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Effect of glutathione depletion on the conversion of xanthine dehydrogenase to oxidase in rat liver*

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Abstract—The ability of endogenous glutathione (GSH) to modify the activity of the enzyme xanthine oxidase (XO) in rat liver was investigated. The effect of hepatic GSH depletion on the conversion of xanthine dehydrogenase (XDH) (EC 1.1.1.204) to XO (EC 1.1.3.22) was determined 10 min after i.p. administration of different amounts of diethylmaleate to fasted rats. After administration of 400 mg/kg, total hepatic non-protein GSH (reduced + oxidized GSH) decreased significantly to 14% of controls. In this condition the level of oxidized GSH was unchanged and no lipid peroxidation was observed, while a significant increase of reversible XO and a minor increase of the irreversible form of the enzyme was detected.

The tripeptide glutathione (GSH†), an important intracellular antioxidant agent, exists in the cells as cytosolic, mitochondrial, nuclear and lysosomal pools [1, 2]. GSH is a scavenger for many kinds of oxygen-derived free radicals and displays a broad range of vital functions including the maintenance of the activity of enzymes containing sulphydryl groups [3]. Xanthine oxidase (XO), an enzyme of purine catabolism, contains 14 sulphydryl groups per subunit [4] and exists as a NAD^+-reducing form (xanthine dehydrogenase, XDH) which can be converted to an oxygen radical-producing form (XO) either by oxidation and blockage of sulphydryl groups (reversible XO, XOrev) or by limited proteolysis (irreversible XO, XOirr) [5, 6]. The XOrev can be reversed to XDH by dithiothreitol (DTT) while the XOirr cannot [5]. Since XDH–XO conversion has been observed in conditions in which a decrease of hepatic GSH level occurs such as long-term *in vivo* and *in vitro* ischemia [7, 8], we have undertaken the present study in order to correlate the simple decrease of hepatic non-protein sulphydryl level with the transformation of XDH to the two different XO forms. For this purpose, to modify the redox system provided by GSH and glutathione disulphide (GSSG), different amounts of diethylmaleate (DEM), a chemical GSH depletor, were injected to fasted rats and the effects on XDH to XO forms conversion were determined.

Materials and Methods

Male Sprague–Dawley rats (200–250 g), six rats in each group, were fasted for 15 hr before the experiments and killed between 8:00 and 10:00 a.m.

DEM-treatment protocol. DEM was administered to rats in a single i.p. injection. For low doses (25–100 mg/kg) DEM was dissolved in corn oil (0.2 mL).

Time course of GSH depletion. Animals treated with DEM (400 mg/kg) were killed at timed intervals after injection.

Dose-dependent GSH depletion. DEM was administered in a range of doses from 25 to 400 mg/kg. Animals were killed 10 min after i.p. injection. Control fasted rats were similarly treated with corn oil only.

Enzyme assay. Livers were washed and homogenized in 50 mM K_2HPO_4 buffer (pH 7.4) containing 1 mM EDTA (1:5; w/v). In some experiments the homogenization buffer was added, before use, with 10 mM 2-mercaptoethanol and trypsin inhibitor (5 mg/mL; type II-S from the Sigma Chemical Co. Poole, U.K.) [9]. The homogenate was centrifuged at 1500 *g* for 10 min and then at 105,000 *g* for 1 hr at 4°. Supernatant was dialysed up to 4 hr against the same homogenization buffer at 4° in order to remove endogenous purines [5]. The enzyme showed the same activity after 3 or 5 hr dialysis. The enzyme activities were evaluated by HPLC, measuring uric acid formation as reported by Cighetti *et al.* [10]. For the evaluation of total enzyme activity (XDH + XO), 0.2 mL (33 mg tissue) of dialysed enzyme fraction was preincubated for 30 min at 37° in the presence of 10 mM DTT and then diluted aliquots (0.5 mg tissue) were incubated after addition of 60 μM xanthine and 0.67 mM NAD^+ for 10 min at 25° [9]. XOirr and total XO (XOrev + XOirr) activities were determined in the absence of NAD^+, for the evaluation of total XO activity, DTT activation was avoided.

The activity of XOrev was calculated as a difference between total XO (XOrev + XOirr) and XOirr activities.

Thiols determination. The content of hepatic non-protein GSH and GSSG fractions were assayed enzymatically in livers homogenized in 0.1 M phosphate buffer, pH 7.5, containing 5 mM EDTA without and with 10 mM *N*-ethylmaleimide, respectively [11, 12]. *N*-Ethylmaleimide was added to the buffer to avoid GSH auto-oxidation.

Lipid peroxidation. This was determined colorimetrically by measuring the hepatic formation of thiobarbituric acid reactive substances (TBARS), using malondialdehyde as the standard [13].

ALT serum level. This was measured in all groups of rats on a Hitachi 747 discrete automatic analyser (Hitachi Ltd, Japan) with standard reagent supplied by Boehringer-Mannheim (Mannheim, F.R.G.).

Results and Discussion

In preliminary experiments (data not shown), in which fed and 15 hr fasted animals were killed 1 hr after i.p. DEM injection (400 mg/kg), we observed a decrease of non-protein GSH level corresponding to 50% and 85% of the controls, respectively. Fasted rats were chosen instead of fed rats that would require higher doses of DEM, which over 400 mg/kg induces liver damage [14]. Fasted rats treated with DEM (400 mg/kg) showed a GSH depletion (Fig. 1A) corresponding to 14% of the controls 10 min after i.p. injection, while a GSH repletion was observed at 120 and 180 min.

Based on these results, dose-dependent GSH depletion was studied 10 min after DEM administration (Fig. 1B).

* In memory of Prof. Alberto Fiecchi.

† Abbreviations: XDH, xanthine dehydrogenase; XO, xanthine oxidase; XOirr, irreversible XO; XOrev, reversible XO; GSH, glutathione; GSSG, glutathione disulphide; DEM, diethylmaleate; DTT, dithiothreitol; MDA, malondialdehyde; ALT, serum glutamate pyruvate transaminase; TBARS, thiobarbituric acid reactive substances.

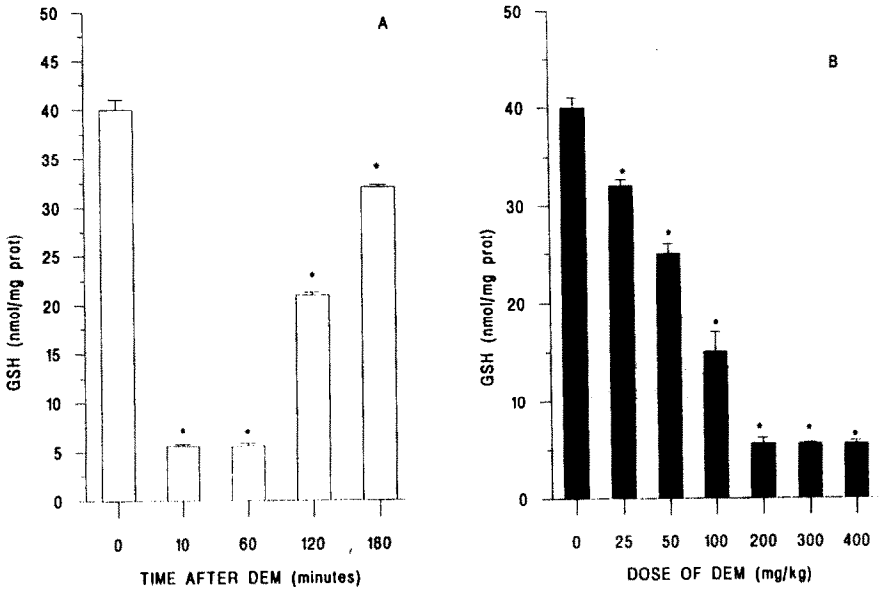

Fig. 1. Time course of GSH content (A) and dose-dependent GSH depletion (B) after DEM administration. (A) Fasted rats were killed at timed intervals after DEM injection (400 mg/kg i.p.). Zero time corresponds to fasted control rats injected with corn oil only. (B) Fasted rats were injected i.p. with the indicated amounts of DEM and killed 10 min later. Each value is the mean ± SE of triplicate determinations from six rats. *$P < 0.01$ vs control by the Student's *t*-test.

The hepatic GSH level was reduced in a dose-dependent manner only when low amounts of DEM were used (25–100 mg/kg), reaching a steady state (14% of the controls) with doses higher than 200 mg/kg.

The evaluation of tissue GSSG showed that the decrease of GSH content *in vivo* was not followed by modification of oxidized GSH level which was 0.35 ± 0.02 nmol/mg protein, both in controls and in maximum GSH-depleted rats. However, as a consequence of GSH depletion, the GSH/GSSG ratio, which was 114 in controls, decreased to 18 in 400 mg/kg DEM-treated animals showing in these rats a significantly more oxidizing intracellular environment.

The time-course of GSH depletion and the doses used are slightly different from those reported recently [15] showing, for fed rats, a maximum hepatic GSH decrease between 30 min and 2 hr after administration of 520 mg/kg of DEM. This minor discrepancy may be attributed to the fact that starved and fed animals react differently to the administration of DEM. Equally, the faster GSH repletion observed in our model may be explained by considering the known activation of hepatic GSH synthesis in fasted animals [16].

Lipid peroxidation (as TBARS) and ALT plasma level, measured in all treated rats as indicators of liver damage, showed no modification with respect to values determined in control rats (TBARS: 1.3 ± 0.2 vs 1.2 ± 0.07 nmol malondialdehyde (MDA)/mg protein and serum glutamate pyruvate transaminase (ALT): 72 ± 5 vs 69 ± 3 U±L).

As far as XDH is concerned, the activity of the total enzyme (XDH + XO), evaluated with and without DTT

Table 1. Effect of GSH depletion on XO in rat liver *in vivo*

DEM (mg/kg)	GSH level (nmol/mg protein)	Without/with*	XOirr ± XOrev (%)	XOirr (%)	XOrev (%)
0	40 ± 1	Without	25 ± 2.5	18 ± 0.8	7 ± 2
		With	23 ± 3	18 ± 1	5 ± 2
100	15 ± 2†	Without	27 ± 2	23 ± 1†	4 ± 1
		With	28 ± 2	23 ± 2†	5 ± 1
400	5.6 ± 0.6†	Without	47 ± 3†	24 ± 0.3†	23 ± 2†
		With	45 ± 2†	23 ± 0.5†	22 ± 1

Animals were killed 10 min after DEM i.p. administration. Livers were homogenized and dialysed in 50 mM K_2HPO_4 buffer without and with* 10 mM 2-mercaptoethanol and trypsin inhibitor (5 mg/mL).

Values of the OX forms have been expressed as percentages of the total enzyme activity (XDH + XO) which was 4.5 ± 6 nmol uric acid/min/mg protein in controls and treated rats.

Each value is the mean ± SE of triplicate determinations from six rats.

† $P < 0.01$ vs controls by the Student's *t*-test.

activation, was 4.5 ± 0.6 nmol uric acid/min/mg protein in controls and in all tested GSH-depleted rats.

Fasted controls showed the percentage of XOirr (18 ± 0.8%) and of total XO (XOrev + XOirr; 25 ± 2.5%) in agreement with values found by others in fed animals [9, 7], indicating that 15 hr fasting alone does not cause conversion of XDH to XO (Table 1). A slight but statistically significant increase of XOirr was observed when depleted GSH reached the value of 15 ± 2 nmol/mg protein while no additional increase was caused by further GSH depletion (Table 1). In contrast, a 3-fold increase of XOrev percentage was evident in rats with the lowest GSH content. After 2 and 3 hr corresponding to GSH repletion, the percentage of the two forms of XO returned to the control values (data not shown).

To ensure that the observed XO increase was not due to oxidative artefacts occurring during enzyme preparation which could also activate protease enzymes [17], in some experiments, livers were homogenized and dialysed in the presence of trypsin inhibitor and 2-mercaptoethanol which prevents XDH to XO transformation without promoting the conversion of XO into XDH [9]. However, even in these conditions similar XO increases (unmodified after different dialysis times) were observed, indicating that *in vivo* GSH depletion and modification of GSH/GSSG ratio produce a XDH to XO conversion (Table 1).

A suitable explanation for the observed increase in XOrev could involve a thiol–disulphide exchange reaction of the thiol enzyme groups catalysed by a GSSG-dependent thiol:disulphide oxidoreductase [18, 19]. This speculation is in agreement with recent conclusions of Battelli *et al.* [20] who reported that XO activity *in vitro* depends on the concentration of GSSG and *in vitro* may depend on the GSH/GSSG ratio.

In conclusion, we set up conditions to modulate the hepatic GSH level and the GSH/GSSG ratio over a short time, treating fasted rats with low amounts of DEM and we observed that depletion of GSH level to a threshold value of 5 nmol/mg protein is associated with a significant conversion of XDH to reversible XO and a minor transformation to the irreversible XO form.

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