Variation of Cysteine Level by Chlorobenzene-Induced Perturbation of Glutathione Metabolism in Rat Liver

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(Received June 16, 1984)

Summary Effects of chlorobenzene-induced alteration in glutathione levels on cysteine metabolism in rat liver were investigated. Male Wistar rats were intraperitoneally injected with chlorobenzene (0.2, 0.5, 1.0 or 2.0 mmol/kg body weight). Both hepatic glutathione and cysteine levels were dose-dependently decreased by the chlorobenzene 6 h after the injection. However, at 24 h, the glutathione in the rats with chlorobenzene increased significantly as compared to that in the rats without chlorobenzene. Concomitant to the elevation in glutathione levels, hepatic glutathione synthesis activities were increased by the chlorobenzene by 68–111%. On the other hand, no significant difference between the rats with and without chlorobenzene was observed as regards cysteine levels at 24 h. Hepatic glutamate, glycine, methionine and serine levels were unaltered but hepatic taurine levels were significantly decreased by the chlorobenzene at both 6 and 24 h. Chlorobenzene administration had no effect on hepatic cystathionine synthase and cystathionase activities. These results indicate that a transient loss of hepatic glutathione, caused by the administration of chlorobenzene, resulted in an acceleration of glutathione synthesis and an increase of cysteine demand in the liver.

Key Words cysteine, glutathione, taurine, glutathione synthesis, cysteine degradation, cysteine synthesis, chlorobenzene

In xenobiotics metabolism, one of the functions of hepatic glutathione is to conjugate with electrophilic foreign compounds or their metabolite enzymatically (1). When animals are administered with these compounds, hepatic glutathione transiently decreases with formation of glutathione-S derivatives and then increases to a significantly higher level than the initial one (2–4). The glutathione-S derivatives formed are further metabolized to mercapturic acid *via* cysteine-S derivatives in kidney or are directly excreted into bile or urine (5). Among these compounds,

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chlorobenzene has been widely used as a solvent and as a chemical intermediate in the chemical industry. There remains a potential risk for people engaged in the manufacture and the handling of this compound. In a previous report (6), we indicated that activities of hepatic glutathione synthesis and of glutathione reductase in rats were elevated by intraperitoneal injection of chlorobenzene. And we also observed the variation of hepatic cysteine levels in chlorobenzene-treated rats. However, there is little information to hand on the metabolism of hepatic cysteine and its related compounds in xenobiotics-administered animals. In the present study, to clarify the effects of xenobiotics-induced perturbation of glutathione metabolism on hepatic cysteine synthesis and degradation, levels of hepatic sulfur-containing amino compounds and activities of enzymes involved in the transsulfuration pathway were measured in rats injected with chlorobenzene.

MATERIALS AND METHODS

Male 6-week-old Wistar rats weighing 140 to 160 g were housed in stainless-steel wire cages at a temperature of 22–24°C and a humidity of 60% in a room with a controlled 12-h light (08:00–20:00 h)-dark cycle, and were fed on a commercial stock diet (Type MF, Oriental Yeast Co., Tokyo) and given drinking water *ad libitum*. Contents (%) of protein and sulfur amino acids in the stock diet were as follows: protein, 24.0; methionine, 0.28; cystine, 0.30. After 5 days feeding, the rats were intraperitoneally injected with chlorobenzene (0.2, 0.5, 1.0 or 2.0 mmol/kg body weight) dissolved in olive oil and were sacrificed by decapitation 6 and 24 h after the injection. Control rats were injected with olive oil without chlorobenzene and all the injections were made at 10:00 h.

Immediately after the death of the animals, the livers were excised rapidly, washed, blotted and weighed. An approximate 1 g portion of the liver was homogenized in 4 volumes of 10% trichloroacetic acid (TCA). The TCA-soluble fraction obtained by centrifugation was assayed for total glutathione, cysteine, taurine and other amino acids. Total glutathione was measured by the recirculating assay using 5,5'-dithio-bis (2-nitrobenzoic acid) and glutathione reductase [EC 1.6.4.2] (from yeast, Sigma type III) (7). Cysteine was determined by the acid-ninhydrin method (8), and taurine and other amino acids were determined by Hitachi 835 amino acid autoanalyzer.

Another 1 g portion of the liver was homogenized in 9 volumes of saline and the homogenate was used for enzyme assay. Glutathione synthesis activity was measured according to the method of Mooz and Meister (9) as modified previously (6). Although two different enzymes are known to be involved in the synthesis of glutathione, synthetic activity was not characterized by alterations in individual enzyme activities but by the glutathione-synthesizing capacity of an enzyme system. Cystathionine synthase [EC 4.2.1.22] activity was assayed by colorimetric determination of the cystathionine formed (10). Cystathionase [EC 4.2.1.15] activity was assayed using L-homoserine as a substrate (11). Protein was

determined by the method of Lowry *et al.* (12) with bovine serum albumin as a standard.

Experimental data were statistically tested by variance analysis or by Student's *t*-test (13).

RESULTS AND DISCUSSION

Figure 1 depicts hepatic total glutathione levels in the rats 6 and 24 h after injection. The glutathione levels of the rats injected with chlorobenzene were significantly lower at 6 h and higher at 24 h than those of the control rats. The magnitude of the variation in glutathione levels was apparently dependent on the level of chlorobenzene injected. Recently, Ogata and Shimada have reported that 21% of the chlorobenzene injected into rats intraperitoneally was found in urine as *p*-chlorophenylmercapturic acid (14). The decrease of hepatic glutathione at 6 h implies a conjugation of chlorobenzene-metabolite with reduced glutathione, which is the first step in the formation of mercapturic acid. In Table 1 are shown hepatic glutathione synthetic activities 24 h after injection. The synthetic activities of the

Fig. 1. Alteration of hepatic total glutathione content in rat, 6 (○) and 24 (●) h after intraperitoneal injection with chlorobenzene. After being fed on a commercial stock diet for 5 days, rats were injected with graded levels of chlorobenzene intraperitoneally. The rats were treated 6 and 24 h after the injection and hepatic total glutathione was determined by recirculating assay (7). Circles and bars express means and SEM ($n=5$), respectively. Circles in the same frame not sharing a common superscript differ significantly ($p<0.05$).

Table 1. Elevation of hepatic glutathione synthesis activity by injection with chlorobenzene in rat.

After being fed on a commercial stock diet for 5 days, rats were intraperitoneally injected with chlorobenzene dissolved in olive oil. Glutathione synthesis activities in the rats 24 h after the injection were measured as described in MATERIALS AND METHODS.

Chlorobenzene injected (mmol/kg body weight)	Glutathione synthesis activity (unit/mg soluble protein)
0	0.57 ± 0.10^a
0.2	1.05 ± 0.16^b
0.5	1.10 ± 0.22^b
1.0	0.96 ± 0.06^b
2.0	1.20 ± 0.08^b

A unit is 1 nmol of glutathione formed per min. Values are means $\pm$ SEM ($n=5$); means in the same column not sharing a common superscript differ significantly ($p<0.05$).

chlorobenzene-treated rats were 68–111% higher than those of the control rats irrespective of the level of chlorobenzene injected. This indicates that synthetic activities were compensatorily elevated by the transient decrease in the glutathione level.

It is well known that hepatic glutathione levels increase after an initial transient decrease caused by an administration of reagents which conjugate with or oxidize glutathione (2–4,15). In addition, it has been reported that the occurrence of increased glutathione levels may reflect the increase in synthesis of γ -glutamylcysteine synthase [EC 6.3.2.2] *de novo* in rats injected with selenite, which is an oxidizing reagent for glutathione (16). The elevation of the activity of glutathione synthesis caused by the administration of chlorobenzene whose metabolite conjugate with glutathione, is thought to occur *via* an increase in γ -glutamylcysteine synthase protein.

Figure 2 shows hepatic cysteine levels in the rats 6 and 24 h after injection. Similarly to glutathione, the chlorobenzene injection caused a dose-dependent decrease in cysteine levels at 6 h. Glutathione regulates its own synthesis by inhibiting the γ -glutamylcysteine synthase reaction (17). It is considered that the absence of feedback regulation was caused by the decrease of glutathione levels and consequently the increase in utilization of the sulfur amino acid for the glutathione synthesis occurred. On the other hand, no difference between the cysteine levels in the chlorobenzene-administered rats and those in the control rats was observed at 24 h while the glutathione synthesis activity and the glutathione level were elevated by chlorobenzene administration. This implies that factors other than the alteration in synthetic activity contribute to the regulation of cysteine levels.

In Table 2 are summarized the contents of some amino acids and taurine in the livers of rats with or without 2.0 mmol/kg of chlorobenzene. The levels of glutamate and glycine, being two other components of glutathione, were not changed by the

Fig. 2. Alteration of hepatic cysteine content in rat, 6 (○) and 24 (●)h after intraperitoneal injection with chlorobenzene. Animals were the same as in Fig. 1. Hepatic cysteine was measured by the acid-ninhydrin method (8). Circles and bars express means and SEM ($n=5$), respectively. Circles in the same frame not sharing a common superscript differ significantly ($p<0.05$).

Table 2. Levels of amino acids and taurine in liver of rat with or without chlorobenzene.

Rats were fed on a commercial stock diet for 5 days and then injected with olive oil containing 2.0 mmol/kg body weight of chlorobenzene. Individual amino acids and taurine were determined using an amino acid autoanalyzer.

	6 h after injection		24 h after injection	
	Control	Chlorobenzene	Control	Chlorobenzene
Glutamate	2.54 ± 0.17	$3.24 \pm 0.46^{\text{NS}}$	2.22 ± 0.14	$2.48 \pm 0.45^{\text{NS}}$
Glycine	1.44 ± 0.09	$1.60 \pm 0.18^{\text{NS}}$	1.52 ± 0.29	$1.51 \pm 0.30^{\text{NS}}$
Methionine	0.66 ± 0.10	$0.60 \pm 0.06^{\text{NS}}$	0.43 ± 0.05	$0.52 \pm 0.06^{\text{NS}}$
Serine	0.77 ± 0.02	$0.83 \pm 0.05^{\text{NS}}$	0.46 ± 0.05	$0.53 \pm 0.12^{\text{NS}}$
Taurine	2.14 ± 0.25	$1.10 \pm 0.06^{**}$	2.88 ± 0.70	$0.98 \pm 0.10^*$

Values ($\mu\text{mol/g}$ wet weight) are means $\pm$ SEM ($n=4$). Significant difference from control group at: **, $p<0.01$; *, $p<0.05$; $^{\text{NS}}$, $p>0.1$ (not significant).

Table 3. Effect of chlorobenzene injection on hepatic cystathionine synthase and cystathionase activities in rat.

Rats were injected with chlorobenzene (2.0 mmol/kg body weight) intraperitoneally, and were sacrificed 24 h after the injection. Cystathionine synthase and cystathionase were assayed by the methods of Matsuo and Kashiwamata, respectively.

	Control	Chlorobenzene
Cystathionine synthase (nmol cystathionine formed per min per mg protein)	13.8 ± 0.7	15.6 ± 1.4
Cystathionase (nmol α -ketobutyrate formed per min per mg protein)	44.3 ± 2.5	44.3 ± 1.3

Values are means $\pm$ SEM ($n=4$).

chlorobenzene; only cysteine levels varied with glutathione levels. This may owe to the smaller size of the hepatic cysteine pool. The taurine contents were decreased by injection with chlorobenzene. The pathway of the oxidative degradation in which cysteine is metabolized to taurine *via* cysteine sulfinic acid is enhanced by dietary supplementation with methionine or cysteine (18–20); hepatic cysteine levels regulate cysteine dioxygenase [EC 1.13.11.20] activity (21). The lowered levels of hepatic cysteine may depress cysteine degradation and result in the low hepatic taurine levels in the chlorobenzene-treated rats.

Cysteine is biosynthesized from methionine *via* homocysteine and cystathionine, and serine also takes part in this transsulfuration. In Table 2, methionine and serine levels are also listed. Additionally, the activities of hepatic cystathionine synthase and cystathionase, two major enzymes catalyzing transsulfuration, are shown in Table 3. Both the levels of the two amino acids and the activities of the two enzymes were unaltered by the treatment with chlorobenzene. It seems that the transsulfuration was not enhanced by the decrease in cysteine levels.

The results obtained in this study indicate that a depression of the oxidative degradation of cysteine might be caused by the increase of the cysteine demand resulting from the chlorobenzene-induced loss of hepatic glutathione. This supports a hypothesis proposed by Kohashi *et al.*; cysteine dioxygenase may function as a valve for adjusting intracellular pools of sulfur amino acids involving glutathione (18). On the other hand, the enhancement of the transsulfuration pathway was not observed in the xenobiotics-administered rats. However, it has been reported that cystathionine synthase is inhibited by cystine *in vitro* and that the activity of this enzyme is lowered by dietary supplementation with cystine (22). Accordingly, the possibility that an acceleration of cysteine biosynthesis from methionine is brought about by lowered cysteine levels remains. In the present study, we did not examine an uptake of cysteine into hepatocytes. Thor *et al.*

observed that the rate of uptake of cysteine was moderately enhanced in hepatocytes with a lowered level of glutathione as compared to cells with normal glutathione concentrations (23). It is necessary to investigate further the uptake of cysteine in xenobiotics-administered rats.

The authors are grateful to Prof. Kyoden Yasumoto, Dr. T. Suzuki and Mr. T. Higasa of the Research Institute for Food Science, Kyoto University, for their useful advise in the measurement of amino acids. This work was supported by Grant-in-Aid No. 59770409 for Encouragement of Young Scientists from the Ministry of Education, Science and Culture of Japan.

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