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Abstract

Increased activities of liver glucose-6-phosphate dehydrogenase (G6PD, EC 1.1.1.49) and 6-phosphogluconate dehydrogenase (6PGD, EC 1.1.1.44) in the pentose phosphate cycle were accompanied with a depletion of reduced glutathione (GSH) following an intragastric administration of carbon tetrachloride (CCl4) to rats. Oxidized glutathione (GSSG) also decreased remarkably, keeping the GSSG: GSH ratio constant. No significant alteration of glutathione reductase (EC 1.6.4.2.), glutathione peroxidase (EC 1.11.1.9) and malic enzyme (EC 1.1.1.40) activities in the supernatant and gamma-glutamyl transpeptidase (gamma-GTP, EC 2.3.2.2) activity in the homogenate of the injured liver were observed. Furthermore, no marked difference in the GSH-synthesizing activity was found between control and CCl4-intoxicated liver. An intraperitoneal injection of GSH produced a significant increase in liver GSH content in control rats but not in CCl4-treated rats; G6PD activity was not affected. Intraperitoneal injections of diethylmaleate resulted in continuously diminished levels of liver GSH without any alteration of liver G6PD activity. In vitro disappearance of GSH added to the liver homogenate from CCl4-treated rats occurred enzymatically and could not be prevented by the addition of a NADPH-generating system. The results suggest that increased G6PD activity in CCl4-injured liver does not play an important role in the maintenance of glutathione in the reduced form and that the decreased GSH content in the injured liver might be caused by enhanced GSH catabolism not due to gamma-GTP.

KEYWORDS: G6PD, glutathione, GSH, GSSG, CCL, liver injury, diethylmaleate

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GLUTATHIONE METABOLISM AND GLUCOSE 6-PHOSPHATE DEHYDROGENASE ACTIVITY IN EXPERIMENTAL LIVER INJURY

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Abstract. Increased activities of liver glucose-6-phosphate dehydrogenase (G6PD, EC 1.1.1.49) and 6-phosphogluconate dehydrogenase (6PGD, EC 1.1.1.44) in the pentose phosphate cycle were accompanied with a depletion of reduced glutathione (GSH) following an intragastric administration of carbon tetrachloride (CCl_4) to rats. Oxidized glutathione (GSSG) also decreased remarkably, keeping the GSSG : GSH ratio constant. No significant alteration of glutathione reductase (EC 1.6.4.2.), glutathione peroxidase (EC 1.11.1.9) and malic enzyme (EC 1.1.1.40) activities in the supernatant and γ-glutamyl transpeptidase (γ-GTP, EC 2.3.2.2) activity in the homogenate of the injured liver were observed. Furthermore, no marked difference in the GSH-synthesizing activity was found between control and CCl_4-intoxicated liver. An intraperitoneal injection of GSH produced a significant increase in liver GSH content in control rats but not in CCl_4-treated rats ; G6PD activity was not affected. Intraperitoneal injections of diethylmaleate resulted in continuously diminished levels of liver GSH without any alteration of liver G6PD activity. *In vitro* disappearance of GSH added to the liver homogenate from CCl_4-treated rats occurred enzymatically and could not be prevented by the addition of a NADPH-generating system. The results suggest that increased G6PD activity in CCl_4-injured liver does not play an important role in the maintenance of glutathione in the reduced form and that the decreased GSH content in the injured liver might be caused by enhanced GSH catabolism not due to γ-GTP.

Key words : G6PD, glutathione, GSH, GSSG, CCl_4 liver injury, diethylmaleate.

While testing a series of compounds for their ability to transform the three microheterogeneous forms of G6PD in rat liver (1), we found that the faster moving components clearly appeared in CCl_4-injured liver, probably due to the decreased hepatic GSH levels (2). G6PD may have some physiological importance in keeping glutathione in the reduced form. The participation of the pentose

Abbreviations : G6PD, glucose 6-phosphate dehydrogenase ; 6PGD, 6-phosphogluconate dehydrogenase ; GSH, reduced glutathione ; GSSG, oxidized glutathione ; CCl_4, carbon tetrachloride ; γ-GTP, γ-glutamyl transpeptidase ; G6P, glucose 6-phosphate ; $NADP^+$, nicotic adenine dinucleotide phosphate, and NADPH, nicotinic adenine dinucleotide phosphate (reduced form).

phosphate pathway in the regulation of GSH levels has been reported in red cells and brain (3, 4). G6PD activity was shown to be markedly increased in CCl_4-injured liver (1, 2), but the physiological significance in glutathione metabolism has never been delineated (1).

There is some *in vitro* and *in vivo* evidence that the disappearance of GSH added to the liver homogenate is mainly due to the enzymatic oxidation associated with CCl_4-induced lipid hydroperoxide (5). These observations led us to study the metabolic relationship between GSH metabolism and G6PD activity in CCl_4-injured rats and to investigate whether GSH injections to CCl_4-injured rats can prevent the decrease in GSH content and after the elevation of G6PD activity in the damaged liver. The mechanisms of decreased GSH content in the injured liver were also explored from the aspects of the GSH-synthesizing and degrading systems.

MATERIALS AND METHODS

Animals. Male Sprague-Dawley rats weighing 150-200 g were used throughout the experiment. Rats were fasted and given only water for 48 h after an intragastric administration of 1 ml of 20 % CCl_4 solution in liquid paraffin per 100 g body weight. Control rats were treated with liquid paraffin alone. After 48 h the rats were fed freely with Laboratory Chow until sacrifice. Twenty mg/100 g body weight of GSH was intraperitoneally injected every 6 h for the initial 24 h and thereafter every 12 h for 1 to 4 days following CCl_4 treatment. Diethylmaleate was injected intraperitoneally twice at 0 and 24 h, each time at a dose of 0.1 ml per 100 g body weight.

Liver homogenate and supernatant. Liver GSH contents (average of 3 rats) were 20, 116, 148, 52, 101 and 150 μg/100 mg liver 3, 6, 24, 27, 33 and 48 h, respectively, following the diethylmaleate treatment. Liver hemogenate in 0.154 M KCl containing 5 mM Tris-HCl (pH 8.0) and 1 mM EDTA was used for the measurement of GSH in the standard incubation system and for that of γ-GTP activity. Particle-free supernatant was separated from the homogenate by centrifuging at 25000 g for 60 min and used for assays of various enzyme activities and determination of liver GSH contents.

Enzyme assays. The activities of G6PD, 6PGD and malic enzyme were determined as described earlier (6). The following enzymes were assayed glutathione reductase, by the method of Mize and Langdon (7) ; glutathione peroxidase, by the method of Pinto (8), and γ-GTP, by the method of Orlowski (9). The enzyme activities were expressed as mU/mg protein. The rate of GSH synthesis catalized by two ATP-dependent reactions, those involving γ-glutamylcysteine synthetase (EC 6.3.2.2) and glutathione synthetase (EC 6.3.2.3), was estimated by determining the release of inorganic phosphate in the presence of glutamate, cysteine and glycine according to Tateishi *et al.* (10). The supernatant used for this experiment was dialyzed against 0.01 M Tris-HCl buffer (pH 7.1) containing 1 mM EDTA. The results were expressed as nmoles of Pi released/min/mg protein. Protein contents were determined by the method of Lowry *et al.* (11).

Standard incubation system for determination of GSH disappearance in liver homogenate. An aliquot of 0.5 ml of either liver homogenate or supernatant was incubated with or without GSH (0.87 μmole for Experiment I and II in Table 3, and 1.09 μmoles for Experiment III) and other compounds (0.3 μmole of $NADP^+$, 4.95 μmoles of G6P or 19 μmoles of CCl_4) in 5 mM Tris-HCl

(pH 8.0) in a final volume of 1.5 ml for 30 min at 37 ℃ with constant shaking of 70 strokes per min. The reaction was terminated with 1.5 ml of 4 % metaphosphoric acid. GSH content was determined by the method of Owens and Belcher (12). The determination of GSSG content was made by the method of Tietze (13). The results were expressed as μg per 100 mg wet liver weight.

Chemicals. Nucleotides, sugar phosphates, phosphoenol-pyruvate, 5, 5'-dithiobis-(2-nitrobenzoic acid), diethylmaleate, GSH and GSSG were obtained from Sigma Chemical Co. The purified enzymes were the products of Boehringer Mannheim GmbH.

RESULTS AND DISCUSSION

Liver G6PD activity and GSH metabolism in CCl_4-intoxicated rats. After CCl_4 administration, the liver GSH concentration decreased gradually, reached a minimum 24 h after treatment and returned to the original concentration by the 4 th day (Fig. 1).

These changes in GSH content appeared to correlate inversely with marked increases in the specific activities of G6PD and 6PGD (Table 1). G6PD and 6PGD may function *in vivo* as regulators by increasing the low GSH levels by converting GSSG back to GSH coupled with NADPH generation from the pentose phosphate pathway. However, high GSSG levels were not found in CCl_4-injured liver even though the GSH level remained low 2 days after the CCl_4 administration (Table 1). Moreover, glutathione reductase, glutathione peroxidase and malic enzyme activities in CCl_4-injured liver did not change. Significant changes in

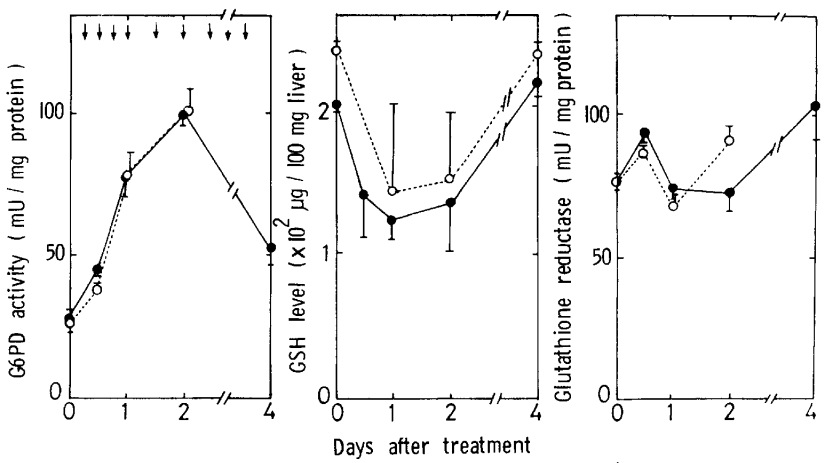

Fig. 1. Liver GSH, G6PD and glutathione reductase activities in CCl_4-injured rats treated with or without GSH. Rats were injected intraperitoneally with GSH (•) or saline (○) every 6 h for the initial 24 h and thereafter every 12 h for 1 to 4 days after the CCl_4 injection, as indicated by the arrows. Rats were sacrificed at the time indicated. Four rats were used in all groups. Vertical lines indicate the standard error of the mean. Other details are described under Materials and Methods.

TABEL 1. METABOLISM OF GLUTATHIONE IN CCL_4-INJURED RAT LIVER

	Control	CCl_4-injured
GSH (μg/100 mg liver)	187 ± 38 (4)	118 ±28 (3)*
GSSG (μg/100 mg liver)	2 ± 1 (4)	1 ± 1 (3)
Glutathione reductase	77 ± 1 (4)	73 ± 6 (4)
Glutathione peroxidase	719 ±26 (3)	800 ± 57 (3)
GSH synthesis (nmoles Pi released /min/mg protein)	22 ± 4 (3)	21 ± 10 (3)
γ-GTP**	1.3 ± 0.3 (3)	1.3 ± 0.6 (3)
G6PD	30 ± 2 (4)	96 ± 3 (4)*
6PGD	53 ± 2 (4)	71 ± 2 (4)*
Malic enzyme	11 ± 1 (4)	10 ± 1 (4)

Enzyme activity was expressed as milliunits (mU)/mg protein. Values are given as the mean ± SEM with the number of rats shown in parentheses. The asterisks (*) denote $p < 0.05$ over the controls. The values were obtained 24 h after CCl_4 treatment. ** γ-GTP activity was determined using the liver homogenate and expressed as mU/mg homogenate protein.

γ-GTP, an important GSH-degrading enzyme, activity in the liver homogenate were not observed in CCl_4-treated rats. No marked difference in the overall activities of GSH synthesis from the three constituent amino acids, glutamate, cysteine and glycine, was found in normal and CCl_4-injured liver (Table 1). Most amino acid levels increased in the injured liver (14). The Km value of the GSH-synthesizing system for cysteine (2.5×10^{-3} M) is known to be higher than hepatic cysteine levels under physiological conditions, but the increased amino acid levels in the injured liver may favor GSH synthesis. Another regulating factor of GSH synthesis is ATP. ADP and AMP are reported to inhibit one or both of GSH-synthesizing reactions (15). The ATP/ADP ratio, since it does not decrease in CCl_4-injured liver (16), can not contribute to the lowering of the GSH level.

Further study was carried out to elucidate whether the diminished GSH level can initiate an increased G6PD activity or not. However, the diminished content of liver GSH following intraperitoneal injections of diethylmaleate failed to change liver G6PD activity for the 48 h of the experiment. A previous communication (10) also reported that actinomycin D inhibited the dietary induction of liver G6PD without affecting liver GSH content.

Although CCl_4 administration to rats is known to produce a decrease in the NADPH/$NADP^+$ ratio (17), corresponding alterations in the GSH/GSSG ratio did not occur. This suggests that the glutathione reductase reaction is not at quilibrium. The Km of rat liver glutathione reductase with respect to NADPH and GSSG has been reported to be 3 and 55 μM, respectively (7). The concentration of NADPH and GSSG in the normal liver is approximately 300 (18) and 60 μM (19), respectively, and 42 h after CCl_4 administration the liver NADPH level decreased only about 45 % of the normal level (17). It is therefore apparent that

the rate of the GSSG reductase modulated reaction is almost entirely dependent on liver GSSG content and not the NADPH level.

From these observations, it may be concluded that G6PD does not participate in the maintenance of glutathione in the reduced state. Furthermore, the decreased GSH level in the damaged liver is probably caused by enhancement of GSH catabolism, not by impairment of the GSH-synthesizing mechanism.

In vivo effect of GSH injection on G6PD activity and glutathione metabolism in CCl_4-injured rat liver. The intraperitoneal injection of GSH produced a significant increase in the liver GSH content ($p < 0.05$) in control rats but not in CCl_4-treated rats (Table 2). The specific activities of G6PD and glutathione reductase were not altered throughout the period studied (Fig. 1). In CCl_4-injured rats injected with GSH, serum GPT activity was slightly lower than in the rats without the GSH injection, although the difference was not significant. The loss of GSH added to liver homogenate is known to be mainly due to its enzymatic oxidation to GSSG (20, 21) by glutathione peroxidase (22), xanthine oxidase (21) or other related enzymes associated with the formation of CCl_4-induced lipohydroperoxide (20, 22). Christophersen suggested (23) that as a free radical scavenger GSH alleviates CCl_4 toxicity by coupling to lipoperoxide through lipid peroxidation in the microsomal fraction. If GSH is oxidized to GSSG by the coupling reaction, neither total glutathione nor the GSH level should decrease since the reduction of GSH from GSSG occurs rapidly depending upon the concentration of GSSG formed. Our observations that there was a marked decrease in the total glutathione pool in the damaged liver led us to examine the disappearance of GSH from the liver homogenate *in vitro.*

In vitro enzymatic disappearance of GSH with the liver homogenate from CCl_4-injured rats. The rate of GSH disappearance with the liver homogenate from CCl_4-intoxicated rats was much higher than with that from control rats (Table 3). The boiled homogenate and particle-free supernatant, even from the damaged liver,

TABLE 2. EFFECT OF GSH INJECTION ON LIVER G6PD ACTIVITY AND GSH LEVELS IN CCL_4-TREATED RATS

Treatment (No. of rats)	GSH injection	Serum GPT (KU)	Liver G6PD (mU/mg protein)	Liver GSH (μg/100 mg liver)
None (4)	−	26 ± 8	30 ± 6	201 ± 2
None (4)	+	33 ± 5	31 ± 5	242 ± 6*
CCl_4 (8)	−	1430 ± 186	94 ± 15	136 ± 33
CCl_4 (8)	+	1182 ± 262	108 ± 24	140 ± 34

Rats were intraperitoneally injected with GSH or saline alone as described under Material and Methods. Rats were sacrificed 24 h following CCl_4 treatment. *$p < 0.05$

TABLE 3. REDUCTION OF GSH CONCENTRATION WITH LIVER HOMOGENATE OR SUPERNATANT FROM CONTROL AND CCL_4-TREATED RATS

Liver preparation	Initial GSH (μmoles)	GSH removed (%)
I. Supernatant		
"Control"	1.47	3
"CCl_4"	1.28	7
II. Homogenate		
"Control"	0.62	6
"Control"	1.49	9
"Control" + $NADP^+$ + G6P	1.49	5
"Control" + CCl_4	0.62	21
"CCl_4"	1.30	79
"CCl_4" + $NADP^+$ + G6P	1.30	68
"CCl_4 (boiled)"	1.30	6
III.		
GSH	1.09	1
GSH + CCl_4	1.09	4

"Control" and"CCl_4" were the liver supernatant or homogenate from 24 h-starved rats treated with paraffin alone and from CCl_4-treated rats, respectively. Rats were sacrificed 24 h after the treatment. Initial GSH indicates the sum of endogeneous GSH contained in the liver preparation and exogeneous GSH added to the system. In Experiment III, the incubation was carried out without the liver homogenate or supernatant.

however, had little ability to lower the GSH content, suggesting that the disappearance of GSH occurs with the subcellular particles and may be mainly enzymatic in nature. The possibility of direct GSH conjugation with CCl_4 seems impossible. Using the dialyzed liver supernatant from starved rats, the stoichiometric formation *in vitro* of GSH from GSSG can be obtained only in a complete system with $NADP^+$ and G6P. Therefore, if the disappearance of GSH in CCl_4-intoxicated liver is mainly due to the accelerated oxidation to GSSG and diminished reduction of GSSG during the incubation of the homogenate under aerobic condition, GSH disappearance should not be enhanced in the presence of a NADPH-generating system. Indeed, the results indicated that $NADP^+$ and G6P hardly affected the rate of GSH disappearance. This suggests that there may exist another mechanism for GSH removal in the case of injured liver. The GSH disappearance increased with the time of incubation to 30 min, with increased concentrations of the homogenate to 75 μg/1.5 ml and with increasing temperatures up to 50 ℃ (data not shown). The incubation of the normal liver homogenate with CCl_4 enhanced the reduction of GSH concentration (Table 3), suggesting that the disappearance of GSH in the homogenate may be coupled to an early event in CCl_4-liver damage. It has been determined by measuring the malonaldehyde and

diene conjugation absorption that liver microsomal lipid peroxidation occurs extremely rapidly, within 5 min following CCl_4 administration both *in vivo* and *in vitro* (24). It is also known that lipid hydroperoxide in the liver increased 3-fold 2 h after the administration of CCl_4 to rats and was still high 24 h after the treatment (25). Several liver enzymes are known to specifically require GSH as a substrate for the conjugation reaction (26). Therefore, there is a possibility that GSH conjugates enzymatically with some unknown intermediates in the chain of reactions related to CCl_4 toxicity. The exact mechanisms of the GSH-degrading system in the injured liver are now under study.

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