Effect of glycyrrhizin on glutathione S-transferase activity and glutathione in rats intoxicated with carbon tetrachloride

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Abstract

The restoring effect of glycyrrhizin (GLZ) on glutathione S-transferase (GST) activity in Y-protein fraction and glutathione (GSH) content in liver was examined in rats intoxicated with carbon tetrachloride (CCl_4) and the protective effect of GLZ was similarly examined in rats given GLZ and CCl_4 concomitantly. GST activities toward the substrate 1-chloro-2,4-dinitrobenzene (CDNB) and 1,2-dichloro-4-nitrobenzene (DCNB) were decreased significantly ($p<0.01$) by CCl_4-intoxication, but were restored to the control level by GLZ administration (4 mg/kg/day for 6 weeks). A similar restorative effect of GLZ was also observed for Y-protein and GSH contents, which were decreased significantly ($p<0.01$) by the intoxication. In the case of GLZ and CCl_4 co-administration, GST activities toward CDNB and DCNB and GSH content remained at the control level, but Y-protein content decreased significantly ($p<0.05$) from the control. GLZ itself did not have any effect on GST, GSH, and Y-protein in control rats.

Thus, GLZ has significant restorative and protective effects for GST activity, and GSH content in liver, while there was a restorative but not a protective effect in the case of Y-protein content.

Key words glycyrrhizin, CCl_4-intoxicated rat, glutathione S-transferase, Y-protein, glutathione.

Abbreviations AST, alanine transaminase ; ALT, aspartate transaminase ; BSP, sulfobromophthalein ; CCl_4, carbon tetrachloride ; CDNB, 1-chloro-2,4-dinitrobenzene ; DCNB, 1,2-dichloro-4-nitrobenzene ; ECA, ethacrynic acid ; GLZ, glycyrrhizin ; GLZ-NH_4, monoammonium glycyrrhizinate ; GOT, glutamic oxaloacetic transaminase ; GPT, glutamic pyruvic transaminase ; GSH, glutathione ; GST, glutathione S-transferase ; *p*-NBC, *p*-nitrobenzyl chloride ; TPBO, *trans*-4-phenyl-3-buten-2-one.

Introduction

Glycyrrhizin (GLZ) has frequently been used in the treatment of chronic hepatitis,[1] allergic disorder,[2-4] inflammation,[2,5] and gastric ulcers.[6] GLZ has been reported to have the detoxifying actions for tetrodotoxin,[7] strychnine,[8] tetanal toxin,[9] and trimeresurus venom[10] in mice. Glutathione S-transferase (GST) and glutathione (GSH) participate in detoxication by GSH conjugate formation.[11-13] However, no data on the effects of GLZ upon GST and GSH have been reported so far.

Levi *et al*.[14] reported that Sephadex G75 gel filtration of rat liver supernate to which sulfobromophthalein (BSP) had been added gave three peaks of BSP-binding protein, designated as X, Y, and Z. Thereafter, Habig *et al*.[15] found that GST activity is present only in the Y-protein fraction. GST activity[16] and GSH content[17] in rat liver cytosol are reduced by carbon tetrachloride (CCl_4)-intoxication.

In the present study, we examined the restora-

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tive effect of GLZ on GST activity of Y-protein and GSH content in liver of rats intoxicated with CCl_4. Further, the protective effect of GLZ against CCl_4 was examined similarly by measuring GST activity and GSH content in GLZ- and CCl_4 co-administered rats.

Materials and Methods

Materials : Monoammonium glycyrrhizinate ($GLZ-NH_4$) was used as supplied by Minophagen Pharmaceutical Ind. Ltd. (Tokyo, Japan). Transaminase CII-test Wako (Wako Pure Chemical Ind. Osaka, Japan), glutathione reductase (Boehringer Mannheim GmbH, Germany), and nicotinamide-adenine dinucleotide phosphate, reduced form, (Oriental Yeast Co., Ltd., Tokyo, Japan) were purchased. All other reagents were commercial products of analytical grade.

Animals and treatments : Male Wistar rats, weighing 120-130 g, were used. During the experiments, the animals were kept in restraining cages with free access to water and a commercial pellet diet (Oriental Yeast Co., Ltd.) under normal housing conditions. CCl_4 in olive oil (3 : 4 v/v), 0.1 ml/100 g of body weight, was injected under the skin of the back twice a week (Monday and Thursday). CCl_4-intoxicated rats were produced by repeated injection of CCl_4 for 6 weeks.[18] Control rats were produced by repeated injection of equivalent amounts of olive oil. $GLZ-NH_4$ (4 mg/kg as GLZ) in 5% glucose solution or 5% glucose solution alone (1 ml each) was administered into the caudal vein daily for 6 weeks. The initial dose of CCl_4, olive oil, $GLZ-NH_4$ or 5% glucose solution was given on a Monday. The animals were divided into seven groups of five rats as follows : group I (control), the control rats ; group II (intoxication), the intoxicated rats ; group III (CCl_4+GLZ), the treatment with GLZ and CCl_4 was started on the same day (Monday) ; group IV (CCl_4→GLZ), GLZ administration to the intoxicated rats was started on the Monday after the last CCl_4 dosing ; group V (CCl_4→), the intoxicated rats were maintained without GLZ treatment for 6 weeks from the Monday after the last CCl_4 dosing ; group VI (normal), 5% glucose solution alone was administered ; group VII (GLZ-alone), GLZ alone was given. The abbreviations in parentheses are used to indicate the groups in the text. Rats of all groups were killed on the Monday after the last treatment in all cases in order to obtain blood and liver samples.

Preparation of liver supernatant fraction : Fifty % homogenate was prepared by the method of Iga *et al.*,[19] during which process, blood was also collected for the preparation of plasma samples. A portion of the homogenate was used for GSH determination. The remainder was centrifuged at 105,000 *g* for 120 min in a Hitachi 65p-7 ultracentrifuge (Hitachi Koki Co. Ltd., Tokyo, Japan) at 4℃. The supernatant fraction was immediately subjected to gel filtration after measuring the volume.

Elution of supernatant : Three ml of supernatant was applied to a Sephadex G-75 column (2.5×100 cm). Elution was performed with 0.01 M phosphate buffer (pH 7.4) using pump-driven downward flow (2 ml/40 min/tube) at 4℃ Aliquots of 200 μl from all tubes that might correspond to the Y-fraction[14] were assayed for GST activity using 1-chloro-2,4-dinitrobenzene (CDNB) as a substrate. All tubes showing GST activity were combined, freeze-dried at −80℃ and stored at −40℃ until required.

Analytical method : The dried Y-protein was dissolved in an appropriate volume of phosphate buffer (pH 7.4) and GST activity was determined according to the method of Habig *et al.*[20] using CDNB, 1,2-dichloro-4-nitrobenzene (DCNB), *trans*-4-phenyl-3-buten-2-one (TPBO), *p*-nitrobenzyl chloride (*p*-NBC), and ethacrynic acid (ECA) as substrates. Y-Protein and GSH concentrations in the liver homogenate were determined by the Lowry-Folin method[21] and the Ellman method,[22] respectively. Plasma alanine transaminase (ALT) and aspartate transaminase (AST) activities were determined by the pyruvate oxidase-*p*-chlorophenol method.[23]

Statistical analysis : All means are presented with their standard error (mean±S.E.). Student's *t* test was utilized to determine the significance of differences between the control and intoxicated or GLZ-treated groups.

Results

GST activities with the substrates CDNB, DCNB, TPBO, *p*-NBC and ECA in the Y-fraction of groups I-VII are shown in Table I. The activity with CDNB was higher than those with the other substrates in all groups, e.g., in group I (control), the activity with CDNB was approximately 60 times higher than that with DCNB, and 100-700 times higher than those with TPBO, *p*-NBC, and ECA. GST activities toward CDNB and DCNB in groups II (intoxication) and V ($CCl_4\rightarrow$) were decreased significantly ($p<0.01$ or 0.05) as compared with the control (approximately 56 and 68% of the control, respectively), while those with other substrates showed no significant difference among all groups. The absence of any significant difference in GST activities toward CDNB and DCNB in groups III (CCl_4+GLZ) and IV ($CCl_4\rightarrow$GLZ) indicates significant protective and restorative effects of GLZ on GST activity, respectively. GLZ does not have a significant inducing effect on any GST activity, because GST activities toward all substrates showed no significant difference among groups I (control), VI (normal), and VII (GLZ-alone).

Fig. 1 shows Y-protein contents in liver of groups I-VII. The Y-protein content decreased significantly ($p<0.01$ or 0.05) in groups II (intoxication), III (CCl_4+GLZ), and V ($CCl_4\rightarrow$) to approximately 65, 86, and 61% of the control, respectively. The significant decrease in group

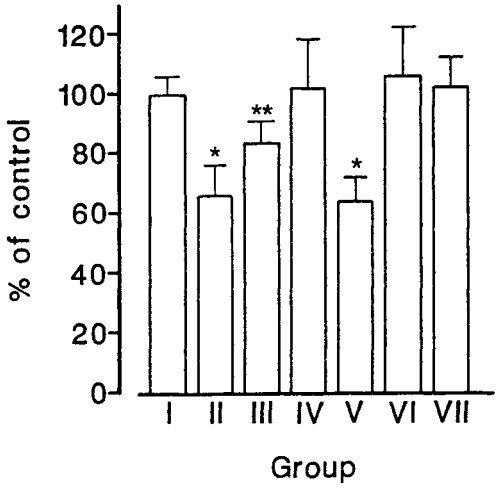

Fig. 1 Effect of glycyrrhizin on Y-protein content in rat liver. The mean (±S.E.) contents of Y-protein were 25.2 (±2.0), 16.4 (±4.8), 21.8 (±2.6), 25.8 (±6.0), 15.3 (±4.2), 28.2 (±5.6) and 25.6 (±4.2) mg/g liver in groups I, II, III, IV, V, VI and VII, respectively. Percentages were calculated from these data, taking the group I (control) value as 100%. For details of groups I-VII, see Table I. The bars indicate S.E..
*$p<0.01$ and **$p<0.05$ vs group I.

Table I Glutathione S-transferase activities[a] toward selected substrates in Y-fraction[b] of rat liver.

Substrate	Group[c]						
	I	II	III	IV	V	VI	VII
CDNB	1.020	0.570*	0.965	0.945	0.690*	0.957	1.095
	±0.196	±0.190	±0.095	±0.115	±0.190	±0.078	±0.125
DCNB (×10)	0.175	0.116*	0.150	0.140	0.132**	0.177	0.165
	±0.025	±0.028	±0.024	±0.032	±0.028	±0.033	±0.047
ECA (×10^2)	1.165	0.995	1.020	1.110	0.925	1.150	1.300
	±0.165	±0.124	±0.114	±0.228	±0.137	±0.181	±0.142
p-NBC (×10^2)	0.765	0.716	0.734	0.805	0.710	0.707	0.780
	±0.085	±0.048	±0.090	±0.076	±0.097	±0.122	±0.170
TPBO (×10^2)	0.145	0.128	0.140	0.153	0.133	0.136	0.150
	±0.025	±0.037	±0.032	±0.044	±0.028	±0.042	±0.034

Results are given as the mean±S.E. of five rats.
[a] μmol/min/mg protein. Determined by the method of Habig *et al.*[20]
[b] Obtained by gel filtration of 105,000 *g* liver supernatant on a Sephadex G75 superfine column (2.5×100 cm). For details, see the text.
[c] I, control; II, CCl_4-intoxication; III, CCl_4+GLZ; IV, $CCl_4\rightarrow$GLZ; V, $CCl_4\rightarrow$; VI, normal; VII, GLZ-alone. For details, see the text.
*$p<0.01$ and **$p<0.05$ vs group I.

III shows no protective effect of GLZ against CCl_4. From the comparison with group V, the absence of a significant decrease in Y-protein content of group IV (CCl_4→GLZ) shows a restoration effect of GLZ. The Y-protein content showed no significant difference among groups I, VI and VII, indicating that GLZ itself does not affect Y-protein content.

Table II shows GSH contents in liver of groups I-VII. The contents in groups II and V decreased significantly ($p<0.01$) from the control (approximately 59 and 65% of the control, respectively), but there was no significant difference between groups III and IV. This shows that there are significant protective and restorative effects of GLZ on GSH content. The absence of a significant difference among the control, normal, and GLZ alone groups I, VI and VII indicates that GLZ does not have an inducing effect on GSH content.

Table III shows the body weight, liver wet weight, and plasma AST and ALT activities in groups I-VII. There was no significant difference of body weight among groups I-VII, in spite of the different experimental durations of 6 (groups I-III, and VI-VII) and 12 (groups IV-V) weeks. The liver weight was significantly decreased ($p<0.01$) only in the intoxicated rats (group II), compared with the control. These results indicate a protective effect by GLZ and by the rats themselves after the intoxication, a restorative effect of GLZ, and no significant influence of the drug on liver weight within the GLZ-treatment period (6 weeks). Plasma AST and ALT activities in groups II and V increased significantly ($p<0.01$) compared with the control. In group III, only ALT activity increased significantly ($p<0.05$) from the control, but with a significant decrease as compared with those of groups II and V ($p<0.01$ or 0.05). This indicates a protective effect of GLZ against CCl_4. The AST and ALT activities in group IV showed no significant difference from those in the control, indicating an effect of GLZ treatment, in comparison with the result in group V. The two enzyme

Table II Glutathione (GSH) contents[a] in rat liver.

	Group[b]						
	I	II	III	IV	V	VI	VII
GSH, mg/g liver	2.098 ±0.493	1.234* ±0.360	1.645 ±0.394	1.752 ±0.282	1.360* ±0.353	2.168 ±0.437	2.058 ±0.320

Results are given as the mean±S.E. of five rats.
[a]Determined by the method of Ellman.[22]
[b]See Table I.
*$p<0.01$ vs group I.

Table III Patho-physiological changes in all groups.

	Group[a]						
	I	II	III	IV	V	VI	VII
Body weight (g)	267.5 ± 2.5	252.0 ± 2.5	265.0 ± 2.5	282.5 ± 3.0	270.0 ± 2.0	266.5 ± 3.3	267.5 ± 2.5
Liver wet weight (g)	12.9 ± 0.3	10.0* ± 0.4	12.2 ± 0.5	13.3 ± 0.3	12.8 ± 0.5	12.4 ± 0.4	12.1 ± 0.2
Plasma AST[b] (IU/l)	27.4 ± 2.1	391.6* ±31.5	40.1 ± 7.3	33.9 ± 4.1	82.9* ± 11.7	22.9 ± 5.8	28.0 ± 7.9
Plasma ALT[c] (IU/l)	8.8 ± 1.6	149.0* ± 13.7	19.0** ± 4.2	14.0 ± 3.4	33.6* ± 7.8	7.2 ± 1.9	11.6 ± 3.8

Results are given as the mean±S.E. of five rats.
[a]See Table I.
[b]Aspartate transaminase activity.
[c]Alanine transaminase activity.
AST and ALT activities were determined by the pyruvate oxidase-*p*-chlorophenol method.[23]
*$p<0.01$ and **$p<0.05$ vs group I.

activities showed no significant difference among the control, normal, and GLZ-alone groups I, VI, and VII, indicating that AST and ALT activities are not influenced by GLZ itself.

Discussion

Booth *et al*.[11] reported that GST activity in liver exists almost wholly in the soluble fraction (approximately 80%), where its level is 30-200 times higher than those in the heart, kidney, lung, spleen, and blood. Jakoby[12] reported that at least five different types of GST exist in rat hepatic supernatant. These enzymes show specific activities toward appropriate substrates.[12] Habig *et al*.[20] showed that GST activity toward substrate CDNB in rat liver is higher than those toward other substrates, DCNB, TPBO, *p*-NBC, and ECA. In this study too, the activity towards CDNB was higher than those with the other substrates in the control rats (Table I). Srivastava *et al*.[16] reported that rats treated with CCl_4 (twice a week for 14 days) exhibited a decrease of 46.4% in GST activity towards CDNB. An almost identical decrease was found in the intoxicated rats here (mean, 55.9%). The restorative and protective effects of GLZ were readily apparent in the GST activities toward CDNB and DCNB, but were not observed in the activities toward other substrates, which were lower than those toward CDNB and DCNB. The fact that the levels were lower could explain the lack of a significant decrease caused by the intoxication. A significant decrease of Y-protein in CCl_4-intoxicated rats has been reported by Sugiyama *et al*.[24] In this study, the decreased levels of Y-protein contents in groups II and V induced by the intoxication was restored by GLZ administration to the control level (Fig. 1). Overall, Y-protein content corresponded well to GST activity in all groups except group III (Table I and Fig. 1). As shown in Table II, GSH content (mean, 1.234 mg/g liver) in the intoxicated rats was almost identical with that (mean, 0.937 mg/g liver) found by Ferreyra *et al*.[17] and was restored to the control level by GLZ-treatment (group IV, Table II). The observed restorative effect on both GST activity and GSH content suggests a detoxifying action of GLZ (GSH-conjugation). A protective effect of the drug on GST activity and GSH content upon co-administration of CCl_4 and GLZ (group III) was indicated, but not on Y-protein content (Fig. 1). Consequently, Y-protein content in group III did not correspond to GST activities toward CDNB and DCNB. The reason for this is obscure. Plasma AST and ALT activities in the intoxicated rats (Table III) were almost identical with the GOT (230.4 IU/l as AST) and GPT (106.0 IU/l as ALT) activities in rats intoxicated with CCl_4 (16−18 times, twice a week) reported by Iga *et al*.[19] A restorative effect of GLZ on plasma AST and ALT activities increased by the intoxication was observed. A protective effect for both enzyme activities were also observed, but the effect for ALT activity was less than that for AST activity.

Thus, it was found that GLZ has significant restorative and protective effects on GST activities toward CDNB and DCNB in CCl_4-intoxicated rats, and also on GSH content. However, there was a restorative, but not a protective effect in the case of Y-protein content.

和文抄録

四塩化炭素 (CCl_4) 処理ラット肝におけるY-たん白分画中のグルタチオンS-トランスフェラーゼ (GST) 活性及びグルタチオン (GSH) 含量に対するグリチルリチン (GLZ) の回復効果を検討した。また，ラットへGLZとCCl_4を同時に投与し，CCl_4に対するGLZの予防効果についても検討した。1-クロロ-2,4-ジニトロベンゼン (CDNB) 及び1,2-ジクロロ-4-ニトロベンゼン (DCNB) を基質とするGST活性は，CCl_4処理により有意に低下した ($p<0.01$) が，GLZ投与 (4 mg/kg/day, 6 weeks) によりコントロールレベルまで回復した。また，CCl_4処理により有意に低下した ($p<0.01$) Y-たん白及びGSH含量においてもGLZによる回復効果が観察された。GLZとCCl_4の同時投与のケースにおいて，CDNBとDCNBに対するGST活性及びGSH含量はコントロールレベルに留まったが，Y-たん白含量は有意に低下した ($p<0.05$)。GLZそれ自体，コントロールラットにおけるGST活性及びGSH，Y-たん白含量への影

響はなかった。

かくして，GLZは肝GST活性，GSH含量の回復及び予防効果を有し，Y-たん白含量の回復効果も示したが，予防効果は見られなかった。

References

1) Suzuki, H., Ohta, Y., Takino, T., Fujisawa, T., Hirayama, C., Shimizu, N. and Aso, Y. : Therapeutic effects of stronger neo-minophagen C on human chronic hepatitis. *Igaku no Ayumi* **102**, 562-578, 1977.

2) Sotomatsu, S., Takahashi, Y., Hiroi, J., Namikata, A. and Okano, N. : Glycyrrhizin and skin. *Skin and Urology* **21**, 138-144, 1959.

3) Kuroyanagi, T., Kurusu, A., Sugiyama, H. and Saito, M. : Studies of experimental allergic hepatitis. 2. Effect of predonisolone and glycyrrhizin on allergic hepatic lesion. *Jpn. J. Med. Progress* **49**, 458-465, 1962.

4) Kuroyanagi, T. and Saito, M. : Effect of predonisolone and glycyrrhizin on passive transfer of experimental allergic encephalomyelitis. *Allergy* **15**, 67-74, 1966.

5) Yamamoto, S., Miyamoto, A., Awachi, Y. and Hiroshima, Y. : Anti-inflammatory activity of stronger Neo-Minophagen C by granulation tissue test. *J. Therapy* **45**, 157-158, 1963.

6) Takagi, K., Okabe, S. and Saziki, R. : A new method for the production of chronic gastric ulcer in rats and the effect of several drugs on its healing. *Jpn. J. Pharmacol.* **19**, 418-426, 1970.

7) Miyoshi, H. : Antidotal value of glycyrrhizin. *Jpn. J. Med. Progress* **39**, 1-7, 1952.

8) Kuboki, N. and Hoshizaki, K. : On the detoxicating effect of glycyrrhizin extracted from radix liquiritiae. *Sogo Igaku* **12**, 792-795, 1955.

9) Sindo, T. and Haga, K. : The effect of glycyrrhizin on the tetanal toxin-antitoxin neutralization. *Allergy* **2**, 332-337, 1954.

10) Sawai, Y., Makino, M. and Kawamura, Y. : Antitoxic action of glycyrrhizin against trimeresurus venom. *Minophagen Med. Rev.* **6**, 78-81, 1961.

11) Booth, J., Boyland, E. and Sims, P. : An enzyme from rat liver catalysing conjugations with glutathione. *Biochem. J.* **79**, 516-524, 1961.

12) Jakoby, W.B. : The glutathione S-transferase : a group of multifunctional detoxification proteins. *Adv. Enzyme.* **46**, 383-414, 1977.

13) Kaplowitz, N. : Physiological significance of glutathione S-transferases. *Am. J. Physiol.* **239**, G 439-444, 1980.

14) Levi, A. J., Gatmaintan, Z. and Arias, I.M. : Two hepatic cytoplasmic protein fractions, Y and Z, and their possible role in the hepatic uptake of bilirubin, sulfobromophthalein and other anions. *J. Clin. Inv.* **48**, 2156-2167, 1969.

15) Habig, W.H., Pabst, M.J., Fleischner, G., Gatmaitan, Z., Arias, I.M. and Jakoby, W.B. : The identity of glutathione S-transferase B with ligandin, a major binding protein of liver. *Proc. Nat. Acad. Sci. USA.* **71**, 3879-3882, 1974.

16) Srivastava, S.P., Singh, K.P., Saxena, A.K., Seth, P.K. and Ray, P.K. : In vivo protection by protein A of hepatic microsomal mixed function oxidase system of CCl_4-administered rats. *Biochem. Pharmacol.* **36**, 4055-4058, 1987.

17) Ferreyra, E.C., Villarruel, M.C., Bernacchi, A.S., Fernandez, G., Feros, O.M. and Castro, J.A. : Late preventive effects against carbon tetrachloride-induced liver necrosis of the calcium chelating agent Calcion. *Arch. Toxicol.* **63**, 450-455, 1989.

18) Tsurufuji, S. and Ohuchi, K. : Enzymatic activities of collagen metabolism in isolated hepatocytes. *J. Jpn. Biochem. Soc.* **44**, 721, 1972.

19) Iga, T., Sugiyama, Y., Yokota, M., Tomono, Y., Awazu, S. and Hanano, M. : Pharmacokinetic aspects of sulfobromophthalein transport in chronically carbon tetrachloride-intoxicated rats. *Biochem. Pharmaclo.* **26**, 1867-1875, 1977.

20) Habig, W.H., Pabst, M.J. and Jakoby, W.B. : Glutathione S-transferase, the first enzymatic step in mercapturic acid formation. *J. Biol. Chem.* **193**, 265-275, 1951.

21) Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. : Protein mesurement with the Folin phenol reagent. *J. Biol. Chem.* **193**, 265-275, 1951.

22) Ellman, G.L. : Tissue sulfhydryl groups. *Arch. Biochem. Biophys.* **82**, 70-77, 1959.

23) Ohkawa, J., Miyoshi, Y., Matsuura, H. and Misaki, H. : A new colorimetric determination of transaminase activity using pyruvate oxidase. *Jpn. J. Clin. Path.* supplement **26**, 7130-7139, 1978.

24) Suyama, A., Sugiyama, Y., Iga, T., Awazu, S. and Hanano, M. : The binding and conjugating abilities of the Y-fraction for sulfobromophthalein (BSP) in chronic carbon tetrachlorideintoxicated rats. *J. Pharmacobio-Dyn.* **2**, 105-112, 1979.