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MECHANISM OF ACTIVATION OF RAT LIVER MICROSOMAL GLUTATHIONE TRANSFERASE BY NORADRENALINE AND XANTHINE OXIDASE

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Abstract—Activation of glutathione transferase activity in rat liver microsomes under a variety of conditions producing oxidative stress was investigated. Neither hydrogen peroxide (10 mM) (added or produced endogenously by glucose + glucose oxidase) nor duroquinone together with an NADPH-regenerating system (which generates the superoxide anion radical) had any significant effect on the glutathione transferase activity towards 1-chloro-2,4-dinitrobenzene. On the other hand, incubation of microsomes with 1 mM noradrenaline (which autooxidizes and generates superoxide anion radical) gave a 160% activation, as shown earlier (Aniya and Anders, *J Biol Chem* **264**: 1998–2002, 1989). This was taken as an indication that microsomal glutathione transferase could be activated by oxidative stress. Here, we demonstrate that activation by this compound is due to covalent binding (presumably of the quinone formed during autooxidation). The xanthine/xanthine oxidase system, which generates the superoxide anion radical and hydrogen peroxide, increases microsomal glutathione transferase activity, but this activation was not dependent on the presence of xanthine. Western blots of microsomes treated with xanthine oxidase revealed that activation was due to proteolysis (presumably by contaminating proteases in the xanthine oxidase). In conclusion, there is no firm evidence that rat liver microsomal glutathione transferase is activated directly by reduced oxygen species in the microsomal system. The possibility remains that oxidative stress triggers secondary mechanisms such as generation of reactive intermediates and/or activation of proteolysis, which can in turn increase enzyme activity.

Liver microsomal glutathione transferase has been characterized in great detail (see Ref. 1 for a review) and shares the functional characteristics of the glutathione transferases (EC 2.5.1.18) in general, i.e. a broad substrate specificity towards hydrophobic electrophiles [2]. Many genotoxic, toxic and pharmacologically active compounds are detoxified [3] by glutathione conjugation. Microsomal glutathione transferase also displays the unusual property of activation by electrophiles [4] and proteolysis [5].

Recent evidence [6] suggests that the glutathione peroxidase activity (towards lipid hydroperoxides) and the hydroxyalkenal-conjugating activity of the microsomal enzyme might protect the liver against lipid peroxidation, which is a consequence of oxidative stress. It has also been found that the enzyme is activated by reduced oxygen species [7] formed by autooxidation of noradrenaline. Since the glutathione peroxidase activity of microsomal glutathione transferase is increased by activation of the enzyme [6], it is an attractive hypothesis that oxidative stress up-regulates the enzyme which could thereby afford increased protection against the deleterious effects of oxidative stress (e.g. lipid peroxidation).

This work was prompted by an inconsistency in activation of microsomal glutathione transferase by noradrenaline and duroquinone (in the presence of an NADPH-regenerating system), i.e. the former yielded activation and the latter did not, although both produce superoxide anion radicals. Based on the ability of catecholamines to yield species that can bind covalently to proteins [8], an alternate hypothesis for activation was tested here. Furthermore, other systems that produce hydrogen peroxide and the superoxide anion radical did not activate the microsomal glutathione transferase.

MATERIALS AND METHODS

Materials. Rainbow marker molecular mass standards were purchased from the Radiochemical Center (Amersham, U.K.). Xanthine oxidase (cow milk; Boehringer, Mannheim, Germany) and glucose oxidase (Sigma Chemical Co., St Louis, MO, U.S.A.) were bought from the sources indicated. [^{3}H]Noradrenaline, levo-[7-^{3}H] (13 Ci/mmol) was obtained from NEN Research Products (Germany).

Methods. Rat liver microsomes and microsomal glutathione transferase were prepared from male Sprague–Dawley rats (180–200 g) as described earlier [9].

Microsomes (1–2 mg protein/mL) were incubated with the various agents as indicated in the Tables at 37° in 50 mM potassium phosphate buffer, pH 7.4, 25 mM sucrose and 30 μM EDTA. An NADPH-regenerating system [5 mM isocitrate, 1 mM NADP, isocitrate dehydrogenase (0.6 U/mL) and 10 mM $MgCl_2$] was added as indicated and aliquots were removed for determination of glutathione transferase activity. Some samples were preincubated for 5 min

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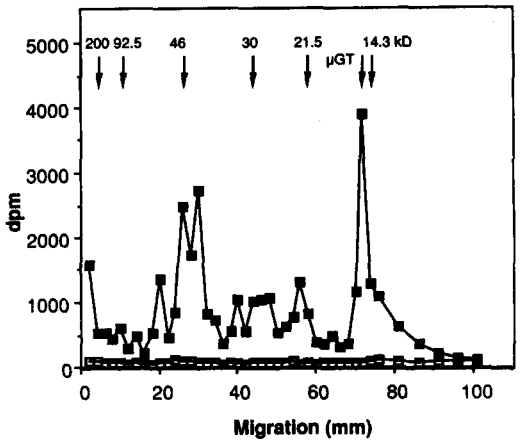

Fig. 1. The radioactivity profile of microsomal proteins separated by SDS–PAGE after incubation with [^{3}H]-noradrenaline (0.5 mM) for 0 (□) and 30 (■) min at 37°. Arrows indicate the positions of microsomal glutathione transferase (μGT) and rainbow marker molecular mass standards (kDa). Details are described in Materials and Methods.

with 10 mM dithiothreitol (final concentration) before assay as indicated.

Microsomal glutathione transferase activity was measured according to Habig *et al.* [10] using 0.5 mM 1-chloro-2,4-dinitrobenzene as the second substrate.

[^{3}H]Noradrenaline was diluted to 0.5 Ci/mmol with cold noradrenaline and incubated with microsomes at a concentration of 0.5 mM as described above. Samples were withdrawn at 0 and 30 min and 10 mM dithiothreitol was added to stop the reaction. The microsomes were washed by centrifugation in 0.15 M Tris–HCl, pH 8, and subsequently subjected to SDS–PAGE (15%) as described by Laemmli [11]. The gel lane was cut into 2-mm sections and the pieces were digested with 600 μL hydrogen peroxide (30%) at 60° overnight. After addition of 4 mL Instagel, radioactivity was determined by scintillation counting and the data converted to dpm by correction for quenching. Surrounding lanes with standards (rainbow markers and purified microsomal glutathione transferase) were stained and their positions are indicated in Fig. 1.

For determination of proteolysis, samples were withdrawn and analysed by SDS–PAGE (15%) followed by Western blotting. Immunodecoration and peroxidase anti-peroxidase staining was performed as described earlier [12].

Protein was determined by the method of Peterson [13] using bovine serum albumin as standard.

RESULTS AND DISCUSSION

Rat liver microsomes were subjected to a variety of conditions known to produce oxidative stress and the glutathione transferase activity was determined (Table 1). Activation by hydrogen peroxide was not observed, as evidenced by the lack of effect of the compound itself and of a H_2O_2-generating system (glucose/glucose oxidase). Inclusion of sodium azide to prevent the removal of hydrogen peroxide by contaminating catalase did not alter the results.

Treatment with noradrenaline or xanthine/xanthine oxidase causes activation of the enzyme. On the surface, this finding would seem to indicate a role for the superoxide anion radical formed by both these systems. However, this conclusion is contradicted by the lack of effect by duroquinone together with an NADPH-regenerating system, which also generates the superoxide anion radical [14]. Since, upon oxidation of noradrenaline, a

Table 1. Effect of oxidative stress on glutathione transferase activity towards 1-chloro-2,4-dinitrobenzene in rat liver microsomes

Treatment (20 min)	% of control activity ± SD (N = No. of experiments)
Control	100*
Hydrogen peroxide (10 mM)	97 ± 12 (N = 3)
Glucose (10 mM) + glucose oxidase (50 mU/mL)†‡	108 ± 4 (N = 4)
Glucose (10 mM) + glucose oxidase (50 mU/mL) + sodium azide (10 mM)	84, 100
Xanthine (0.1 mM) + xanthine oxidase (30 mU/mL)‡	193 ± 74 (N = 3)
Xanthine (0.1 mM)	96 ± 8 (N = 3)
Xanthine oxidase (30 mU/mL)	184 ± 105 (N = 3)
Xanthine oxidase (60 mU/mL)	445 ± 55§ (N = 3)
Duroquinone (0.2 mM) + NADPH-generating system	94 ± 17 (N = 3)
Noradrenaline (1 mM)	160 ± 6§ (N = 3)

Details are described in Materials and Methods.

* The control incubation activity at 20 min is taken as 100%. When compared to 0-min incubations the activity is increased by 134 ± 17% (N = 6).

† Glucose and glucose oxidase had no effect separately.

‡ Glucose + glucose oxidase and xanthine + xanthine oxidase had no effect at lower enzyme concentrations spanning two orders of magnitude.

§ Significantly different from control ($P < 0.001$, Students *t*-test).

Table 2. Effect of dithiothreitol treatment (5 min) on the glutathione transferase activity in noradrenaline-activated (30 min) microsomes

Treatment	% of initial activity
Control	126 ± 20 (N = 4)
Control + dithiothreitol (10 mM)	100 ± 16 (N = 4)*
Noradrenaline (1 mM)	310 ± 21 (N = 4)†
Noradrenaline (1 mM) + dithiothreitol (10 mM)	366 ± 47 (N = 4)*

Details are described in Materials and Methods.

* Not significantly different from dithiothreitol-untreated.

† Significantly different from control ($P < 0.001$, Students t-test).

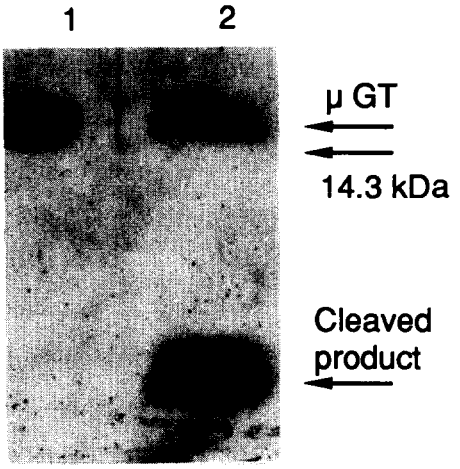

Fig. 2. Immunoblot analysis of proteolytic modification of microsomal glutathione transferase in microsomes. Lane 1, control; lane 2, microsomes treated with xanthine oxidase (40 mU/mL for 20 min). The position of a molecular mass marker is indicated. Details are described in Materials and Methods.

quinone [15] that can react covalently with nucleophilic groups in proteins or DNA is formed [8, 16], and since it is known that rat liver microsomal glutathione transferase is activated by electrophiles, including benzoquinone [17], it was reasonable to investigate this possibility. Incubation of microsomes with 0.5 mM [^{3}H]noradrenaline leads to covalent binding of radioactivity to protein (Fig. 1). Of the incorporated label, 20% comigrates with the microsomal glutathione transferase, which corresponds to a binding of 0.1 mol radioactive substance per mol enzyme subunit. The degree of modification of microsomal glutathione transferase agrees well with the extent of activation. Furthermore, microsomal glutathione transferase appears to be attacked preferentially, since 20% of the label binds to this enzyme which represents 3% of the protein. The latter observation underlines the idea that microsomal glutathione transferase is very efficient in binding reactive intermediates, thereby protecting other proteins. This is, however, not a suicidal function, as suggested for certain cytosolic glutathione transferases [18], since activation ensues. In some cases (when the electrophile is a substrate for the enzyme), this might lead to more efficient detoxification.

Earlier work [7] showed that noradrenaline activation was prevented by including dithiothreitol in the incubation. It was speculated that dithiothreitol prevented oxidation of the sulfhydryl group of microsomal glutathione transferase. However, when dithiothreitol is added after incubation with noradrenaline, there is no reversal of the activation (Table 2). The concentration of dithiothreitol used (5 mM) effectively reduces the mixed disulfide formed between microsomal glutathione transferase and 5,5′-dithiobis-(2-nitrobenzoic acid) [19]. This rules out the formation of a readily reducible, oxidized thiol group in the enzyme and is consistent with covalent modification, since dithiothreitol could scavenge electrophilic quinones when added during the incubation.

Activation by high amounts of xanthine oxidase does not require the presence of xanthine. Therefore, an explanation not involving the formation of oxygen radicals was tested. Immunoblot analysis of microsomal glutathione transferase after incubation of microsomes with xanthine oxidase revealed extensive proteolysis (Fig. 2) which is known to activate the enzyme [5]. It is known that this commercial preparation of xanthine oxidase contains low levels of proteases, the activity of which become significant when large amounts are added. It is also possible that some component in the enzyme preparation activates an endogenous protease. However, activation is clearly not due to reduced oxygen species.

The hypothesis that microsomal glutathione transferase can be activated during oxidative stress is attractive, since this enzyme can reduce lipid hydroperoxides and conjugate hydroxyalkenals [6] that may be formed. We have indications that the activity can be increased in isolated liver cells by t-butylhydroperoxide.* Whether the enzyme is activated directly by the hydroperoxide or whether oxidative stress triggers some endogenous activation mechanism remains to be elucidated. At this point an indirect mechanism appears more likely. There are many candidates for such an activation mechanism, including: (1) modification by a reactive metabolite, (2) triggering of thiol–disulfide interchange (which activates the enzyme *in vitro* [19, 20]) and (3) activation of proteolysis [5]. Future studies on the activation of microsomal glutathione transferase in more complex systems will have to take all these possibilities into account.

Similar conclusions regarding the activation of microsomal glutathione transferase by metabolites of α-methyldopa have been reported recently by Haenen *et al.* [21].

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