Polymorphic glutathione S-transferase subunit 3 of rat liver exhibits different susceptibilities to carbon tetrachloride: differences in their interactions with heat-shock protein 90

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Rat glutathione S-transferase (GST) subunit 3 gene has polymorphism, one type encoding Asn¹⁹⁸-Cys¹⁹⁹ (NC type) and another encoding Lys¹⁹⁸-Ser¹⁹⁹ (KS type). To examine whether the two types of GST 3-3 exhibit different susceptibilities to oxidative stress *in vivo*, rats were administered with CCl₄, a hepatotoxin causing severe oxidative stress, and its effect on liver GST 3-3 was compared. Decrease in GST activities in liver due to CCl₄ administration was more evident in NC type rats than in KS type rats, and most GST activities of KS type rats were confined to S-hexylglutathione–Sepharose, whereas those of NC type rats were not. Decreases in GST subunits 1 and 3 were more marked in NC type rats and glutathiolated NC type GST 3-3 was also detected. These results indicated that KS and NC type GST 3-3

of rat livers exhibited different susceptibilities to CCl₄ *in vivo*. A protein consisting of a subunit with molecular mass of 90 kDa was shown to bind to KS type GST 3-3 but not to NC type. This protein was identified as heat-shock protein (HSP) 90 β by N-terminal amino acid sequencing and immunoblotting. A specific HSP90 inhibitor geldanamycin released their binding. There was no difference in the binding of apoptosis signal-regulating kinase 1 to GST 3-3 between NC and KS type rats. These findings suggest that HSP90 interacts with KS type GST 3-3 and thereby protects it from inactivation due to CCl₄.

Key words: glutathiolation, glyoxalase I, mitogen-activated protein kinase, oxidative stress.

INTRODUCTION

Glutathione S-transferases (GSTs) are a group of multifunctional enzymes that catalyse the conjugation of many electrophiles with glutathione [1]. They have been classified into seven groups: Alpha, Mu, Pi, Theta, Sigma, Zeta and Omega [2,3]. Hepatic GSTs are involved in detoxication and conjugation reactions of GST 3-3, 3-4 and 4-4, the major forms of rat liver GSTs in the Mu class, and are activated by reactive oxygen species [4].

Hirosaki hairless rats (HHRs) are a mutant strain established from the Sprague–Dawley strain at the Institute for Animal Experiments, Hirosaki University School of Medicine [5]. In our earlier study [6], rat GST subunit 3 gene has polymorphism, one type encoding Asn¹⁹⁸-Cys¹⁹⁹ (NC type) and another encoding Lys¹⁹⁸-Ser¹⁹⁹ (KS type). HHRs are homozygous for the NC type subunit 3 gene, whereas some Sprague–Dawley rats are homozygous for the KS type gene. An *in vitro* study has revealed that NC type GST 3-3 is glutathiolated at Cys¹⁹⁹ when exposed to GSSG, whereas KS type GST 3-3 is activated by reactive oxygen species [6]. However, it remains to be clarified whether these two types of GST 3-3 exhibit different susceptibilities to oxidative stress *in vivo*. Furthermore, such polymorphism of GST subunit 3 may be a factor to determine the susceptibility of individual rats to hepatotoxic substances.

Heat-shock protein (HSP) 90 is an ATP-dependent molecular chaperone involved in the folding and activation of a number of substrate proteins. These include protein kinases [7,8] and transcription factors such as steroid hormone receptors [9] and p53 [10]. HSP90 is known to interact with non-native or destabilized proteins and suppresses their aggregation [11,12]. Disruption of

these complexes is reported to promote proteolytic degradation of substrate proteins [13,14].

In the present study, we administered CCl₄, a typical hepatotoxin causing severe oxidative stress [15,16], to both NC and KS type rats and compared its effect on liver GST 3-3 in both types of rats. More hepatic GST activities were lost in NC type rats than in KS type rats by CCl₄ administration; NC type GST 3-3 lost its affinity for S-hexylglutathione–Sepharose, whereas some NC type enzyme bound to the affinity matrix was glutathiolated at the Cys residue. Thus the NC type enzyme of rat liver was found to be affected severely by CCl₄ treatment. We also found that HSP90 β bound to the KS type GST subunit 3, but not to the NC type.

EXPERIMENTAL

Materials

CCl₄ was obtained from Wako Pure Chemical Industries (Osaka, Japan). Glutathione and 1-chloro-2,4-dinitrobenzene (CDNB) were purchased from Sigma–Aldrich (St. Louis, MO, U.S.A.), and acetonitrile was from Kanto Chemicals (Tokyo, Japan). Sepharose 4B, Polybuffer exchanger 118 and Pharmalyte 8–10.5 were obtained from Pharmacia Biotech (Uppsala, Sweden), and dithiothreitol (DTT) was from Boehringer Mannheim (Tokyo, Japan). Trifluoroacetic acid and CNBr were obtained from Nakalai Tesque (Kyoto, Japan). Geldanamycin (GA) was a gift from National Cancer Institute (Bethesda, MD, U.S.A.). All other chemicals were of analytical grade.

Abbreviations used: ASK1, apoptosis-signal-regulating kinase 1; CDNB, 1-chloro-2,4-dinitrobenzene; DTT, dithiothreitol; GA, geldanamycin; GST, glutathione S-transferase; HHR, Hirosaki hairless rat; HSP, heat-shock protein.

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Administration of CCl₄

Since we have established a homozygous strain for KS type GST subunit 3 gene from Sprague–Dawley rats and a homozygous strain for NC type gene from HHRs, rats which are homozygous for the individual genes were used in the present study. GST subunit 3 genotypes of rats used were confirmed by PCR with primers covering polymorphic nucleotide sequences followed by digestion with a restriction enzyme *Eae*I. Briefly, the 5' and 3' primers were common for KS and NC type subunit 3 genes and had the sequences 5'-AGGGCCTGAAGAAGATCTCTGCC-3' and 5'-TGACAAAGCTGGAGAAGGGAGCA-3' respectively. A mixture of CCl₄ and corn oil (1:1, v/v; 3.0, 4.5 and 6.0 ml/kg body weight) was administered to male KS and NC type rats (250–300 g body weight) using a stomach tube. Rats used as controls received corn oil alone (4.5 ml/kg body weight). Rats were killed by decapitation 48 h after CCl₄ administration [17], and the livers were analysed.

Purification of GSTs from rat liver

Livers from KS or NC type rats as described above were homogenized with 9 vol. of 10 mM Tris/HCl (pH 7.8) in 0.2 M NaCl. The supernatant obtained by centrifugation at 105 000 g for 45 min was subjected to S-hexylglutathione–Sepharose affinity chromatography and the GST forms were eluted with 5 mM S-hexylglutathione/10 mM Tris/HCl (pH 7.8)/0.2 M NaCl, as described by Guthenberg and Mannervik [18]. GST 3-3 from control KS and NC type rats was further purified by chromatofocusing (pH 10.5–7.0), as described previously [6].

Assay for GST activity

GST activity was assayed using CDNB as a substrate by the method of Habig et al. [19]. The supernatant centrifuged at 105 000 g for 45 min was used as the enzyme source.

Electrophoresis and immunoblotting

SDS/PAGE was performed by the method of Laemmli [20]. Protein was stained with Coomassie Brilliant Blue R-250 (Nakalai Tesque). Immunoblotting was performed by the method of Towbin et al. [21], using polyclonal anti-HSP90 β and anti-ASK1 (where ASK1 stands for apoptosis signal-regulating kinase 1) antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.).

Separation of GST subunits by HPLC

Individual GST subunits were separated by HPLC, essentially as described by Ostlund-Farrants et al. [22]. HPLC was performed using a μ -Bondasphere 5 μ C18 column (3.9 mm $\times$ 150 mm; Waters, Milford, MA, U.S.A.). The following gradient system

of solvent A (water) and solvent B [80% (v/v) acetonitrile and 0.1% trifluoroacetic acid] at the flow rate of 1.2 ml/min: 0–5 min, 100% solvent A; 5–15 min, 0–44% solvent B; 15–90 min, 44–74% solvent B was used for elution [6]. All runs were performed at 35 °C, and column effluent was monitored at 220 nm.

Treatment with DTT

GST forms eluted from S-hexylglutathione–Sepharose columns were mixed with 10 mM DTT/0.1 M Tris/HCl (pH 7.8) and then incubated at 25 °C for 30 min.

Proteins bound to KS type GST 3-3–Sepharose

GST 3-3 (10 mg each) purified from KS and NC type rat livers by chromatofocusing [6] was dialysed against 0.1 M NaHCO₃/0.5 M NaCl (pH 8.3; buffer A), and then incubated at 4 °C overnight with 20 ml Sepharose 4B activated by CNBr [23]. After blocking the resins with 1 M Tris/HCl (pH 8.0) at 4 °C overnight, they were washed with 0.1 M acetic acid/NaOH (pH 4.0) followed by 20 mM Tris/HCl (pH 7.8; buffer B), and set into columns (2 cm $\times$ 5 cm) equilibrated with buffer B. Liver supernatant preparations from KS or NC type rats were applied to these columns, and proteins were eluted with 0.2 M NaCl in buffer B. Column effluent was monitored at 280 nm and eluted proteins were analysed by SDS/PAGE and other methods. In some experiments, elution was performed with 5 μ M GA instead of 0.2 M NaCl. For this experiment, control KS type rat liver supernatant-derived bound fractions of KS type GST 3-3–Sepharose were used as the protein source. These samples were dialysed against buffer B, and then incubated with 100 μ l of 50% (v/v) suspension of KS or NC type GST 3-3–Sepharose for 30 min. After washing three times with buffer B, GST 3-3–Sepharose was incubated with 5 μ M GA in buffer B for 1 h. All incubations and elutions were performed at 4 °C.

N-terminal amino acid sequence analysis

Edman degradations were performed in a gas-phase protein sequencer (model 492; Applied Biosystems, Foster City, CA, U.S.A.). Phenylthiohydantoin derivatives were identified and quantified as reported previously [24].

RESULTS

Effects of CCl₄ administration on liver GST activities in NC and KS type rats

To examine whether NC and KS types of GST 3-3 of rat liver exhibit different sensitivity to hepatotoxic agents, one of them, CCl₄, was administered to both types of rats, and GST 3-3 remaining in the liver as well as liver GST activities was evaluated. As shown in Table 1, administration of CCl₄ (1.5 ml/kg body weight) to NC type rats resulted in approx. 30% loss of activity

Table 1 Comparison of GST activities after CCl₄ administration between NC and KS type rat livers

Different doses of CCl₄ (1.5, 2.25 and 3.0 ml/kg body weight) were administered to NC or KS type rats. Control rats received corn oil without CCl₄. GST activity (units/g of tissue) of liver supernatant after centrifugation at 105 000 g for 45 min was assayed with CDNB. The results represent the means $\pm$ S.E.M. Relative ratios towards control NC and KS type rats are shown in parentheses ***P* < 0.005, ****P* < 0.002, and *****P* < 0.0005 compared with control rats without CCl₄ administration. ‡*P* < 0.02, ‡‡*P* < 0.005 between GST activities of NC type and KS type rat livers at doses of 2.25 and 3.0 ml of CCl₄.

Rat	Dose of CCl ₄ (ml/kg body weight)...	GST activity (units/g of tissue)			
		0	1.5	2.25	3.0
NC type		344 $\pm$ 34 (100)*	244 $\pm$ 31 (71)**	196 $\pm$ 15 (57)***	155 $\pm$ 7 (45)***
KS type		314 $\pm$ 22 (100)	313 $\pm$ 53 (100)	232 $\pm$ 17 (74)***‡	197 $\pm$ 13 (63)***‡‡

Table 2 Unbinding of CCl₄-treated NC type rat liver GSTs to S-hexyl-GSH-Sepharose

Liver supernatant fractions obtained from NC and KS type rats with and without administration of CCl₄ (3.0 ml/kg body weight) were applied to an S-hexyl-GSH-Sepharose column (3 cm × 5 cm) equilibrated previously with 10 mM Tris/HCl (pH 7.8)/0.2 M NaCl. Unbound and bound fractions were assayed for GST activity towards CDNB. Percentage of activity value for the respective supernatants applied is given in parentheses.

Rat	CCl ₄ (ml/kg body weight)	Total GST activity (units)		
		Supernatant applied	Unbound	Bound
NC type	0	2250 (100)	238 (11)	1481 (65)
	3.0	1240 (100)	988 (80)	57 (5)
KS type	0	2290 (100)	184 (8)	1570 (69)
	3.0	1620 (100)	297 (18)	859 (53)

Figure 1 SDS/PAGE of eluates from S-hexylglutathione-Sepharose affinity chromatography of CCl₄-administered NC and KS type rat liver supernatants

SDS/PAGE (12.5 % gel) was performed and the protein was stained with Coomassie Brilliant Blue R-250. Lane M, molecular-mass markers; lanes 1 and 4, CCl₄-administered NC and KS type rat liver supernatants respectively (30 µg of protein each); lanes 2 and 5, fractions bound to S-hexylglutathione-Sepharose (30 µg of protein each) for NC and KS type rat livers respectively; lanes 3 and 6, unbound fractions (100 µg of protein each) for NC and KS type rat livers respectively. GST subunit 3 is indicated by an asterisk.

when compared with control NC type rats without CCl₄ treatment, whereas KS type rats exhibited almost the same activity as control KS type rats. Although at higher concentrations, dose-dependent loss of activity was observed for both types of rats, the degree of loss was higher in NC type rats. At CCl₄ doses of 2.25 and 3.0 ml/kg body weight, GST activity was significantly different for NC and KS type rats. When liver GST activities were expressed as units/mg of protein, NC type rats also exhibited lower activity after CCl₄ treatment when compared with KS type rats (results not shown). To identify GST forms involved in decreasing GST activities after CCl₄ administration, GST forms were purified from liver supernatant through S-hexylglutathione-Sepharose affinity chromatography and subjected to SDS/PAGE and HPLC analyses. Most of the GST activities of both KS and NC type rat livers without CCl₄ administration were confined to the column. In CCl₄-administered KS type rats, most of the activities were bound to S-hexylglutathione-Sepharose, whereas activities were not confined for NC type rat liver (Table 2).

SDS/PAGE of CCl₄-administered NC and KS type rat liver supernatants revealed that the protein content of GST subunits with molecular mass in the range 25–28 kDa was not significantly different for the two types (Figure 1, lanes 1 and 4), but bound

Figure 2 HPLC analysis of S-hexylglutathione-Sepharose-bound fractions of CCl₄-administered NC and KS type rat liver supernatants

Liver supernatants from control and CCl₄-administered NC type rats and those from KS type rats were subjected to S-hexylglutathione-Sepharose affinity chromatography, and the bound fractions were subjected to HPLC. Elution was performed as described in the Experimental section. (A, C) Results from control NC (A) and KS type (C) rat liver supernatants. (B, D) Results from CCl₄-administered NC (B) and KS type (D) rat liver supernatants. CCl₄ was administered at the dose of 3.0 ml/kg body weight. Numbers on peaks denote the respective GST subunits. Because of different retention times, NC and KS type GST subunits 3 are described as 3a and 3b respectively. Peak X in (B) was eluted before the NC type GST subunit 3 (3a) and peak Y in (B) was identified as glyoxalase I by N-terminal amino acid sequencing.

fractions derived from NC type rat liver showed decreased GST subunits 1 and 3/4, compared with those for KS type rat liver (lanes 2 and 5). These subunits were found in unbound fractions for CCl₄-administered NC type rat liver, but rarely found for KS type rat liver (lanes 3 and 6). Levels of bound fractions for GST subunit 2 were not different for NC and KS type rat livers.

HPLC analysis indicated that all GST subunits of bound fractions derived from NC and KS type rat livers were decreased after CCl₄ administration (Figure 2). Decrease in GST subunits 1 and 3 was more marked in NC type rat liver when compared with KS type rat liver. A peak corresponding to subunit 3 was rarely

Figure 3 Conversion of peak X of CCl₄-administered NC type rat into GST subunit 3 by DTT treatment

Liver supernatant from CCl₄-administered (3.0 ml/kg body weight) NC type rats was subjected to S-hexylglutathione–Sepharose affinity chromatography and the bound fractions were incubated (A) without or (B) with 10 mM DTT at 25 °C for 30 min. These samples were analysed by HPLC for alteration of GST subunits. Elution conditions were the same as in Figure 2.

detected in CCl₄-administered NC type rat liver, and a minor peak (named X in Figure 2B), preceding subunit 4, was observed. Peak X showed a different retention time from NC type subunit 3 (peak 3a) but a similar retention time as KS type subunit 3 (peak 3b). In CCl₄-administered NC type rat liver, another peak (named Y in Figure 2B), eluted at 35 min, was detected and was identified as glyoxalase I by N-terminal amino acid sequencing (results not shown). In our previous study [6], glutathiolated NC type subunit 3 at Cys¹⁹⁹ was shown to be eluted faster when compared with the unmodified subunit. This suggested that peak X may correspond to a glutathiolated NC type subunit 3. To examine this possibility, bound fractions derived from CCl₄-administered NC type rat liver containing peak X were treated with 10 mM DTT and then subjected to HPLC analysis. This treatment resulted in complete disappearance of peak X and an increase in NC type subunit 3 (Figure 3B, peak 3a). Other GST subunits were not altered after the treatment. These results suggested that peak X is probably a glutathiolated NC type subunit 3. These results indicated that most GST subunits 1 and 3 of NC type rat liver were affected by CCl₄ treatment and lost their binding affinity to S-hexylglutathione–Sepharose. Those GST forms recovered from unbound fractions, however, exhibited enzyme activity when assayed for GST activity (Table 2).

Comparison of proteins bound to KS and NC type GST subunit 3

To determine the factors contributing to the different sensitivities of NC and KS type GST 3-3 to CCl₄, we examined whether proteins showed any preferential binding affinity for either NC or KS type GST subunit 3-3. KS or NC type GST 3-3 was purified by chromatofocusing, and then conjugated to CNBr-activated Sepharose 4B as described in the Experimental section. Liver supernatants of control KS or NC type rats were applied to KS type GST 3-3–Sepharose column. After washing with 20 mM Tris/HCl (pH 7.8), bound proteins were eluted with 0.2 M NaCl

Figure 4 SDS/PAGE of rat liver proteins bound to KS and NC type GST 3-3–Sepharose

KS and NC type GST 3-3 were purified from control rat liver and coupled with Sepharose 4B activated by CNBr, as described in the Experimental section. Supernatant from KS and NC type rat livers was applied to each GST 3-3–Sepharose column. Bound proteins were analysed by SDS/PAGE. (A) KS or NC type rat liver supernatant was applied to KS type GST 3-3–Sepharose column (2 cm × 5 cm). Elution was performed with 0.2 M NaCl in 20 mM Tris/HCl (pH 7.8). SDS/PAGE was performed as described in Figure 1. Lane M, molecular-mass markers; lanes 1 and 4, KS and NC type rat liver supernatants (100 µg of protein) respectively; lanes 2 and 3, unbound (100 µg of protein) and bound fractions (30 µg of protein) of KS type GST 3-3–Sepharose for KS type supernatant respectively; lanes 5 and 6, unbound and bound fractions of KS type GST 3-3–Sepharose for NC type supernatant respectively. The band, indicated by an asterisk, with subunit molecular mass of 90 kDa was recovered in the bound fractions. (B) Supernatant from KS (lanes 1 and 3) and NC type rat livers (lanes 2 and 4) were applied to KS (lanes 1 and 2) and NC type GST 3-3–Sepharose (lanes 3 and 4). Proteins eluted with the buffer as described above were examined by SDS/PAGE. (C) Immunoblotting was performed using an anti-HSP90β antibody. Lanes were the same as in (B). The band, indicated by an asterisk, with subunit molecular mass of 90 kDa bound to KS type GST 3-3–Sepharose was stained with the antibody.

Figure 5 SDS/PAGE of protein eluted with GA from KS type GST 3-3-Sepharose

A protein with subunit molecular mass of 90 kDa, partially purified as described in Figure 2, was incubated with NC or KS type GST 3-3-Sepharose. After unbound proteins were removed, individual GST-Sepharose matrices were washed with 20 mM Tris/HCl (pH 7.8) and then eluted with or without 5 μ M GA in the same buffer. Lane M, molecular-mass markers; lane 1, purified protein used in the experiment (20 μ g of protein); lanes 2 and 3, proteins unbound to NC and KS type GST 3-3-Sepharose respectively; lanes 4 and 5, proteins eluted with GA from NC and KS type GST 3-3-Sepharose respectively; lanes 6 and 7, proteins eluted with 20 mM Tris/HCl (pH 7.8) from NC and KS type GST 3-3-Sepharose respectively.

Figure 6 Binding of ASK1 to both NC and KS type GST 3-3-Sepharose

Immunoblotting was performed using anti-ASK1 antibody. Lanes 1 and 2, KS and NC type liver supernatants (100 μ g of protein each) respectively; lanes 3 and 4, KS and NC type liver supernatants respectively, both bound to KS type GST 3-3-Sepharose (30 μ g of protein each); lanes 5 and 6, KS and NC type liver supernatants respectively, both bound to NC type GST 3-3-Sepharose (30 μ g of protein each); lanes 7 and 8, KS and NC type liver supernatants respectively, both unbound to KS type GST 3-3-Sepharose (100 μ g of protein each); lanes 9 and 10, KS and NC type liver supernatants respectively, both unbound to NC type GST 3-3-Sepharose (100 μ g of protein each).

in 20 mM Tris/HCl (pH 7.8), and then subjected to SDS/PAGE. As shown in Figure 4(A), a protein with molecular mass of 90 kDa was detected as the major band in bound fractions (band marked with asterisk, lanes 3 and 6), but not in unbound fractions (lanes 2 and 5). Other proteins with subunit molecular masses of 30, 32 and 55 kDa were observed in bound fractions (Figure 4B).

Furthermore, liver supernatant preparations from control KS and NC type rat livers were applied to KS and NC type GST 3-3-Sepharose columns (Figure 4B). A 90 kDa protein derived from either KS or NC type liver supernatant was bound to KS type GST 3-3-Sepharose (lanes 1 and 2), but not to NC type GST 3-3-Sepharose (lanes 3 and 4). Even after treatment with 10 mM DTT in 0.1 M Tris/HCl (pH 7.8), NC type GST 3-3-Sepharose did not retain the 90 kDa protein. When liver cytosol prepared with 30 mM potassium phosphate buffer (pH 7.4) was applied to a GST 3-3-Sepharose column previously equilibrated with the same buffer, the 90 kDa protein also exhibited preferential binding to KS type GST 3-3.

To identify the 90 kDa protein, N-terminal amino acid sequencing was performed. It revealed the protein to have the sequence Glu-Glu-Glu-Val-Glu-Thr-Phe-Ala, consistent with the sequence Glu⁸ to Ala¹⁵ of rat HSP90 β [25]. This protein was identified as HSP90 β by immunoblotting with anti-HSP90 β antibody (Figure 4C, lanes 1 and 2). Immunoblotting also supported that the protein was weakly bound to NC type subunit 3 (lanes 3 and 4). Because of negligible amounts, it was not possible to determine N-terminal amino acid sequences of other proteins bound to GST 3-3-Sepharose.

Effect of GA treatment on HSP90 β binding to GST subunit 3

In addition to HSP90 β , many proteins were eluted with 0.2 M NaCl in 20 mM Tris/HCl (pH 7.8) from KS type GST 3-3-Sepharose. This raised the possibility that HSP90 β did not bind directly to KS type GST subunit 3, but binds to the GST via other proteins. To rule out this possibility, we examined whether HSP90 β and other proteins could be eluted with 5 μ M GA from KS type GST 3-3-Sepharose. As shown in Figure 5, HSP90 β was detached and no other major proteins were eluted from KS type GST 3-3-Sepharose with GA (lane 5). These results indicated that HSP90 β was directly bound to KS type GST 3-3-Sepharose.

ASK1 binding to both KS and NC type GST subunit 3

Since mouse GSTM1-1, corresponding to rat GST 3-3, is reported to bind to ASK1 [26], we examined whether KS and NC type GST 3-3 showed different binding activities to ASK1. Similar to the binding experiment for HSP90 β , liver supernatant preparations from KS and NC type rats were applied to KS and NC type GST 3-3-Sepharose, and bound fractions eluted with 0.2 M NaCl in 20 mM Tris/HCl (pH 7.8) were subjected to immunoblotting with anti-ASK1 antibody. As shown in Figure 6, ASK1 was detected in bound fractions of KS and NC type GST 3-3-Sepharose (lanes 3-6), but not in unbound fractions (lanes 7-10), indicating that ASK1 was bound to both types of GST 3-3.

DISCUSSION

The present study revealed that GST 3-3 retained enzyme activity in KS type rat liver after CCl₄ administration, whereas NC type GST 3-3 lost affinity for S-hexylglutathione-Sepharose but exhibited catalytic activity when assayed with glutathione. These results indicated that KS and NC types of GST 3-3 from rat liver exhibited different susceptibilities to CCl₄ *in vivo*.

The KS type GST 3-3, and not the NC type, was shown to bind to HSP90 β . The interaction between KS type GST and HSP90 seemed to be specific, since a specific HSP90 inhibitor GA released their binding. Since replacement of Lys¹⁹⁸-Ser residues of GST 3-3 with Asn¹⁹⁸-Cys residues resulted in a loss of affinity for HSP90 β , the residues seemed important for its binding. HSP90 has been demonstrated to function as a chaperone to fold destabilized proteins, including heat-denatured citrate synthase [27]. These findings suggest that HSP90 interacts with KS type GST 3-3 and thereby protects it from inactivation or folds inactive forms to the native form, whereas NC type GST 3-3 is modified by CCl₄ because of lack of interaction with HSP90. Although not bound to S-hexylglutathione-Sepharose, NC type GST exhibited enzyme activity when assayed with glutathione, suggesting a reversible nature of the modification. This modified form, however, differs from NC type enzyme glutathiolated at Cys¹⁹⁹, because the glutathiolated form is bound to S-hexylglutathione-Sepharose [6]. Such a glutathiolated form was detected in CCl₄-administered NC type rat liver. Glutathiolation of NC type GST 3-3 was suggested due to its complete conversion into the unmodified subunit after DTT treatment (Figure 3). Furthermore, HPLC and mass chromatography analyses revealed the release of glutathione after DTT treatment, confirming the result obtained from glutathiolated NC type GST 3-3 in our previous study [6]. These results indicate that glutathiolation of NC type GST 3-3 occurs after CCl₄ administration *in vivo*.

There may be a difference in the susceptibility of NC and KS type rats to liver damage induced by CCl₄. Loss of liver GST

activity due to CCl₄ administration was more obvious in NC type rats than in KS type rats, and most GST activity of NC type rats was not bound to S-hexylglutathione–Sephadex. Glyoxalase I, an enzyme involved in the detoxification of a reactive carbonyl, methylglyoxal [28], was detected in CCl₄-administered NC type rat liver. Methylglyoxal binds to cellular proteins at their Lys, Arg and Cys residues [29,30], and the reaction with Cys residue is reversible [31]. Increase in glyoxalase I in CCl₄-treated NC type rat liver may suggest increased production of methylglyoxal. Thus the carbonyl may be partly involved in the production of modified NC type GST 3-3, which is not bound to S-hexylglutathione–Sephadex.

ASK1, a mitogen-activated protein kinase kinase kinase (MAPKKK) that activates c-Jun N-terminal kinase and p38 signalling cascades, is activated in response to various cytotoxic stresses and induces apoptotic cell death [32,33]. In the present study, since there was no difference in ASK1 binding to GST 3-3 between NC and KS type rats, ASK1 was not expected to contribute to the different susceptibilities to CCl₄ observed in the two types of rats.

In conclusion, KS and NC types of GST 3-3 in rat liver exhibited different susceptibilities to CCl₄ *in vivo*. KS type GST was bound to HSP90 β , whereas NC type GST did not. Interaction between KS type GST and HSP90 β , probably contributes to the protection of the GST form from inactivation by CCl₄.

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