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STUDIES ON THE ACTIVATION OF RAT LIVER MICROSOMAL GLUTATHIONE TRANSFERASE IN ISOLATED HEPATOCYTES

GERD LUNDQVIST and RALF MORGENSTERN*
Department of Toxicology, Karolinska Institutet, Box 60400, S-10401 Stockholm, Sweden

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Abstract—The mechanism of activation of microsomal glutathione transferase in isolated liver cells by diisapropylidene acetone (phorone) was investigated. Phorone (1 mM) causes a time-dependent increase (up to 2.6-fold) in the glutathione transferase activity of microsomes isolated from treated hepatocytes. Since phorone reacts with sulfhydryl groups, the possibility that this compound activated microsomal glutathione transferase directly was studied. It was found that neither the activity of the purified enzyme nor that in isolated microsomes is affected by phorone. It has been suggested [Masukawa T and Iwata H, *Biochem Pharmacol* **35**: 435–438, 1986] that activation of microsomal glutathione transferase by phorone *in vivo* is mediated through thiol–disulfide interchange involving oxidized glutathione (GSSG). It is shown here that the glutathione transferase activity of isolated microsomes, which was increased by the addition of 10 mM GSSG, can be decreased to the basal level with 0.1 M dithioerythritol. Dithioerythritol, on the other hand, only marginally decreases the glutathione transferase activity in microsomes isolated from phorone-treated hepatocytes. This finding argues against a role for thiol–disulfide interchange in the activation of the enzyme by phorone. Furthermore, the glutathione depletion caused by phorone does not seem to be responsible for activation *per se*, since other thiol depletors [e.g. diethylmaleate (DEM)] do not affect the activity of the enzyme. Immunoblot analysis of microsomes isolated from phorone-treated hepatocytes did not reveal any partial proteolysis which might have accounted for the activation. It is suggested that activation of microsomal glutathione transferase by phorone proceeds through a mechanism which might reflect an *in vivo* regulation of this enzyme.

Additional compounds which have been shown to activate the microsomal glutathione transferase *in vivo* were also tested and significant activation was obtained with 1,2-dibromoethane (1.4-fold) but not with DEM or carbon tetrachloride. Activation was also obtained with 1-chloro-2,4-dinitrobenzene (CDNB) (1.6-fold) and to a small extent with *t*-butyl hydroperoxide (1.2-fold). The activation by 1,2-dibromoethane and CDNB is probably mediated through covalent binding, considering the known alkylating properties of these compounds. CDNB is the first substrate shown to activate the microsomal glutathione transferase implying that electrophilic compounds which are substrates can increase the rate of their own elimination by reacting with this enzyme. In addition, activation by *t*-butyl hydroperoxide indicates that oxidative stress can activate microsomal glutathione transferase.

Rat liver microsomal glutathione transferase (EC 2.5.1.18) [1] is a membrane-bound member of the glutathione transferase family [2]. These enzymes catalyse the conjugation of glutathione to electrophilic molecules with hydrophobic properties [3] and thus serve in detoxication. Among other characteristics, microsomal glutathione transferase is unique in its ability to be activated *in vitro* by electrophiles (through binding to cys-49) [4], thiol–disulfide interchange [5–7], proteolysis [8] and, non-covalently, by bromosulfophtalein [9] or removal of an endogenous microsomal inhibitor of unknown nature [10]. It should be noted that the activity of the modified enzyme only increases with substrates that are comparatively strong electrophiles [11].

Thus, there are numerous possibilities for regulation of this enzymatic activity. Evidence has accumulated for activation of the enzyme by reactive intermediates or direct alkylators, both *in vivo* and *in vitro* [6, 12–16], which supports the idea that toxic compounds can activate the enzyme and thereby, in some cases, their own metabolism. Whether there is also endogenous regulation of the microsomal glutathione transferase is highly interesting, both with regard to possible endogenous substrates and the possibility of manipulating this enzyme *in vivo*.

The present study was performed to investigate previous claims [6] that microsomal glutathione transferase can be activated *in vivo* by phorone through thiol–disulfide interchange and to examine the effect of a range of other compounds on the activity of this enzyme in isolated rat hepatocytes. The results show for the first time that activation of microsomal glutathione transferase can be mediated by a substrate [1-chloro-2,4-dinitrobenzene (CDNB†)] which, thus, increases the rate of its own conjugation.

* Corresponding author: Ralf Morgenstern, Institutionen för Toxikologi, Karolinska Institutet, Box 60400, S-10401 Stockholm, Sweden. Tel. (46) 8-7287574; FAX (46) 8-334467.

† Abbreviations: CDNB, 1-chloro-2,4-dinitrobenzene; phorone, diisopropylidene acetone; DEM diethylmaleate; TPA, phorbol 12-myristate 13-acetate; GSH, glutathione; GSSG, oxidized glutathione.

MATERIALS AND METHODS

Materials. Rainbow marker molecular weight standards were purchased from the Radiochemical

Center (Amersham, U.K.). Collagenase type 2, oxidized glutathione (Boerhinger, Mannheim, Germany), phorone, DEM (EGA-Chemie, Steinheim, Germany), hydrogen peroxide, carbon tetrachloride, 1,2-dibromoethane, CDNB (Merck, Darmstadt, Germany), calcium ionophore A 23187 (Calbiochem, U.S.A.) and *t*-butylhydroperoxide, dithioerythritol and TPA (Sigma Chemical Co., St Louis, MO, U.S.A.) were obtained from the sources indicated.

Methods. Rat liver microsomes and microsomal glutathione transferase were prepared from male Sprague–Dawley rats (180–200 g) as described previously [17].

Hepatocytes were prepared as described previously [18] and incubated at 37° (5×10^6 cells/mL) in Krebs–Henseleit buffer in rotating flasks under an atmosphere of 95% O_2/5% CO_2 for the times indicated. All additions were made with dimethyl sulfoxide as solvent and controls received the solvent alone. DEM (2 mM) pretreatment was performed at 37° (5×10^6 cells/mL) for 15 min whereafter cells were pelleted and resuspended. Viability was determined by Trypan blue exclusion [19] and was 70–90% in all experiments.

Microsomes from hepatocytes were prepared according to a previous report [20] and demonstrated glutathione transferase activity similar to or somewhat lower than microsomes prepared by conventional methods [21]. In short, hepatocytes were pelleted at 50 *g* for 5 min, resuspended in 0.25 M sucrose and sonicated twice for 10 sec (4 amps, Branson sonifier equipped with a small tip) on ice with intermittent cooling. The sonicated cells were centrifuged at 10,000 *g* for 10 min and the supernatant obtained was incubated with 8 mM $CaCl_2$ and 5 mM $MgCl_2$ for 10 min. Microsomes were pelleted at 1500 *g* for 10 min, resuspended in 0.15 M Tris–Cl, pH 8, and 1 mM EDTA, and pelleted at 1500 *g* for 10 min after another addition of 8 mM $CaCl_2$ and 5 mM $MgCl_2$. The resulting pellet was resuspended in 0.25 M sucrose-1 mM EDTA, pH 7.5. Thorough mixing using an automatic pipette was used to facilitate the withdrawal of representative samples. Dithioerythritol (final concentration 0.1 M) was added to aliquots of the phorone-activated and control microsomes and incubated at 4° for 5–10 min after which activity was determined.

Activation of microsomal glutathione transferase in microsomes by GSSG was performed by incubating microsomes (10 mg/mL) in 0.25 M sucrose, 50 mM potassium phosphate, pH 8, and 50 mM GSSG at room temperature. Aliquots were withdrawn at the time points indicated and assayed for glutathione transferase activity. 0.1 M Dithioerythritol was added to parallel incubations as indicated and assayed in the same fashion.

Treatment of purified microsomal glutathione transferase with phorone (2 mM) was performed at room temperature in 10 mM potassium phosphate, pH 8, 1 mM GSH, 0.1 mM EDTA, 1% Triton X-100, 20% glycerol and 0.1 M KCl for up to 3 hr. Treatment of isolated microsomes (10 mg/mL) with phorone (1 mM) was performed at 37° in 0.125 M sucrose, 75 mM Tris–Cl, pH 7.5, with or without the addition of cytosol (5 and 10 mg/mL), and with or without the addition of an NADPH-generating system (5 mM isocitrate 1 mM NADP isocitrate dehydrogenase, 0.6 U/mL and 10 mM $MgCl_2$) for up to 4 hr.

Microsomal glutathione transferase activity was measured as described previously [22] using 0.5 mM CDNB as the second substrate.

SDS–PAGE was performed according to Laemmli [23] in 15% polyacrylamide gels. Western blotting and immunodecoration followed by peroxidase anti-peroxidase staining were performed as described [24].

Acid-soluble sulfhydryl concentration (mainly GSH) was determiend as described previously [25]. DEM, phorone, 1,2-dibromoethane and CDNB at the concentrations used here depleted thiols from 35–50 down to 2–8 nmol/10^6 cells in control experiments.

Protein was determined by the method of Peterson [26] using bovine serum albumin as the standard.

RESULTS AND DISCUSSION

A number of compounds (phorone, 1,2-dibromoethane, CCl_4 and DEM [6, 13]) have been shown to increase the activity of microsomal glutathione transferase *in vivo* after a short exposure. Since microsomal glutathione transferase can be activated by a number of conditions *in vitro*, including covalent modification, thiol–disulfide interchange and proteolysis, several mechanisms might explain the increase in activity *in vivo*. The possibility of induction appears less likely in view of the short time of treatment and the consistent lack of induction of the microsomal glutathione transferase by common inducers of drug metabolism [22].

In order to investigate the mechanism of activation, these compounds were added to isolated hepatocytes and it was found that phorone consistently activated the enzyme in a time-dependent manner (Fig. 1) while 1,2-dibromoethane had a lesser effect (Table 1). DEM and CCl_4 did not increase the activity.

When microsomes from phorone-treated cells and controls were treated with *N*-ethylmaleimide

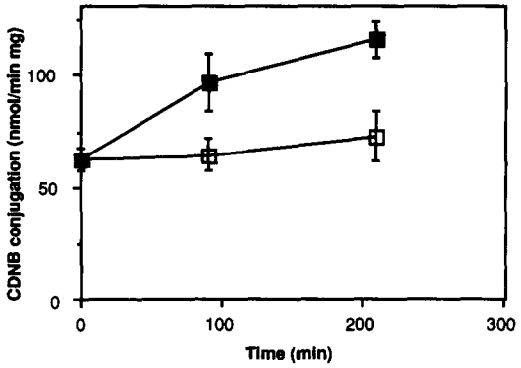

Fig. 1. Microsomal glutathione transferase activity towards CDNB in microsomes prepared from isolated hepatocytes treated with phorone (1 mM) for different periods of time (■) as compared to control (□) (N = 4).

Table 1. Activity towards CDNB in microsomes isolated from hepatocytes

Treatment	Incubation time (min)	Activity (%)*	Activity (%) after DTE treatment	Activity (%) after NEM treatment
Control	285	100 ± 11 (N = 4)	90 ± 0 (N = 2)	1160 ± 107 (N = 3)
Phorone (1 mM)	285	262 ± 14 (N = 4)†	210 ± 34 (N = 2)‡	949 ± 138 (N = 3)§
Phorone (0.5 mM)	285	144 ± 17 (N = 4)†	168 ± 36 (N = 2)	
Control (DEM-pretreated)	143	100 ± 8 (N = 2)		
Phorone (1 mM) (DEM-pretreated)	143	165 ± 14 (N = 2)†		
Control	200	100 ± 10 (N = 4)		
DEM (2 mM)	200	112 ± 4 (N = 4)		
DEM (1 mM)	200	103 ± 23 (N = 4)		
1,2-Dibromoethane (1 mM)	200	140 ± 25 (N = 4)†		
1,2-Dibromoethane (0.5 mM)	200	138 ± 8 (N = 4)†		
Control	210	100 ± 15 (N = 4)		
CCl_4 (1 mM)	210	87 ± 11 (N = 4)		
Control	105	100 ± 6 (N = 2)		
CDNB (0.3 mM)	105	161 ± 18 (N = 2)†		
Control	140	100 ± 7 (N = 2)		
Hydrogen peroxide (5 mM)	140	102 ± 10 (N = 2)		
t-Butylhydroperoxide (0.5 mM)	140	116 ± (N = 4)†		
Bromosulfophtalein (0.1 mM)	140	105 ± 7 (N = 4)		
TPA (50 nM)	140	111 ± 11 (N = 6)		
Vasopressin (100 nM)	140	99 ± 9 (N = 2)		
Ca-ionophore (100 nM)	140	98 ± 0 (N = 2)		

Details are described in Materials and Methods.

* CDNB activity expressed as percentage of control. Control specific activities ranged between 50 and 100 nmol/min mg in different experiments.

† Significantly different from the respective control at $P < 0.05$ or less (Student's *t*-test). Other values were not significantly different from controls.

‡ Significantly different from DTE-untreated at $P < 0.05$ (Students *t*-test).

§ Not significantly different from NEM-treated control.

N, number of observations.

(activation according to Ref. 16), the microsomal glutathione transferase activities were increased to the same maximum level (Table 1). This result rules out any increase in the level of microsomal glutathione transferase protein.

It has been suggested that activation by phorone *in vivo* is mediated by the observed increase in GSSG and subsequent thiol–disulfide interchange [6]. If this were the case, it should be possible to reverse the activation by a reductant such as dithioerythritol, as shown in Fig. 2 using GSSG-activated microsomes *in vitro*. However, in isolated hepatocytes treated with phorone there is only a small decrease in microsomal glutathione transferase activity upon exposure to dithioerythritol (0.1 M) (Table 1). This finding rules out formation of any substantial amounts of an easily reducible mixed disulfide between the microsomal glutathione transferase and GSH after phorone treatment.

Pretreatment of the hepatocytes with DEM, which effectively depletes GSH, before phorone addition did not decrease activation (Table 1). This indicates that it is not the phorone–GSH conjugate that mediates activation. Furthermore, GSH depletion *per se* is not responsible for activation, since DEM had no activating effect.

Turning to other possibilities, we observed by immunoblot analysis (now shown) that there is no increased proteolysis of the microsomal glutathione transferase after phorone treatment, which might

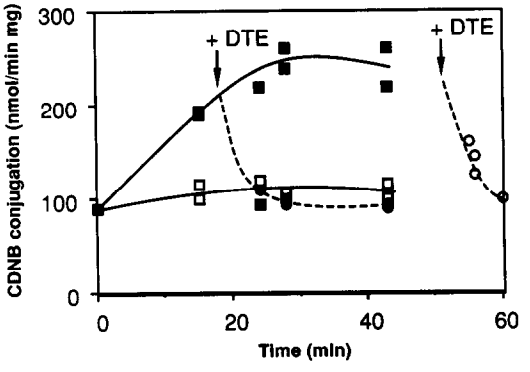

Fig. 2. Activation of liver microsome glutathione transferase activity towards CDNB by GSSG (50 mM) (■) as compared to controls (□) and reversion of the activation by addition of dithioerythritol (0.1 M) to parallel GSSG incubations at the times indicated (18 min, ●), (52 min, ○). Controls that received dithioerythritol (0.1 M) alone showed a slight decrease in activity (10%) which was not statistically significant. Each result shown is one of three experiments with essentially identical results.

explain the activation. We also investigated the possibility that phorone is metabolized to a reactive metabolite that could covalently modify the cys-49 in the microsomal glutathione transferase or that this compound reacts with the enzyme directly (bearing in mind that it is a sulfhydryl reagent). Incubation of the purified enzyme or liver microsomes with up to 5 mM phorone for as long as 3 hr yielded no activation, thus, excluding a direct effect. Incubation of microsomes with phorone (1 mM) in the presence of an NADPH-regenerating system did not indicate the formation of a reactive metabolite that could activate the enzyme. Repeating these incubations with microsomes in the presence of increasing amounts of 100,000 *g* supernatant, to better approximate intracellular conditions, gave similar results. In the intact cell or animal, local concentration differences and enzyme distributions could yield activation by a reactive metabolite; this possibility should, therefore, not be dismissed altogether. In conclusion, activation by phorone potentially mimics endogenous regulation and further studies are clearly warranted.

It has been shown in a number of studies [6, 12,–16] that microsomal glutathione transferase can be activated by reactive intermediates formed from xenobiotics or directly by electrophiles, both *in vivo* and *in vitro*. Activation by 1,2-dibromoethane and CDNB (shown in Table 1) is probably also mediated by covalent modification, considering the electrophilic nature of these compounds. CDNB is also a substrate for the microsomal glutathione transferase demonstrating that activation by electrophiles that are substrates can occur. This type of regulation offers an instant response to toxic insult.

Compounds that cause an increase in intracellular calcium (27, 28] and protein phosphorylation [29] could conceivably regulate the microsomal glutathione transferase through phosphorylation. However, neither TPA, vasopressin nor Ca-ionophore had any significant effect on the enzyme activity in isolated hepatocytes (Table 1). These studies are now being extended by investigating the effect of purified protein kinase C on the activity of the isolated microsomal glutathione transferase.

Bromosulfophtalein, which can activate the enzyme by non-covalent binding in a certain concentration range [9, 30] and increases the microsomal glutathione transferase sensitivity to protelolysis by trypsin [8], was tested to see whether either of these properties of the compound could mediate activation of the enzyme. This was not the case indicating that the endogenous proteolysis, which has been noted in microsomes under certain conditions (unpublished observation), is not augmented.

Microsomal glutathione transferase is active as a glutathione peroxidase towards lipid hydoperoxides and can conjugate hydroxyalkenals [31] which are formed during lipid peroxidation. Since the glutathione peroxidase activity is increased upon activation, such activation of the microsomal glutathione transferase might be a useful response in counteracting the toxic effects of oxidative stress. We observe here a small but significant increase in microsomal glutathione transferase activity in hepatocytes treated with *t*-butylhydroperoxide (Table 1), but not with hydrogen peroxide. This small effect obviously requires further optimization in order to establish its significance in cell defenses and to facilitate studies of the mechanism of activation.

In summary, isolated hepatocytes offer a model system that mimics the *in vivo* situation regarding activation of microsomal glutathione transferase by phorone and 1,2-dibromoethane. Thus, the possible mechanism of activation *in vivo* can be sought. There are quite a few examples of activation via a reactive metabolite or electrophile. Whether a physiological mechanism also exists remains a question of great interest.

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