/kg) 1 hr prior to the probe implantation.

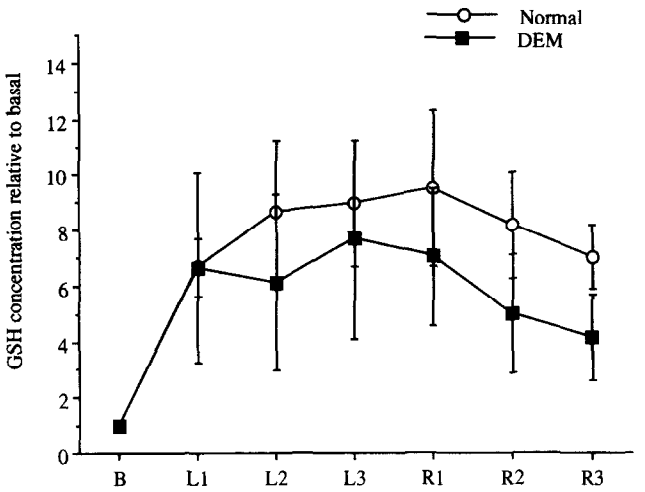

FIG. 3. Effect of DEM on extracellular GSH concentration after global liver ischemia/reperfusion. Data were obtained from 8 different rats and are presented as means ± SEM. Key: B: basal; L1, L2, and L3: 10, 20, and 30 min after ligation; and R1, R2, and R3: 10, 20, and 30 min after reperfusion. Absolute values for GSH in DEM-treated and untreated rats were 4.3 ± 2.4 and 25.2 ± 8.7 μM, respectively.

Tissue GSH levels, before and after global liver ischemia, were examined in both DEM-treated and untreated rats. Global liver ischemia for 30 min significantly reduced tissue GSH levels (50%). Reperfusion (for 30 min) did not alter the GSH levels further (Fig. 4). In DEM-treated rats, global liver ischemia for 30 min also decreased tissue GSH levels significantly (to about 30% of the basal value). Interestingly, the GSH levels in DEM-treated rats were higher after 30 min of reperfusion than after ischemia (Fig. 4).

Mortalities occurred following DEM treatment and global liver ischemia. The rate for DEM-treated rats was 33%. However, there were no mortalities among the untreated rats following global liver ischemia for 30 min and reperfusion for 30 min.

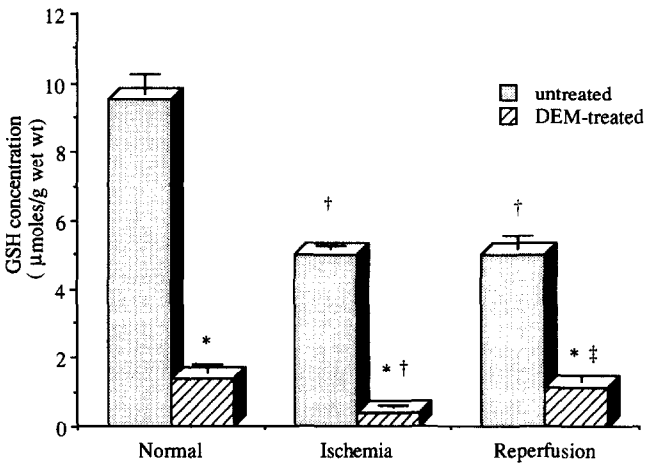

FIG. 4. Effect of DEM on tissue GSH concentration after global liver ischemia/reperfusion. Data were obtained from 8 different rats and are presented as means ± SEM. Key: *$P < 0.05$ vs corresponding untreated rats, †$P < 0.05$ vs normal, and ‡$P < 0.05$ vs ischemia (ANOVA).

DISCUSSION

GSH is present in high concentration in the liver. Liver cells can actively transport GSH to the interstitial space, and the overall GSH efflux from the liver is responsible for as high as 85% of the GSH present in extracellular fluids such as plasma. Measurement of hepatic transport of GSH is usually achieved by monitoring the GSH level in sinusoidal efflux from an isolated liver or from the liver of an anesthetized animal. However, frequent sampling and relatively large blood volumes, which are needed for monitoring *in vivo* sinusoidal GSH efflux in an anesthetized animal, make the continuous and long-term monitoring of hepatic GSH export a very difficult task, especially in small animals such as rats. Microdialysis perfusion, through microdialysis probes implanted in the liver, is capable of continuous sampling of liver interstitial fluids. Microdialysis perfusion and on-line HPLC analysis allow for continuous and automatic monitoring of the extracellular GSH level and *in vivo* hepatic GSH efflux in anesthetized rats.

DEM is commonly used to lower tissue GSH [7]. In the present study, we chose DEM rather than buthionine sulfoximine (BSO), another commonly used reagent to lower tissue GSH levels by inhibiting GSH synthesis. DEM forms a conjugate with GSH, and thus has a much faster effect (usually 1 hr after injection) on tissue GSH content [7]. We did observe that when tissue GSH content decreased, following administration of DEM, basal extracellular GSH level decreased proportionally. This finding provides direct and *in vivo* evidence for the interrelation between liver extracellular GSH level and tissue GSH content. Tissue GSH levels, which actually represent the combination of intracellular and extracellular GSH levels, were used in the present study instead of the intracellular GSH levels due to two reasons. One is that extracellular GSH levels are usually two to three orders of magnitude lower than that in the intracellular space. For example, hepatocyte intracellular GSH levels are estimated to be in the millimolar range, while the extracellular GSH levels are usually in the micromolar range [11]. The other reason is that it is more convenient to measure the tissue GSH levels.

In the present study, we observed that the decrease of extracellular GSH was proportional to the tissue decrease of GSH following DEM administration. Therefore, extracellular GSH determined by microdialysis and on-line HPLC analysis might be used to represent tissue GSH levels. This is a reasonable observation as liver cells have active transporters for GSH [19, 20], and therefore, lowered tissue GSH would result in decreased export of GSH. Microdialysis and on-line HPLC analysis are of value in *in vivo* investigations targeting the role of GSH in the liver.

Although basal extracellular GSH content decreases after the administration of DEM, global liver ischemia can cause an increase in extracellular GSH content in DEM-treated rats. When presented as fold increase relative to basal, elevated GSH content in the liver extracellular space showed no significant difference among DEM-treated and untreated rats. These results suggest that although tissue GSH levels decrease following DEM administration, global liver ischemia can induce the dramatic release of GSH into the interstitial space, regardless of lowered tissue GSH content. GSH is a major free radical scavenger, and lowered GSH content following administration of DEM has been shown to exacerbate the damage caused by ischemia/reperfusion to organs such as the brain [21] and heart [22]. However, in the liver, GSH may be released to protect the organ against increasing levels of ROS, even in the presence of lowered tissue GSH.

The high mortality rate for DEM-treated rats after hepatic ischemia/reperfusion suggests that liver GSH is a factor of protection against the oxygen-derived free radicals.

In conclusion, we used microdialysis perfusion and an on-line HPLC technique to observe that DEM administration lowered both tissue and extracellular GSH levels in livers of anesthetized rats, and the decreases in the GSH levels were approximately on the same scale. Additionally, we observed that relative GSH release induced by liver ischemia was not affected by DEM administration.

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