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OXIDATIVE STRESS-INDUCED ACTIVATION OF MICROSOMAL GLUTATHIONE *S*-TRANSFERASE IN ISOLATED RAT LIVER

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Abstract—The activation of microsomal glutathione (GSH) *S*-transferase in isolated rat liver by oxidative stress was investigated using both ischemia/reperfusion and perfusion with hydrogen peroxide. When the isolated liver was reperfused for 30 min and 60 min after 90 min ischemia, microsomal GSH *S*-transferase activity, but not cytosolic transferase activity, was increased 1.2-fold and 1.3-fold, respectively. In addition, microsomal GSH peroxidase activity was also significantly increased after 60 min reperfusion following ischemia. The increase in microsomal GSH *S*-transferase activity by ischemia/reperfusion was reversed by dithiothreitol. When *N*-ethylmaleimide, which activates microsomal GSH *S*-transferase by covalent binding to the cysteine residue of the enzyme, was incubated with microsomes, transferase activity was increased to 526% in control microsomes and to 399% in liver that underwent ischemia/reperfusion. A small amount of a dimer protein of microsomal GSH *S*-transferase was detected in ischemia/reperfusion liver. These data indicate that microsomal GSH *S*-transferase is activated by ischemia/reperfusion of the liver by means of disulfide bond formation. When rats were pretreated with a catalase inhibitor 3-amino-1,2,4-triazole for 6 weeks, microsomal GSH *S*-transferase activity was increased 1.4-fold by ischemia/reperfusion, corresponding to a 1.8-fold increase as compared to the non-perfused liver of untreated rats. Perfusion of the isolated liver with hydrogen peroxide (1 mM, 15 min) also caused a significant increase in microsomal GSH *S*-transferase activity with a concomitant decrease in GSH content, confirming that liver microsomal GSH *S*-transferase in rats was activated *in vivo* by oxidative stress.

Glutathione (GSH†) *S*-transferase (EC 2.5.1.18) catalyses the reaction of GSH conjugation with electrophiles and also functions as GSH peroxidase [1–4]. In contrast to cytosolic GSH *S*-transferase, rat liver microsomal GSH *S*-transferase contains only one cysteine residue per subunit [5, 6], and is activated by a modification of the sulfhydryl group with *N*-ethylmaleimide [7], by limited proteolysis [8] or by thiol–disulfide exchange [9, 10]. We reported recently that microsomal GSH *S*-transferase is also activated by oxygen radicals *in vitro* [11, 12]; thus, it is assumed that the microsomal GSH *S*-transferase is activated *in vivo* by oxygen radicals generated endogenously or during oxidative stress. To confirm this assumption, the effect of ischemia/reperfusion of the isolated rat liver on GSH *S*-transferase activity was examined since reperfusion followed by ischemia produces oxygen radicals [13, 14]. Furthermore, perfusion of the isolated liver with hydrogen peroxide and ischemia/reperfusion of the liver pretreated with the catalase inhibitor, 3-amino-1,2,4-triazole (3-AT) were investigated.

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† Abbreviations: GSH, reduced glutathione; GSSG, oxidized glutathione; 3-AT, 3-amino-1,2,4-triazole.

MATERIALS AND METHODS

Chemicals. Reduced glutathione (GSH), GSH reductase, 3-AT and cumene hydroperoxide were purchased from the Sigma Chemicals Co. (St Louis, MO, U.S.A.). NADPH and heparin were obtained from Oriental Yeast and Nacalai Tesque (both Tokyo, Japan), respectively. 1-Chloro-2,4-dinitrobenzene was obtained from Wako Pure Chemicals (Tokyo, Japan). Hydrogen peroxide and sodium pentobarbital were purchased from Santoku-Kagaku (Tokyo, Japan) and Abbott Laboratories (North Chicago, IL, U.S.A.) respectively. All other chemicals used were of analytical reagent grade.

Animals and liver perfusion. Male Sprague–Dawley rats (150–300 g) that had been starved overnight were used. In cases where pretreatment of rats with a catalase inhibitor was necessary, 3-AT (0.2%, w/v) was dissolved in drinking water for 6 weeks whereas control animals received untreated water for the same period. After anesthetizing the rat with sodium pentobarbital (50 mg/kg, i.p.), the cannula was inserted into the portal vein followed by infusion of heparin (250 U/mL) and the liver was removed after ligation of the superior vena cava. Perfusion of the liver was carried out continuously from the portal vein to the inferior vena cava (control) with Krebs–Henseleit solution (pH 7.4) gassed with 95% O_2/5% CO_2 or reperfused for certain periods of time after a flow stop of 90 min (ischemia) at a flow rate of 40 mL/min/liver at 37°.

Table 1. Alteration of enzyme activity in and GSH content of ischemia/reperfused liver

Treatment	GSH (N = 22) (nmol/mg protein)	GSSG (N = 19) (nmol/mg protein)	Microsomes: GSH S-transferase (N = 24) (μmol/mg/min)	Microsomes: GSH peroxidase (N = 16) (μmol/mg/min)	Cytosol: GSH S-transferase (N = 28) (μmol/mg/min)	Cytosol: GSH peroxidase (N = 10) (μmol/mg/min)
Control	5.5 ± 1.7	0.21 ± 0.17	0.073 ± 0.013	0.057 ± 0.013	1.575 ± 0.389	0.276 ± 0.054
Ischemia	6.0 ± 2.5	0.29 ± 0.15†	0.094 ± 0.026*	0.064 ± 0.015*	1.658 ± 0.520	0.308 ± 0.101

Isolated rat liver was perfused continuously (Control) or reperfused for 60 min after 90 min of ischemia (Ischemia). GSH or GSSG content of the liver homogenate and activities of microsomal and cytosolic enzymes were measured as described in Materials and Methods.

Values are the means ± SD. *P < 0.01, †P < 0.05, Control vs Ischemia.

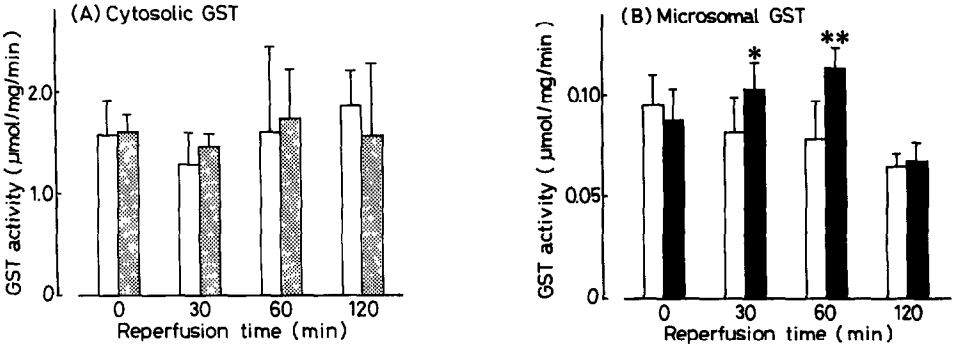

Fig. 1. Time course of the effect of ischemia/reperfusion of the isolated liver on GSH S-transferase activity. Isolated rat liver was perfused continuously (control) or reperfused for an indicated time after 90 min ischemia (ischemia). GSH S-transferase activity in cytosol (A) and microsomes (B) was measured as described in Materials and Methods. Values are presented as the means ± SD for three to six rats. (□) Control, (▨, ■) ischemia. *P < 0.05, **P < 0.01, control vs treated.

Hydrogen peroxide (H_2O_2) perfusion of the isolated liver was carried out either with or without 1 mM H_2O_2 dissolved in Krebs–Henseleit solution at certain indicated times under the same conditions given for ischemia/reperfusion.

After perfusion, the liver was homogenized with 2 vol. of ice-cold potassium chloride solution (1.15%), and cytosol and microsomes were prepared as described previously [11].

Measurement of GSH content and enzyme activity. The GSH content of liver homogenates was measured by the method of Reed *et al.* [15] using HPLC. Non-protein thiol in the homogenate and protein thiol in the microsomes of the liver were also measured spectrophotometrically using 5,5′-dithiobis-2-nitrobenzoic acid. Briefly, the acid extract from the homogenate and SDS-solubilized microsomes were incubated in 5,5′-dithiobis-2-nitrobenzoic acid and the thiol content of each fraction was calculated. GSH S-transferase and GSH peroxidase activities were measured by the methods of Habig *et al.* [16] and Reddy *et al.* [4] using 1-chloro-2,4-dinitrobenzene and cumene hydroperoxide as substrates, respectively. Catalase activity in liver homogenates was measured by the method of Beers and Sizer [17]. Protein concentration was determined by the method of Lowry *et al.* [18].

Gel electrophoresis and immunoblotting. Gel electrophoresis and immunoblotting were carried out using anti-microsomal GSH S-transferase antibody, which was prepared in our laboratory, as described previously [12].

Statistical analysis. Data were expressed as the means ± SD. Significance of difference was calculated using Student's *t*-test, where P values <0.05 were taken as significant.

RESULTS

Ischemia/reperfusion

When the isolated liver was reperfused from 0 to 120 min after 90 min ischemia, microsomal GSH S-transferase activity increased 1.2-fold at 30 min and 1.3-fold at 60 min reperfusion. However, no increase in cytosolic GSH S-transferase activity was observed during reperfusion (Fig. 1). As shown in Table 1, GSH peroxidase activity in microsomes also significantly increased after a 60 min reperfusion. A small increase in the oxidized glutathione (GSSG) content of the liver homogenate was observed after a 60 min reperfusion of the ischemic liver. The increased activity of microsomal GSH S-transferase after a 60 min reperfusion of the ischemic liver was reversed by treatment with dithiothreitol; activation

Table 2. Effect of dithiothreitol or *N*-ethylmaleimide on microsomal GSH *S*-transferase activity

Treatment	Microsomal GST activity (μmol/mg/min) None	Dithiothreitol	*N*-Ethylmaleimide
Control	0.073 ± 0.011 (100)	0.063 ± 0.011 (87)	0.388 ± 0.119 (526)
Ischemia	0.086 ± 0.009* (100)	0.062 ± 0.011 (72)	0.347 ± 0.121 (399)

Isolated rat liver was perfused continuously (Control) or reperfused for 60 min after 90 min of ischemia (Ischemia). Microsomes prepared from control or ischemia/reperfused liver were incubated with 10 mM dithiothreitol for 10 min or with 1 mM *N*-ethylmaleimide for 2 min at room temperature. Microsomal GSH *S*-transferase activity was measured as described in Materials and Methods.

Values are the means ± SD for eight rats. *$P < 0.01$, Control vs Ischemia.

Values in brackets are percentages (untreated, 100%).

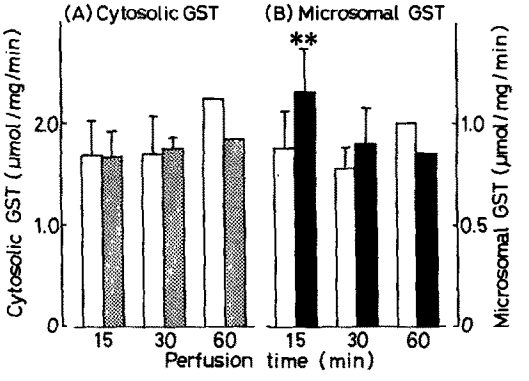

Fig. 2. Time course of the effect of H_2O_2 perfusion of the isolated liver on GSH *S*-transferase activity. Isolated rat liver was perfused with or without 1 mM hydrogen peroxide for the indicated time at 37°. GSH *S*-transferase activity in cytosol and microsomes was measured as described in Materials and Methods. Values are presented as the means ± SD for three to 10 rats. (□) Control, (▨, ■) H_2O_2 perfusion. **$P < 0.01$, control vs H_2O_2 perfusion.

of the transferase caused by *N*-ethylmaleimide was 4-fold in ischemia/reperfusion of the liver as compared to 5.3-fold for the control (Table 2).

Hydrogen peroxide perfusion

Figure 2 shows the time course of the effect of hydrogen peroxide perfusion of isolated liver on GSH *S*-transferase activity. Microsomal GSH *S*-transferase activity was significantly increased by 15 min perfusion of the isolated liver with 1 mM hydrogen peroxide followed by a decrease after 60 min perfusion. The GSH content of the liver was decreased to 80% of the control after 15 min perfusion, whereas GSSG was not detected in H_2O_2-perfused liver. GSH peroxidase activity in neither microsomes nor cytosol was increased by a 15 min perfusion of the liver with hydrogen peroxide (Table 3).

Table 4 shows the relation between microsomal GSH *S*-transferase activity and the content of non-protein thiol of homogenates and protein thiol of microsomes after H_2O_2 perfusion of the liver. Non-protein thiol of liver homogenates was decreased to 76% (nmol/mg protein) of the control by H_2O_2 perfusion and the protein thiol in liver microsomes was also decreased to 94% while GSH *S*-transferase activity was increased to 125%.

Ischemia/reperfusion of 3-AT-pretreated rat liver

When 3-AT was given to rats for 6 weeks, catalase activity in the liver homogenate decreased to 58% as compared to the untreated liver. In the 3AT-pretreated liver, microsomal GSH *S*-transferase activity increased to 132% as compared to the untreated liver by 3-AT treatment alone and to 179% by ischemia/reperfusion (Table 4).

Gel electrophoresis and immunoblotting

Immunoblotting with anti-microsomal GSH *S*-transferase antibodies after electrophoretic separation of the microsomal proteins of ischemia/reperfusion liver showed the presence of proteins with a M_r of 34,000 and 68,000 in addition to the native microsomal GSH *S*-transferase (M_r 17,000) (Fig. 3). In the control liver only native GSH *S*-transferase was detected.

DISCUSSION

Activation of microsomal GSH *S*-transferase by oxidative stress was examined using isolated rat liver. Since it is well known that ischemia/reperfusion of the liver generates oxygen radicals [13, 14], we first studied the effect of ischemia/reperfusion of the isolated rat liver on GSH *S*-transferase activity. Microsomal GSH *S*-transferase activity was significantly increased by 30 min and 60 min reperfusions after 90 min ischemia. The increase in transferase activity caused by ischemia/reperfusion of the liver was reversed by treatment of microsomes with dithiothreitol. In addition, the activity of microsomal GSH *S*-transferase was increased 4-fold by *N*-ethylmaleimide through ischemia/reperfusion of the liver as compared to a 5.3-fold increase in

Table 3. Alteration of enzyme activity and GSH content in H_2O_2-perfused liver

		Microsomes		Cytosol	
	GSH (N = 7) (nmol/mg)	GSH *S*-transferase (N = 10) (μmol/mg/min)	GSH peroxidase (N = 7) (μmol/mg/min)	GSH *S*-transferase (N = 10) (μmol/mg/min)	GSH peroxidase (N = 7) (μmol/mg/min)
Control	13.21 ± 4.07	0.098 ± 0.028	0.037 ± 0.012	1.696 ± 0.353	0.218 ± 0.052
H_2O_2-perfused	10.58 ± 4.18	0.115 ± 0.022*	0.039 ± 0.014	1.669 ± 0.265	0.218 ± 0.046

Isolated rat liver was perfused with or without 1 mM H_2O_2 for 15 min at 37°. GSH content and enzyme activities of microsomes and cytosol were measured as described in Materials and Methods.

Values are the means ± SD. *$P < 0.01$, Control vs H_2O_2-perfused.

Table 4. Alteration of non-protein thiol, microsomal protein thiol and GSH *S*-transferase activity in the liver after H_2O_2 perfusion

	Non-protein thiol		Microsomal protein thiol	GSH *S*-transferase activity
	(nmol/mg)	(μmol/g tissue)	(nmol/mg)	(μmol/mg/min)
Control	22.7	3.54	92.2	0.140
H_2O_2	17.4	3.01	86.7	0.175

Livers were perfused with or without 1 mM H_2O_2 for 15 min at 37° at 20 min intervals. Content of non-protein thiol of the liver homogenate, microsomal protein thiol and GSH *S*-transferase activity were measured as described in Materials and Methods.

Values are the means of the two separated experiments.

Table 5. Effect of 3-AT on enzyme activity in ischemia/reperfused liver

		Microsomes						Cytosol		
	3-AT	GSH *S*-transferase (μmol/mg/min)	(%)		GSH peroxidase (μmol/mg/min)	(%)		GSH *S*-transferase (μmol/mg/min)	(%)	
Control	−§	0.136 ± 0.033	100		0.079 ± 0.013	100		1.713 ± 0.153	100	
	+‖	0.180 ± 0.40†	132	100	0.095 ± 0.026	120	100	2.666 ± 0.258*	156	100
Ischemia	+	0.244 ± 0.044†‡	179	136	0.110 ± 0.030	139	116	2.606 ± 0.174*	152	98

Rats were given 0.2% (w/v) 3-AT dissolved in drinking water for 6 weeks while control animals received water alone. Livers isolated from 3-AT-treated rats were perfused continuously (Control) or reperfused for 60 min after 90 min of ischemia (Ischemia). Activities of microsomal and cytosolic enzymes were measured as described in Materials and Methods.

Values are presented as the means ± SD for four rats. *$P < 0.01$, †$P < 0.05$, Control vs 3-AT-treated. ‡$P < 0.01$, 3-AT Control vs 3-AT Ischemia.

§ No perfusion; ‖ perfusion alone.

the control. Since *N*-ethylmaleimide activates microsomal transferase by covalent binding to the cysteine residue of the enzyme, less activation of the enzyme in ischemia/reperfusion of the liver than in the control implies that the sulfhydryl group in the transferase is consumed during ischemia/reperfusion to form a dimer of microsomal GSH *S*-transferase (Fig. 3). These data show that microsomal GSH *S*-transferase is activated by ischemia/reperfusion of the liver via modification of the sulfhydryl group of the enzyme, forming a disulfide bond.

It has been reported that hydrogen peroxide activates microsomal GSH *S*-transferase *in vitro* [12, 19]. In the present investigation, we examined whether hydrogen peroxide perfusion of the rat liver activates microsomal GSH *S*-transferase. Microsomal GSH *S*-transferase activity was increased after 15 min perfusion with hydrogen peroxide with a concomitant decrease in GSH content in the liver, followed by a decrease in transferase activity after a 60 min perfusion. In addition to a decrease in non-protein thiol in liver homogenates, the microsomal protein

thiol content was also decreased by H_2O_2 perfusion of the liver. Furthermore, a protein dimer of microsomal GSH S-transferase was observed in H_2O_2-perfused liver as was observed in ischemia/reperfused liver (data not shown). Thus, it was clarified that liver microsomal GSH S-transferase was activated by the same mechanism as *in vitro* activation by H_2O_2 in either ischemia/reperfusion or H_2O_2 perfusion liver.

Since microsomal GSH S-transferase activity is decreased under severe oxidative stress such that the GSH content of the liver becomes less than 50% of the control (unpublished data), it seems likely that a moderate oxidative stress may result in an activation of microsomal GSH transferase.

It is assumed that when catalase activity is disturbed *in vivo*, accumulated hydrogen peroxide aggravates oxidative stress, resulting in further activation of microsomal GSH S-transferase. To demonstrate the effect of hydrogen peroxide on the activation of microsomal GSH S-transferase *in vivo*, the liver pretreated with a catalase inhibitor 3-amino-1,2,4-triazole (3-AT) was exposed to ischemia/reperfusion. Microsomal GSH S-transferase activity was increased to 1.3-fold for the liver subjected to 3-AT treatment alone and to 1.8-fold for the liver subjected to 3-AT treatment and ischemia/reperfusion. Since transferase activity was not increased by perfusion alone (data not shown), hydrogen peroxide accumulated by catalase inhibition by 3-AT treatment may activate endogenously microsomal transferase. The net increase in transferase activity of 3-AT-treated liver by ischemia/reperfusion was 1.4-fold compared with a 1.3-fold increase in the untreated liver, reflecting protection of degradation of hydrogen peroxide produced during the reperfusion by catalase inhibition. Considering the 42% decrease in catalase after 3-AT pretreatment, the 1.4-fold increase in transferase activity of 3-AT-treated liver by ischemia/reperfusion seems low. This may mean that only hydrogen peroxide generated around the microsomal transferase contributes to enzyme activation, and that hydrogen peroxide produced in microsomal membranes during ischemia/reperfusion may be capable of activation of the transferase. We are now studying the role of the cytochrome P450 system as an oxygen radical generator.

The 1.3- or 1.4-fold increase in microsomal GSH S-transferase caused by oxidative stress such as ischemia/reperfusion or hydrogen peroxide perfusion was lower than that caused by *in vitro* treatment of microsomes with hydrogen peroxide: a 2-fold increase [12]. The difference in transferase activation between *in vivo* and *in vitro* experiments may be due to the presence of metabolizing enzymes for active oxygens such as catalase or Se-dependent GSH peroxidase in the liver and also to the lability of oxygen radicals in such cellular environments. Moreover, some GSH S-transferases converted to dimers by oxidative stress may be reversed endogenously by enzymes such as thioltransferase or protein disulfide isomerase, resulting in a small increase in transferase activity.

In this study, we used synthetic substrates for the measurement of GSH S-transferase and GSH peroxidase activities such as 1-chloro-2,4-dinitrobenzene and cumene hydroperoxide. Since GSH S-transferase is capable of using 4-hydroxyalkenals or lipid peroxides, which are toxic products of lipid peroxidation [20, 21], as substrates, GSH S-transferase activity increased by oxygen radicals may play an important role in detoxifying toxic metabolites from lipid peroxidation caused during oxidative stress. Indeed, we have observed that GSH peroxidase activity in hydrogen peroxide-treated microsomes *in vitro* was higher in linoleic acid hydroperoxide than in cumene hydroperoxide (unpublished data).

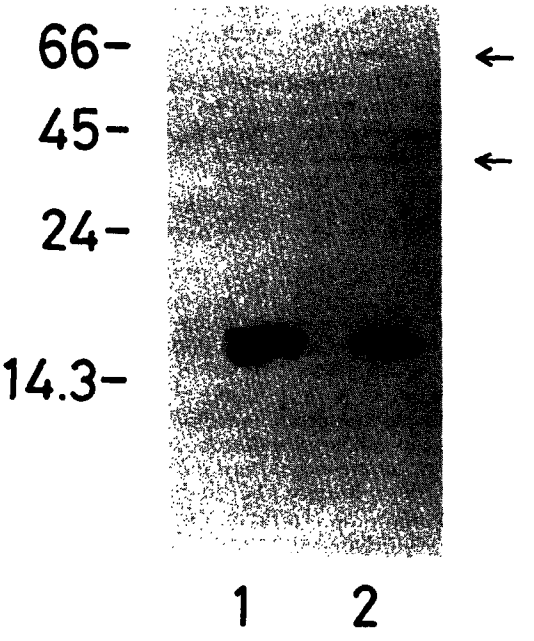

Fig. 3. Gel electrophoresis and immunoblotting of ischemia/reperfusion liver microsomes. Liver microsomes from control (100 μg) and ischemia/reperfusion (90 μg) groups were applied to each lane of 15% SDS–polyacrylamide gels. Electrophoresis was performed according to the method of Laemmli [22] under non-reducing conditions at 6°. Immunoblotting was carried out by transferring proteins from polyacrylamide gels to nitrocellulose paper as described previously [12]. Lane 1, control microsomes; lane 2, ischemia/reperfusion microsomes. Protein standards are lysozyme, M_r 14,300; trypsinogen, M_r 24,000; egg albumin, M_r 45,000 and bovine albumin, M_r 66,000.

In summary, microsomal GSH S-transferase was activated by oxidative stress in the isolated liver of the rat, suggesting detoxification of toxic metabolites of lipid peroxidation caused by oxidative stress.

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