Biochem. J. (1983) **215**, 441–445
Printed in Great Britain

Reduced glutathione protection against rat liver microsomal injury by carbon tetrachloride

Dependence on O_2

Raymond F. BURK, Kuldeep PATEL and James M. LANE
Department of Medicine, Division of Gastroenterology and Nutrition, University of Texas Health Science Center, 7703 Floyd Curl Drive, San Antonio, TX 78284, U.S.A.

(*Received 6 April 1983/Accepted 27 July 1983*)

Rat liver microsomal membranes contain a reduced-glutathione-dependent protein(s) that inhibits lipid peroxidation in the ascorbate/iron microsomal lipid peroxidation system. It appears to exert its protective effect by scavenging free radicals. The present work was carried out to assess the effect of this reduced-glutathione-dependent mechanism on carbon tetrachloride-induced microsomal injury and on carbon tetrachloride metabolism because they are known to involve free radicals. Rat liver microsomes were incubated at 37°C with NADPH, EDTA and carbon tetrachloride. The addition of 1 mM-reduced glutathione (GSH) markedly inhibited lipid peroxidation and glucose 6-phosphatase inactivation and, to a lesser extent, inhibited cytochrome *P*-450 destruction. GSH also inhibited covalent binding of [^{14}C]carbon tetrachloride-derived ^{14}C to microsomal protein. These results indicate that a GSH-dependent mechanism functions to protect the microsomal membrane against free-radical injury in the carbon tetrachloride system as well as in the iron-based systems. Under anaerobic conditions, GSH had no effect on chloroform formation, carbon tetrachloride-induced destruction of cytochrome *P*-450 or covalent binding of [^{14}C]carbon tetrachloride-derived ^{14}C to microsomal protein. Thus, the GSH protective mechanism appears to be O_2-dependent. This suggests that it may be specific for O_2-based free radicals. This O_2-dependent GSH protective mechanism may partly underlie the observed protection of hyperbaric O_2 against carbon tetrachloride-induced lipid peroxidation and hepatotoxicity.

A GSH-dependent protein is present in rat liver microsomes that inhibits lipid peroxidation caused by ascorbate or NADPH in the presence of ADP-chelated iron (Christopherson, 1968; Burk, 1983). Evidence has been presented that suggests it functions by scavenging free radicals (Burk, 1983).

Carbon tetrachloride injures the microsome through free-radical-induced lipid peroxidation and direct free-radical attack. Most of the injury is considered to be related to $CCl_3^{\bullet}$ production by the cytochrome *P*-450 system (Recknagel *et al.*, 1977). The present study examines the effect of GSH on carbon tetrachloride metabolism by rat liver microsomes and on carbon tetrachloride-induced injury to the microsomes. The aims were to determine whether GSH protected against carbon tetrachloride-derived free radicals and to characterize any GSH effects found.

Abbreviations used: GSH, reduced glutathione; GSSG, oxidized glutathione.

Experimental

Hepatic microsomes were prepared as described previously (Burk, 1983) from 300–400 g male Sprague–Dawley strain rats (Timco Labs, Houston, TX, U.S.A.) that had been fed a nutritionally-adequate semisynthetic diet from weaning (Lawrence & Burk, 1978). Experiments were carried out with microsomes on the day they were prepared.

All incubations were carried out in a volume of 5 ml in 25 ml Erlenmeyer flasks sealed with a rubber septum. They were shaken in a water bath at 37°C. The buffer was 50 mM-Tris/HCl, pH 7.5, also containing 140 mM-NaCl and 50 μM-EDTA. The incubations represented in Fig. 1 and in Tables 1 and 3 used 200 μM-NADPH, 1 mM-carbon tetrachloride, and a microsomal protein concentration of approx. 0.5 mg/ml. The incubations represented in Table 2 used 1 mM-NADPH, 72 μM-[^{14}C]carbon tetrachloride (sp. radioactivity 2.8 Ci/mol), and a microsomal protein concentration of approx. 2 mg/ml.

O_2 was excluded for the anaerobic experiments by bubbling buffers with N_2 for 15 min. Then the complete incubation mixture without carbon tetrachloride and NADPH was gently bubbled with N_2 for 5 min at 37°C before the flask was sealed and carbon tetrachloride (10 μl volume in ethanol) was injected. A 5-min equilibrium period was allowed and then the experiment was started by injection of NADPH.

For the experiment shown in Table 2, 1 ml of flask atmosphere was sampled with a gas-tight syringe immediately after NADPH injection and 15 min later. Carbon tetrachloride and chloroform in the sample were determined immediately. ^{14}C covalent binding to microsomal protein was determined (Sipes *et al.*, 1977) at the 15 min time point. Blank experiments were run with injection of buffer instead of NADPH and used to correct the carbon tetrachloride-disappearance results. No chloroform production or significant ^{14}C covalent binding was detected in the absence of NADPH.

The experiments shown in Tables 1 and 3 were terminated by cooling the open flasks in an ice bucket. Assays were performed within 4 h. Thiobarbituric acid-reactive substances were determined as before (Burk *et al.*, 1980). GSH added to the flask after the incubation was finished had no effect on the thiobarbituric acid-reactive substance assay. Cytochrome *P*-450 was assayed by the method of Raj & Estabrook (1970) and glucose 6-phosphatase by the method described by Glende *et al.* (1976).

Carbon tetrachloride and chloroform were measured using a Porasil C (80/100) column in a Hewlett–Packard 5880A gas chromatograph with a flame-ionization detector. Oven temperature was 85°C. Standard curves were constructed by injecting carbon tetrachloride and chloroform into 25 ml Erlenmeyer flasks containing 5 ml of buffer.

[^{14}C]Carbon tetrachloride was purchased from New England Nuclear, Boston, MA, U.S.A. Carbon tetrachloride was obtained from Aldrich Chemical Co., Milwaukee, WI, U.S.A., and chloroform from American Scientific and Chemical, Portland, OR, U.S.A. NADPH was purchased from Boehringer-Mannheim, Indianapolis, IN, U.S.A. GSH, GSSG, propylthiouracil and dithiothreitol were purchased from Sigma Chemical Company, St. Louis, MO, U.S.A. Other chemicals were reagent grade or better.

Results

GSH inhibits carbon tetrachloride-induced microsomal lipid peroxidation as shown in Fig. 1. In the absence of GSH the onset of lipid peroxidation is preceded by a 2-min lag. Addition of GSH lengthens the lag so that it is 4 min with 0.1 mM-GSH and 6 min with 1 mM-GSH. This effect on the lag is similar to that reported previously with iron-dependent lipid peroxidation systems (Burk, 1982). It appears to be rather specific for GSH because it could not be produced with 1 mM-dithiothreitol, -propylthiouracil or -GSSG (results not shown).

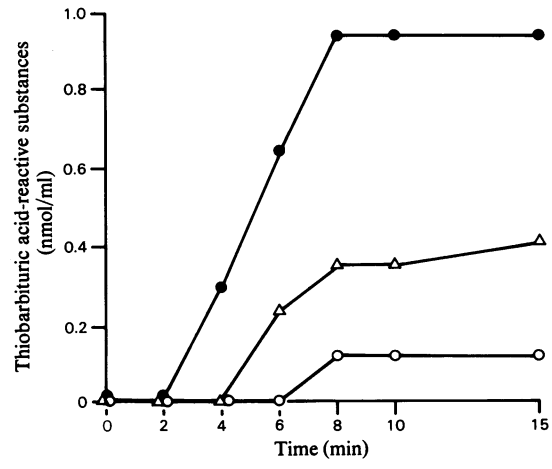

Fig. 1. *GSH protection against 1 mM-carbon tetrachloride-induced microsomal lipid peroxidation*
The incubations were carried out as described in the Experimental section and varied only in GSH content: ●, no GSH; △, 0.1 mM-GSH; ○, 1 mM-GSH. This experiment is representative of a number of experiments. Addition of an NADPH-generating system had no effect on the results (not shown). Also not shown is a control from which carbon tetrachloride was omitted. No lipid peroxidation was detected in it.

The final extent of lipid peroxidation caused by carbon tetrachloride was reduced by GSH (Fig. 1). This contrasts with results obtained using iron-containing systems, where GSH did not decrease the extent of lipid peroxidation. The explanation for this lies in mechanistic differences between the carbon tetrachloride system and the iron-containing systems. The iron-containing systems do not require cytochrome *P*-450 for their activity and lipid peroxidation proceeds until the peroxidizable microsomal lipids are consumed. Kornbrust & Mavis (1980) have shown that carbon tetrachloride-induced lipid peroxidation ceases long before peroxidizable membrane lipids are consumed. This occurs because the cytochrome *P*-450 species that activates carbon tetrachloride is destroyed by the toxic intermediates or by lipid peroxidation initiated by them. In the experiment depicted in Fig. 1 this appears to have occurred at 8 min whether GSH was present or not. Thus, prolongation of the lag led to a shorter time period during which lipid peroxidation could take place and thereby to less lipid peroxidation. From these results we infer that GSH

protects the microsomal membrane better than it protects the activating species of cytochrome *P*-450.

The experiment reported in Table 1 was an attempt to compare the effect of GSH on carbon tetrachloride injury to the membrane with its effect on injury to cytochrome *P*-450. In a preliminary experiment 1 mM-GSH had no effect on cytochrome *P*-450, thiobarbituric acid-reactive substances or glucose 6-phosphatase activity in the control (results not shown) so it was not added here. GSH diminished lipid peroxidation to 16% of that which occurred in its absence and blocked the inactivation of microsomal glucose 6-phosphatase, which indicated substantial protection of the microsomal membrane. However, GSH protection against cytochrome *P*-450 destruction was much less. It diminished cytochrome *P*-450 loss only to 60% of the loss that occurred in the absence of GSH. That is, in the presence of GSH the inactivation of cytochrome *P*-450 was relatively greater than the membrane lipid peroxidation and the glucose 6-phosphatase inactivation when compared with the results obtained when GSH was not present.

Most of the toxic effects of carbon tetrachloride are caused by products of its metabolism (Recknagel *et al.*, 1977). Because GSH decreased some of the toxic effects of carbon tetrachloride, we examined the influence of GSH on carbon tetrachloride metabolism (Table 2). Under aerobic conditions, so little metabolism took place that it could not be detected by measuring carbon tetrachloride disappearance or chloroform production. Covalent binding to protein was detected, however, and GSH inhibited it. The covalent binding could occur by direct free-radical attack or by formation of phosgene from carbon tetrachloride (Kubic & Anders, 1980). This finding and especially the GSH inhibition of carbon tetrachloride-induced lipid peroxidation are evidence that GSH affects carbon tetrachloride metabolism under aerobic conditions.

When carbon tetrachloride metabolism was assessed under anaerobic conditions, the results were different. Extensive metabolism took place as evidenced by carbon tetrachloride disappearance (Table 2). Free-radical attack by $CCl_3^{\bullet}$ took place because it is responsible for chloroform formation (Table 2). Covalent binding to protein occurred. GSH had no effect on any of these measures of carbon tetrachloride metabolism under anaerobic conditions (Table 2). Thus, GSH appears to alter carbon tetrachloride metabolism only if O_2 is present.

Carbon tetrachloride is known to destroy cytochrome *P*-450 under anaerobic conditions (De Groot & Haas, 1980) presumably by direct free-radical attack. We tested the effect of GSH on this anaerobic cytochrome *P*-450 destruction by carbon tetrachloride metabolites. GSH did not protect cytochrome *P*-450 under those conditions (Table 3) even though it had protected in the presence of O_2 (Table 1). This provides further evidence that the protective effect of GSH against carbon tetrachloride is O_2-dependent. Glucose 6-phosphatase, which for unknown reasons had a higher activity in these rats than in those for which results are given in Table 1, was not inactivated by carbon tetrachloride under anaerobic conditions.

Table 1. *Effect of GSH on carbon tetrachloride-induced microsomal lipid peroxidation and enzyme inactivation under aerobic conditions*

Incubations were carried out for 20 min. All flasks contained 1 mM-carbon tetrachloride. No NADPH was added to controls. Values are means ± S.D. ($n = 6$). Abbreviation used: TBA, thiobarbituric acid. Values with the same superscript are significantly different ($P < 0.05$) by the paired *t*-test.

	TBA-reactive substances (nmol/ml)	Cytochrome *P*-450 (nmol/mg)	Glucose 6-phosphatase (μmol/mg per 20 min)
Control	–*	0.87 ± 0.10*	8.4 ± 2.3*
No GSH	1.47 ± 0.59*	0.62 ± 0.07*	5.6 ± 1.8*,†
1 mM-GSH	0.23 ± 0.17*	0.71 ± 0.07*	7.9 ± 2.3†

Table 2. *Effect of GSH on carbon tetrachloride metabolism by microsomes*

Incubations were carried out for 15 min. Values are means ± S.D. ($n = 6$–13); 23 400 pmol of carbon tetrachloride was added to each flask per mg of protein Abbreviation used: n.d., not detectable. * Significantly different ($P < 0.05$) by paired *t*-test.

	Carbon tetrachloride disappearance (pmol/mg of protein)	Chloroform production (pmol/mg of protein)	[^{14}C]Carbon tetrachloride covalent binding (pmol/mg of protein)
Aerobic			
No GSH	n.d.	n.d.	212 ± 90*
1 mM-GSH	n.d.	n.d.	23 ± 8*
Anaerobic			
No GSH	12 700 ± 2970	4280 ± 1220	202 ± 46
1 mM-GSH	15 700 ± 3490	4500 ± 650	218 ± 114

Table 3. *Effect of GSH on carbon tetrachloride-induced enzyme inactivation under anaerobic conditions*
Incubations were carried out as described in the legend to Table 1, except that anaerobic conditions were maintained. Values are means ± S.D. ($n = 3$). Values with the same superscript are significantly different ($P < 0.05$) by the paired t-test.

	Cytochrome *P*-450 (nmol/mg)	Glucose 6-phosphatase (μmol/mg per 20 min)
Control	0.95 ± 0.17*,†	12.1 ± 1.3
No GSH	0.50 ± 0.17*	12.1 ± 1.0
1 mM-GSH	0.51 ± 0.21†	11.9 ± 0.8

Discussion

These results demonstrate that GSH protects against carbon tetrachloride-induced microsomal lipid peroxidation and associated enzyme inactivation. This supplements previous findings of GSH protection against lipid peroxidation in systems dependent on iron (Burk, 1983) and indicates that the GSH-dependent radical-scavenging mechanism may function with free radicals of diverse origins.

The O_2-dependence of the GSH protection and the lack of effect of GSH on carbon tetrachloride metabolism under anaerobic conditions may be clues to the nature of the GSH protection. Early work ascribed carbon tetrachloride-induced injury to $CCl_3^\bullet$ produced by the cytochrome *P*-450 system (Recknagel *et al.*, 1977). More recently, it has been recognized that when O_2 is present $CCl_3^\bullet$ rapidly combines with it to form $CCl_3O_2^\bullet$ (Packer *et al.*, 1978). This suggests that different free radicals are present under aerobic and anaerobic conditions. Since GSH has a measurable effect only in the presence of O_2 and it inhibits the free-radical-initiated process of lipid peroxidation, it seems likely that the GSH-dependent mechanism can scavenge $CCl_3O_2^\bullet$ but not $CCl_3^\bullet$. Aerobic metabolism of carbon tetrachloride yields a small amount of phosgene, which could be the source of some covalent binding of ^{14}C (Kubic & Anders, 1980). GSH can block this binding by conjugation with phosgene (Pohl *et al.*, 1981). An alternative explanation of the O_2 requirement for GSH protection would be that O_2 is a substrate in the protective mechanism. Previous experiments showing that O_2 consumption correlates closely with lipid peroxidation suggest, however, that the only O_2-consuming mechanism in this system is lipid peroxidation (Burk, 1983). Thus, it seems likely that the GSH-dependent free-radical-scavenging mechanism is primarily a peroxy-radical-scavenging system.

The results of Table 1 are similar to those of Poli *et al.* (1981) and indicate that under aerobic conditions GSH protects the microsomal membrane from carbon tetrachloride damage better than it protects cytochrome *P*-450. This is consistent with the hypothesis that one or more species of cytochrome *P*-450 activates carbon tetrachloride to radical products and hence are present at the point of maximum free-radical concentration (Frank *et al.*, 1982). If the GSH protective mechanism is distributed throughout the membrane, it should be most efficient where free-radical concentration is lowest, viz., remote from the cytochrome *P*-450 activating the carbon tetrachloride. Raising GSH concentration increases protection against lipid peroxidation (Fig. 1) presumably by restricting it to a smaller area surrounding the activating species of cytochrome *P*-450. Thus, under aerobic conditions when GSH concentration is high, the activating cytochrome *P*-450 is destroyed with little injury to the membrane.

The O_2-dependence of the GSH protection against carbon tetrachloride injury may help explain the observation that high concentrations of O_2 protect animals from carbon tetrachloride-induced hepatotoxicity (Rapin *et al.*, 1967) and lipid peroxidation (Kieczka & Kappus, 1980). Most of the hepatic injury by carbon tetrachloride occurs in the centrilobular regions where O_2 tension is lowest. If we construct an analogy with our microsomal experiments, the centrilobular region of the liver would lie toward the anaerobic experiments but would contain some O_2. Thus we might expect less efficient GSH-dependent free-radical scavenging while sufficient O_2 remained to support lipid peroxidation. An increase in O_2 concentration would allow destruction of the activating cytochrome *P*-450 species while promoting GSH protection of the rest of the membrane.

Thus, O_2 appears to have two effects that diminish carbon tetrachloride hepatotoxicity. (1) It inhibits carbon tetrachloride metabolism. (2) It allows GSH-dependent free-radical scavenging to occur. This provides the rationale for the observed protective effect of hyperbaric O_2 against carbon tetrachloride poisoning (Truss & Killenberg, 1982).

The authors are indebted to Dr. W. Haas of the University of Düsseldorf, Düsseldorf, Germany, for helpful suggestions. Mrs. Rebecca E. Ortiz provided secretarial assistance. This work was supported by NIH grant ES02497 and Robert A. Welch Foundation grant AQ-870.

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