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GLUTATHIONE AND CYSTEINE DEPLETION IN RATS AND MICE FOLLOWING ACUTE INTOXICATION WITH DIETHYLMALEATE

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Abstract—We examined the dose-dependent glutathione (GSH) depletion in liver, kidney, heart and brain of rats and mice, and cysteine depletion in rat kidney, following i.p. administration of diethylmaleate (DEM). In either rodent, the fall in total GSH concentration in liver and heart reached an upper value of 90 and 80% with 3 and 4 mmol DEM/kg respectively, which did not increase with higher doses. This study suggests that the residual level of GSH corresponds to the mitochondrial pool, in which case DEM might serve as a tool for the measurement of mitochondrial GSH *ex vivo*. In further experiments, we studied the time course of GSH and cysteine after administration of 3 mmol DEM/kg in rat tissues. Maximal depletion was reached approximately 1 hr after the i.p. injection. Subsequent GSH repletion was fast in liver and kidney, whereas it was slow in heart and brain, with a return to control values by 8–12 and by 48 hr after intoxication, respectively. This study provides new data for cardiac GSH and renal cysteine decrease after intoxication with DEM and should help to optimize GSH depletion models for further pharmacological investigations, especially when the use of inhibitors of glutathione metabolic turnover is undesirable and when side-effects other than GSH depletion must be avoided.

Glutathione (GSH‡) is the major non-protein thiol in mammalian cells and tissues. GSH plays an important role in the storage and interorgan transport of cysteine and in cellular defenses against free radicals, peroxides and electrophiles [1] as a cofactor of glutathione-peroxidases [2, 3] and GSTs (EC 2.5.1.18) [4]. The rate and capacity of many GSH protective processes are dependent on the intracellular concentration of this thiol-containing tripeptide.

Modulation of GSH level may be useful in chemotherapy and in radiation therapy and it may help to protect cells against the toxic effects of drugs and xenobiotics [5–9].

Depletion of intracellular GSH, which is in the millimolar range in most tissues, proved to be a useful tool in previous detoxication and drug metabolism studies. The aim of this study was to optimize a model of GSH depletion *in vivo* in heart and other organs, for pharmacological studies.

Depletion of GSH by enzymic and chemical methods has been widely used. In rodents, the use of moderately reactive compounds that require enzymic catalysis is preferable [10] if alteration of protein sulphydryl groups is undesirable.

Depletion of GSH has been attempted with electrophiles that conjugate with GSH in the presence of GSTs, such as DEM [11]. Soluble GSTs are cytosolic enzymes present in all investigated organs of rats and mice. Phorone may also be useful [12] but in preliminary experiments (unpublished results), it appeared not to be convenient because of its higher toxicity. DEM was reported to deplete GSH stores in liver, kidney, brain, lung and erythrocytes of rats [13]. Depletion of hepatic GSH by DEM led to a proportional decrease of plasma GSH in rats [14]. DEM was also known to decrease hepatic GSH levels in species like mouse [15–17], Syrian hamster [18], dog [19], chick [20] and to a lesser extent rabbit [21, 22] and to deplete brain, liver and kidney non-protein thiol levels in mice [23].

Toxic side-effects of DEM must also be considered and avoided. DEM used at high doses was found to have non-specific effects such as impairment of glycogen metabolism [24] and of the "L" system for amino acid transport [25]. DEM (1 mL/kg) inhibited protein synthesis in brain and liver following *in vivo* administration to mice [23]. Controversial observations on DEM-induced LPO *in vivo* were reported in the past [15, 26], and it seems that LPO and GSH depletion induced by DEM were not casually related, but the consequence of other side-effects of DEM such as interaction with the microsomal monooxygenase system [27], which was only observed when this compound was utilized at high doses *in vivo* or *in vitro*. Others reported that moderate concentration of DEM was sufficient to decrease GSH in isolated hepatocytes without additional side-effects [25] and that DEM appeared not to affect hepatic function [28, 29] nor to induce liver damage *in vivo* [21].

In order to determine the dose range of DEM, which would not be irreversibly toxic while resulting

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‡ Abbreviations: GSH, reduced glutathione; GST, glutathione-*S*-transferase; DEM, diethylmaleate; LPO, lipid peroxidation; MBBr, monobromobimane; MPA, metaphosphoric acid.

in substantial GSH decreases, we investigated the dose-dependent depletion of tissue GSH levels after administration of DEM, and we studied the time course of GSH in liver, kidney, heart and brain after intoxication with an optimal dose of DEM.

MATERIALS AND METHODS

Chemicals. GSH, L-cysteine, mercaptosuccinic acid, tetrabutylammonium hydrogen sulfate and DEM were purchased from the Sigma Chemical Co. (St Louis, MO, U.S.A.). MBBr was from France Biochem. All other chemicals and reagents were of analytical grade, obtained either from Merck or from Prolabo.

Animals. Male Sprague–Dawley rats (200–250 g) and Swiss mice (20–25 g) from Iffa-Credo were used in this study. Animals were fed controlled standard diet (UAR, Villemoisson, France) *ad lib.*

Intoxication protocol. DEM was dissolved in corn oil before i.p. administration. Controls rats were similarly injected with corn oil only.

Dose-dependent GSH depletion: DEM was administered in a range of doses up to a maximum of 6 mmol/kg. Animals were killed 60 min after injection.

Time course of GSH depletion: animals were treated with 3 mmol DEM/kg. They were killed at timed intervals after injection.

Preparation of tissue samples. GSH concentrations were measured in whole tissue extracts of liver, kidney, heart and brain.

Cysteine concentration was measured in rat kidney extracts. The animals were killed by decapitation following diethyl ether anaesthesia and perfusion through the left heart with cold physiological saline.

Organs were immediately excised, weighed and homogenized in 5% (w/v) MPA and protein precipitation was achieved by centrifugation at 5000 *g* for 10 mn.

Supernatants were utilized within an hour or stored for one week in liquid nitrogen.

Assay of glutathione and cysteine. GSH and cysteine were measured as the MBBr adducts by ion-paired reversed phase HPLC on a 3 μm Hypersil ODS 2 (4.6 × 150 mm) column with fluorescence detection, according to the method of Burton and Aherne [30] slightly modified as follows:

Thiomalic acid was added in the MPA homogenates as an internal standard.

Aliquots ranging from 25 to 100 μL of the supernatant fraction were brought to a final volume of 200 μL with 5% MPA and then derivatized: 1.8 mL of 0.2 M borate buffer pH 10.5 was added and the reaction was started with 100 μL of 250 μg/mL MBBr in acetonitrile. The mixture (pH 9.3) was incubated for 15 min at room temperature in the dark and 100 μL glacial acetic acid were added to stop the reaction.

The samples were then stored at 0.4° in the dark until injection.

They were stable for 2 days, with the exception of liver extracts which had to be processed within 24 hr.

The mobile phase consisted of methanol–50 mM ammonium dihydrogen orthophosphate (30/70) containing 30 mM tetrabutyl ammonium hydrogen sulphate and adjusted to pH 5.9 with solid ammonium hydrogen carbonate. The flow rate was 0.75 mL/min. Separation was achieved at 32° in 25 min.

The fluorescence detector was set up to 390 nm excitation wavelength and 480 nm emission wavelength.

GSH, cysteine and thiomalic acid standards were prepared in 5% MPA. Standard curves were obtained by plotting the ratio (GSH or cysteine–MBBr adduct peak area/thiomalate–MBBr adduct peak area) against GSH or cysteine concentration.

Results are the means of individual determinations on five rats. For statistical evaluation of the data, paired comparisons were made using Student's *t*-test.

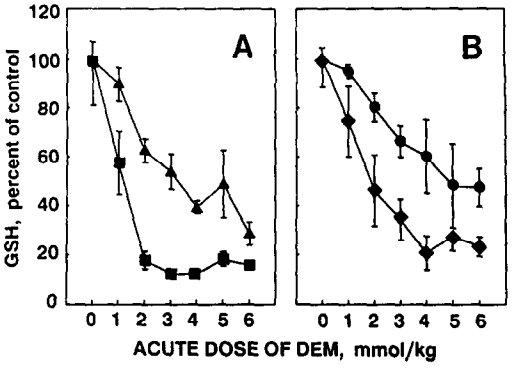

Fig. 1. Dose–response curves of GSH depletion in rat tissues after acute intoxication with diethylmaleate. (A) Liver (■) and kidney (▲) GSH. (B) Heart (◆) and brain (●) GSH. Rats were injected i.p. with DEM in corn oil or corn oil alone (controls) and killed 1 hr later. GSH levels were determined as indicated in Materials and Methods. Results are means ± SD for five rats.

RESULTS

Administration of DEM to rats and mice led to a net decrease of liver, kidney, heart and brain levels of GSH in rats and mice and of kidney cysteine in rats.

Dose-dependent depletion of GSH and cysteine in rats

We found the minimal liver GSH values in the range 1.07 ± 0.11 μmol/g after such treatment as compared to 7.96 ± 1.51 μmol/g for untreated rats (animals killed between 10 a.m. and noon), control levels being 3.97 ± 0.40 μmol/g at 6 p.m.

The maximal decrease was found with 3 mmol DEM/kg (Fig. 1A) and remained unchanged with increasing doses up to 6 mmol/kg. This level represented about 12% of the control one.

In kidney, depletion was dose-dependent (Fig. 1A). The residual amount of GSH remaining after 6 mmol DEM/kg was only 28.47 ± 4.68% of control values (2.50 ± 0.17 μmol/g).

Kidney cysteine levels were decreased to zero

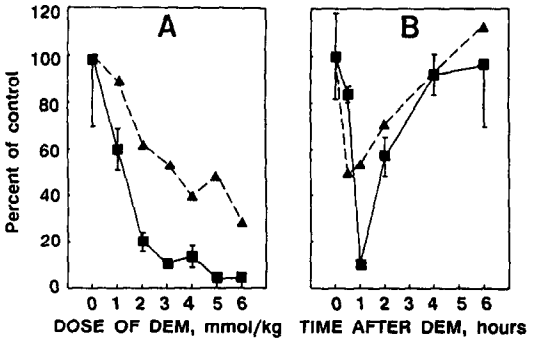

Fig. 2. Effect of diethylmaleate on the cysteine content of rat kidney. (A) Dose–response curves of cysteine (■) and GSH (▲) in kidney. Rats were injected i.p. with DEM in corn oil or corn oil only (controls) and killed 1 hr later. (B) Time course of cysteine (■) and GSH (▲) in kidney after DEM administration. Rats were killed at timed intervals after the administration of 3 mmol DEM/kg i.p. Controls injected with corn oil only were killed at the same times. Thiol levels were determined as indicated in Materials and Methods, results are means ± SD for five rats.

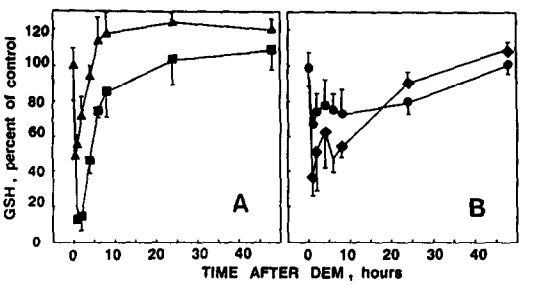

Fig. 4. Time course of GSH content after DEM administration. (A) Liver (■) and kidney (▲). (B) Heart (◆) and brain (●) GSH. Rats were killed at timed intervals after the administration of DEM dissolved in corn oil (3 mmol/kg i.p.) and GSH levels were determined as described in Materials and Methods. Control rats injected with corn oil only were killed at the same times. Each point and vertical bar represents the mean ± SD of determinations from five rats.

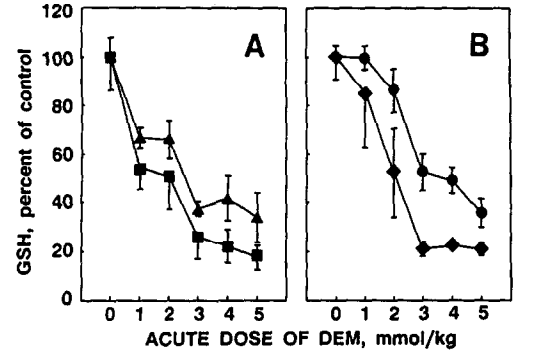

Fig. 3. Dose–response curves of GSH depletion in mouse tissues after acute intoxication with diethylmaleate. (A) Liver (■) and kidney (▲) GSH. (B) Heart (◆) and brain (●) GSH. Mice were injected i.p. with DEM in corn oil alone (controls) and killed 1 hr later. GSH levels were determined as indicated in Materials and Methods. Results are means ± SD for five mice.

after 5 and 6 mmol DEM/kg (Fig. 2A) and were only 10.25 ± 1.80% of the control values (1.05 ± 0.25 μmol/g) after 3 mmol DEM/kg.

In heart (Fig. 1B), the maximal decrease in GSH was observed after 4 mmol DEM/kg: GSH was 20.57 ± 6.89% of the control (1.93 ± 0.22 μmol/g) and was unchanged at higher doses.

In brain (Fig. 1B), administration of 4 mmol DEM/kg led to a decrease of about 53% GSH which was not statistically different after 5 and 6 mmol DEM/kg. The control value was 1.91 ± 0.08 μmol/g.

Dose-dependent depletion of GSH in mice

In liver, kidney (Fig. 3A) and heart (Fig. 3B), a net decrease was obtained with 3 mmol DEM/kg and remained significantly unchanged with higher doses, reaching the lowest values of 17.59 ± 4.99%, 33.79 ± 10.03% and 21.03 ± 2.88% of control, respectively. Control GSH values were 6.70 ± 0.93 μmol/g in liver, 3.86 ± 0.30 μmol/g in kidney and 0.80 ± 0.07 μmol/g in heart.

In brain (Fig. 3B), depletion was dose-dependent and reached 35.58 ± 5.75% of GSH control value (1.90 ± 0.09 μmol/g) following administration of 5 mmol DEM/kg.

Time course of GSH and cysteine in rats

Based on the results of the first experiment, a dose of 3 mmol DEM/kg was chosen to give an overall optimal GSH decrease in all organs studied with no need for a higher dose. This dose resulted in minimal values of 12.21 ± 1.20% GSH in liver, 53.66 ± 7.22% in kidney, 34.23 ± 8.34% in heart and 66.07 ± 6.40% in brain. In kidney, the minimal value of cysteine was very low (10.25 ± 1.80% of the normal values) but not completely depressed.

Rats intoxicated with 3 mmol DEM/kg showed a rapid hepatic GSH depletion (Fig. 4A) which was maximal at 1–2 hr after i.p. injection. GSH was significantly decreased as early as 30 min. 26.51 ± 3.26% GSH remained at 30 min and only 12.21 − 14.02% of the control values at 1–2 hr.

There was some indication of hepatic GSH repletion by 2 hr. GSH was 85.06 ± 13.94% by 8 hr and had returned to normal values by 24 hr.

The time courses of GSH in liver and kidney were similar. Maximal decrease of kidney GSH occurred as soon as 30 min after i.p. injection (Fig. 4A) and remained nearly unchanged at 1 hr (about 50% of control) GSH repletion was then observed. GSH was 93.50 ± 6.46% of the normal content at 4 hr with subsequent increases to approximately 120% of control levels at 8, 24 and 48 hr.

The intoxication resulted in a rapid depletion of kidney cysteine (Fig. 2B) that was maximal by 1 hr (10.25 ± 1.80% of the control value) whereas a

residual cysteine content of 83.90 ± 3.40% was still observed by 30 min; subsequent repletion of cysteine was fast (56.83 ± 8.74% by 2 hr, about 90% by 4–6 hr and 110% by 24 hr).

Administration of DEM to rats resulted in a rapid depletion of heart GSH (Fig. 4B) which fell to 41.17 ± 7.50% of control values as early as 30 min and to 34.23 ± 8.34% by 1 hr. Heart GSH content remained markedly depressed for 5 hr (47.20 ± 8.00%) before rising slowly thereafter. Normal values were only reached by 48 hr.

The time course of GSH in brain was nearly the same as that in heart (Fig. 4B) with a maximal decrease by 30–60 min (67.51–66.07% of control values) which remained significantly unchanged by 8 hr (62.46 ± 12.48%). GSH then steadily increased to 100.90 ± 5.04% of the control value by 48 hr.

DISCUSSION

Our results are in agreement with those reported by others for normal values of tissue GSH in rats [31] and mice [30, 32] and cysteine in rat kidney [33]. As previously reported, circadian variations of hepatic GSH occurred in liver [34, 35] but were not seen in kidney, heart and brain.

In previous reports [11, 15–17, 19, 36] the maximal hepatic GSH decrease was reached 1 hr after treatment, whatever the dose of DEM (from 3.6 to 12 mmol/kg) in rats and mice. Administration of DEM (2.48 mmol/kg) to rats was shown to result in minimal GSH values between 30 min and 2 hr in liver, kidney and brain [13]. This is why we first studied the dose-dependent depletion of GSH 1 hr after i.p. administration.

There was little difference between the susceptibility of rats and mice to DEM intoxication in this study. In the liver of either rodents, maximal depletion of GSH was reached with 2–3 mmol DEM/kg and never exceeded 90%. It should be noted that even with 12 mmol DEM/kg, hepatic GSH in mice did not decrease below 10.5% of the control [16, 17]. DEM was reported to substantially deplete cytosolic GSH but not mitochondrial GSH in isolated hepatocytes [37], and the mitochondrial and cytosolic pools of GSH showed different sensitivities to DEM administered to rats *in vivo* [38]. The transport system responsible for the exchange between the two GSH pools [39, 40] was suggested to function in a way which preserves the mitochondrial GSH pool during cytosolic GSH depletion [9, 40].

It has been claimed that alteration in the mitochondrial pool of GSH may be critical for development of LPO and necrosis [16, 38, 41], which is in possible agreement with the present study. With doses of DEM up to 6 mmol/kg, we observed neither toxicity nor tissue necrosis. We did not find any liver or heart LPO, measured as TBARS, 2 hr after injection of 6 mmol DEM/kg to mice (data not shown).

While further studies would be required [42], the above considerations strongly suggest that the percentage of total hepatic GSH remaining after DEM intoxication *in vivo* is mitochondrial GSH and that DEM might be a useful tool for the measurement of mitochondrial GSH content *ex vivo*.

It may be hypothesized that the lack of mitochondrial GSH depletion by DEM is a general phenomenon in mammalian tissues, due to the low content of GSTs in mitochondria, as previously suggested from data obtained with phorone [43]. In this connection, it is interesting to note that 1-chloro-2,4-dinitrobenzene, the universal substrate for GSTs, was shown to be unable to deplete mitochondrial GSH in synaptosomes [44].

In this study, cardiac GSH was maximally depleted to 20% of control values in either rodents. Whereas GSH depletion following intoxication with diethylmaleate has been well documented, the data presented in this paper are the first demonstration, to our knowledge, of GSH depletion occurring also in heart.

Considering the large volume of the cardiac myocyte occupied by mitochondria, it is possible that GSH depletion in heart again reflects the existence of a mitochondrial pool of GSH that cannot be depleted by DEM.

An optimal dose of 3 mmol DEM/kg was chosen to study the time course of GSH following DEM administration to rats. In every tissue, GSH was nearly maximally depressed 30–60 min after injection of 3 mmol DEM/kg, in agreement with previous reports [11, 15–17, 19, 36]. The time course of GSH was similar to that reported by Richardson and Murphy [13] with 2.48 mmol DEM/kg. While the maximal depletion of kidney GSH occurred by 30 min, the maximal depletion of kidney cysteine was only observed by 1 hr, which suggests that cysteine depletion was mainly a consequence of GSH depletion/repletion rather than due to its direct interaction [42] with DEM.

In the past, toxic side-effects of high doses of DEM led to the conclusion that buthionine-sulfomixine was a preferable GSH depletor [23]. This study shows that DEM may be used to deplete tissue GSH by 50 to 90% *in vivo* with minimal toxic side-effects, while having the pharmacological advantage of leaving γ-glutamyl cysteine synthetase intact. We conclude that an acute intoxication by i.p. administration of 1–3 mmol DEM/kg causes a GSH depletion which is of practical utility for pharmacological studies in rats and mice.

Our model could be especially useful to mimic cardiac GSH decreases such as those observed after ischemia and reperfusion.

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