Role of glutathione in metabolic degradation of dichloromethane in rats

Soo J. Oh, Sang K. Kim, Young C. Kim *

College of Pharmacy, Seoul National University, San 56-1 Shinrim-Dong, Kwanak-Ku, Seoul, South Korea

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Abstract

Dichloromethane (DCM) elimination and carboxyhemoglobin (COHb) generation were examined in adult female SD rats pretreated with a glutathione (GSH) depletor(s). Rats were treated with either buthionine sulfoximine (BSO; 2 mmol/kg, i.p.), diethylmaleate (DEM; 3 mmol/kg, i.p.), phorone (PHO; 1 mmol/kg, i.p.) or BSO plus PHO (BSO; 2 mmol/kg + PHO; 0.5 mmol/kg, i.p.). The hepatic GSH concentration was significantly reduced by each treatment. Decrease in hepatic GSH was maintained at least for 10 h after BSO treatment but recovered rapidly in rats treated with DEM or PHO. The hepatic p-nitrophenol hydroxylase activity was not affected by the GSH depletors at the dose used in this study. Rats were treated with an i.p. injection of DCM (3 mmol/kg) and the concentrations of DCM and the COHb levels in blood were monitored. In rats pretreated with a GSH depletor, the peak COHb level was significantly greater than that of rats treated with DCM only. The peak COHb level attained in each group of rats appeared to be inversely related to the magnitude of reduction in hepatic GSH levels. The half-life of DCM in blood was also increased in rats pretreated with the GSH depletor(s). The results indicate that the GSH-dependent metabolic reaction has an important role in the overall elimination of DCM as well as in the metabolic generation of carbon monoxide (CO) from this solvent. © 2002 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Dichloromethane; Glutathione; Carboxyhemoglobin; Carbon monoxide; Cytochrome P-450; Glutathione depletor

1. Introduction

Dichloromethane (DCM) is widely used in the industry as a degreaser, solvent and extraction medium and also as an important constituent of paint removers. This chemical is considered to be relatively non-toxic except at extremely high exposure levels that may cause narcosis and liver/kidney injuries (Klaassen and Plaa, 1966). The major toxicological hazard from exposure to low concentrations of DCM lies in the formation of carbon monoxide (CO) during its biotransformation (Stewart et al., 1972).

Two major pathways are involved in the metabolic degradation of DCM; a microsomal...
oxidation process and a glutathione (GSH)-dependent cytosolic pathway. The microsomal reaction involves the oxidative dechlorination of DCM resulting in the production of CO and carbon dioxide (CO₂) via the proposed intermediates chloromethanol and formyl chloride (Kubic and Anders, 1978). In inhalation studies the ratio of CO produced per mol DCM oxidized in rats was estimated to be 0.7–0.8 mol (Gargas et al., 1986; Andersen et al., 1994). This pathway is saturated at a concentration exceeding several hundred ppm, which results in a peak carboxyhemoglobin (COHb) level of approximately 10% (McKenna et al., 1982; Kim and Carlson, 1986). It has been suggested that cytochrome P-450 2E1 (CYP 2E1) is the subtype of the cytochrome P-450 family responsible for the metabolic conversion of DCM to CO (Gugenerich et al., 1991). Treatment of rats with an inducer for CYP 2E1, including benzene, toluene and m-xylene, resulted in elevation of the maximum COHb level and reduction of the half-life of DCM in blood (Kim and Kim, 1996).

An alternative metabolic pathway of DCM is a reaction with GSH mediated by a glutathione S-transferase (GST), especially the theta class GST, which produces CO₂ via S-chloromethyl GSH (GSCH₂Cl) and formaldehyde (Blocki et al., 1994; Mainwaring et al., 1996). GSH is required in this reaction, but not consumed (Ahmed and Anders, 1978). This pathway shows no indication of saturation at concentrations of DCM up to 10,000 ppm, indicating that the cytosolic pathway is low affinity, high capacity compared with the cytochrome P-450 dependent reaction (Gargas et al., 1986).

Results of animal studies examining the role of GSH in the metabolic degradation of DCM are controversial. Gargas et al. (1986) demonstrated that when 2,3-epoxypropane was treated to rats for GSH depletion, the maximum COHb level induced by inhaled DCM was increased. But pretreatment with a GSH depletor, such as buthionine sulfoximine (BSO), diethylmaleate (DEM) or phorone (PHO), failed to affect the elevation of the maximum COHb level (Pankow and Jagielki, 1993). In the present study we examined the role of GSH-dependent pathway(s) in the metabolic disposition of DCM by measuring the effect of GSH depletion on the COHb elevation and on the elimination of this solvent in blood.

2. Materials and methods

2.1. Chemicals

Drugs and chemicals, such as NADPH, GSSG reductase, GSH, BSO, DEM, p-nitrophenol, were purchased from Sigma Chemical (St. Louis, MO, USA). Sodium dithionite and DCM were obtained from Fisher Scientific (Fair Lawn, NJ, USA). PHO was purchased from Aldrich Chemical (Milwaukee, WI, USA). All other chemicals and solvents used in this study were reagent grade or better.

2.2. Animals and treatments

Female Sprague–Dawley rats (Yuhan Central Research Institute, Kunpo, Korea), weighing 150–200 g, were used throughout the study. The use of animals was in compliance with the guidelines established by the Animal Care Committee of this institute. Rats were acclimated in environmentally controlled rooms (light: 07:00–19:00 h, dark: 19:00–07:00 h) for at least four weeks prior to experiments. Lab chow and tap water were allowed ad libitum. BSO preparation for injection was made in normal saline. DEM, PHO and DCM were dissolved in corn oil. In preliminary experiments, we examined the time course of hepatic GSH concentrations after administration of each GSH depletor. The maximal decrease was reached 1 h after treatment with DEM (3 mmol/kg, i.p.) or PHO (1 mmol/kg, i.p.). In rats treated with BSO (2 mmol/kg, i.p.) the maximal decrease was observed at t = 4 h. In order to potentiate the depletion of hepatic GSH, an additional dose of PHO (0.5 mmol/kg, i.p.) was administered 3 h following BSO treatment (2 mmol/kg, i.p.) in the PHO + BSO group. The final time point of GSH measurement for each treatment group was set at
17:00 h to avoid involvement of the circadian variation in hepatic levels of this tripeptide. DCM (3 mmol/kg, i.p.) was administered at the time point the greatest GSH depletion was obtained. In rats pretreated with DEM or PHO, DCM was injected 1 h following treatment with the GSH depletor. In BSO only or BSO + PHO treated rats, DCM was administered at \( t = 4 \) h after BSO treatment. The control rats were treated with each vehicle used to prepare the depletor injection. Since no difference in either GSH level, COHb elevation or DCM disappearance between the rats treated with corn oil and those with saline was observed, the control values were all pooled for presentation.

2.3. Measurement of COHb and DCM in blood

Blood samples were withdrawn from an orbital venous plexus. The COHb concentration in blood was determined using a modification of the method of Rodkey et al. (1979). Each blood sample was diluted approximately 1500-fold with 0.01 M Tris solution containing sodium dithionite to prevent dissociation of COHb by oxygen. Absorbance measurements were made at 420 and 432 nm using a spectrophotometer (Model V-550, Jasco Co, Tokyo, Japan). The fraction of the total hemoglobin present as COHb was calculated from these measurements and the molar absorptivities of hemoglobin and carboxyhemoglobin.

The DCM levels in blood were determined using a modification of the method previously reported (Kim and Carlson, 1986). Each 100 µl blood sample in a sealed vial stood at room temperature for 60 min before 100 µl of headspace vapor was injected into a Varian Model 3300 Gas Chromatograph (Varian Instruments Division, Palo Alto, CA, USA) equipped with a flame ionization detector. A 2 m stainless steel column packed with 4% OV-101 and 6% OV-210 (Supelco, Inc, Bellefonte, PA, USA) was used. Nitrogen was the carrier gas (30 ml/min), and air (300 ml/min) and hydrogen (30 ml/min) were utilized in the flame ionization detector. The column was set at 70 °C, the detector at 150 °C, and the injector at 130 °C.

2.4. Measurement of hepatic glutathione content

Rats were sacrificed by decapitation. The liver was homogenized in a 4-fold volume of cold 1 M perchloric acid solution with 2 mM EDTA. After the denatured protein was removed by centrifugation, the supernatant was assayed for GSH according to the method of Griffith (1980). Reaction mixture (final volume of 1 ml) contained 5 mM EDTA, 0.6 mM 5,5′-dithiobis(2-nitrobenzoic acid), 0.2 mM NADPH, 0.2 ml supernatant and 0.5 unit GSH reductase in 0.1 M potassium phosphate buffer (pH 7.5). Incubation was initiated with the addition of GSH reductase (after a 4-min preincubation period) and the rate of formation of 2-nitro-5-thiobenzoic acid was measured at 412 nm.

2.5. Microsomal enzyme assays

The liver was homogenized in a 3-fold volume of an ice-cold buffer consisting of 0.154 M KCl/50 mM Tris–HCl and 1 mM EDTA, pH 7.4. All subsequent steps were performed at 0 to 4 °C. The homogenate was centrifuged at 12 000 × g for 20 min. The supernatant fraction was further centrifuged at 104 000 × g for 60 min. The microsomal pellet was suspended and recentrifuged at 104 000 × g for 60 min. The microsomes were diluted to an equivalent of 0.5 g of liver/1 ml of buffer. Protein content was measured by the method of Lowry et al. (1951). The concentration of cytochrome P-450 was estimated from the CO difference spectrum (Omura and Sato, 1964). p-Nitrophenol (PNP) hydroxylase activity was determined by measuring the formation of 4-nitrocatechol (Koop, 1986). Reaction mixture (final volume of 1 ml) contained 0.1 mM p-nitrophenol, 1.0 mM ascorbic acid, 0.1 ml microsomal suspension, and 1 mM NADPH in 0.1 M potassium phosphate buffer (pH 6.8). Incubation was initiated with addition of NADPH (after a 2-min preincubation period). The temperature was maintained at 37 °C. The reaction was terminated by addition of 0.5 ml ice-cold 10% perchloric acid after a 3 min incubation period.
2.6. Data analysis

All results expressed as the mean ± S.E. were analyzed by a two-tailed Student’s t-test. The acceptable level of significance was established at $P < 0.05$ except when otherwise indicated. The half-life of DCM was determined by linear regression of log blood concentrations of DCM versus time for each animal.

3. Results

3.1. Effects of GSH depletors on hepatic GSH level

Hepatic GSH levels were determined in rats treated with a GSH depletor(s) (Fig. 1). All the depletors used in this study significantly decreased hepatic GSH levels but remarkable differences in the magnitude and duration of reduction were observed. A single dose of DEM (3 mmol/kg, i.p.) or PHO (1 mmol/kg, i.p.) induced a rapid decrease in hepatic GSH levels which recovered to approximately 70–80% of the control level 7 h after the treatment. Hepatic GSH levels were measured every 2 h for 10 h following BSO treatment (2 mmol/kg, i.p.). Hepatic GSH reached the lowest level, approximately one third of the control, at $t = 4$ h, which was maintained at least for 10 h. When an additional dose of PHO (0.5 mmol/kg, i.p.) was administered to rats 3 h following BSO pretreatment (2 mmol/kg, i.p.), hepatic GSH levels were depleted to a level below 5% of the control when measured at $t = 4$ h.

3.2. Effects of GSH depletors on COHb elimination induced by DCM

A 3 mmol/kg dose of DCM caused a significant increase in blood COHb concentrations, reaching a peak of approximately 9% from $t = 2$ to 3 h (Fig. 2). The COHb levels returned to normal 6 h following the treatment. When BSO (2 mmol/kg, i.p.), DEM (3 mmol/kg, i.p.) or PHO (1 mmol/kg, i.p.) was administered prior to DCM, the COHb

![Fig. 1. Effect of a GSH depletor(s) on hepatic GSH contents. Each value represents the mean ± S.E. for more than four rats. The time of each administration prior to sacrifice is indicated in parentheses. **, *** Significantly different from the control group (Student’s t-test, $P < 0.01, 0.001$).](image-url)
elevation was significantly enhanced. The treatment with BSO+PHO that resulted in the most significant decrease in hepatic GSH level showed the greatest induction of COHb. Doubling the DCM dose from 3 to 6 mmol/kg failed to show a corresponding increase in the peak COHb level, suggesting that the conversion of DCM to CO was saturated at the lower dose. However, decline of COHb was significantly delayed as shown by the increase in COHb levels at \( t = 4 \) and 6 h (Student’s \( t \)-test, \( P < 0.01 \)).

3.3. Effects of GSH depletors on half-life of DCM in blood

The effect of GSH depletors on the DCM concentration and half-life in blood was examined in rats treated with a 3 mmol/kg dose of DCM (Fig. 3). The GSH depletor or its combination, which reduced hepatic GSH most effectively in this study, was selected to examine the effect of GSH depletion on DCM elimination in blood. DCM declined in a log–linear fashion in blood. Blood DCM concentrations were significantly greater at \( t = 3 \) h in rats pretreated with BSO (3 mmol/kg, i.p.) compared with rats treated with DCM only. The half-life of DCM in blood was correspondingly prolonged. When an additional
Table 1
Effect of a GSH depletor on microsomal enzyme activity

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>DEM (−1 h)</th>
<th>DEM (−7 h)</th>
<th>PHO (−1 h)</th>
<th>PHO (−7 h)</th>
<th>BSO (−2 h)</th>
<th>BSO (−8 h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytochrome P-450 (nmol/mg protein)</td>
<td>0.73 ± 0.03</td>
<td>0.79 ± 0.03</td>
<td>0.73 ± 0.05</td>
<td>0.80 ± 0.04</td>
<td>0.77 ± 0.01</td>
<td>0.77 ± 0.02</td>
<td>0.77 ± 0.06</td>
</tr>
<tr>
<td>p-Nitrophenol hydroxylase (nmol/min/mg protein)</td>
<td>1.10 ± 0.06</td>
<td>1.30 ± 0.09</td>
<td>1.13 ± 0.04</td>
<td>1.17 ± 0.07</td>
<td>1.15 ± 0.05</td>
<td>1.20 ± 0.03</td>
<td>1.13 ± 0.04</td>
</tr>
</tbody>
</table>

Each value represents the mean ± S.E. for more than four rats. The time of administration prior to sacrifice is indicated in parentheses.

dose of PHO (0.5 mmol/kg, i.p.) was administered to rats, blood DCM concentrations were further elevated from \( t = 2 \) h, however, no differences in either DCM half-life or blood concentration between the BSO-treated rats and the rats treated with BSO + PHO were observed.

3.4. Effects of GSH depletors on \( p \)-nitrophenol hydroxylase activity

The effect of a GSH depletor on \( p \)-nitrophenol hydroxylase activity was examined in rats (Table 1). The microsomal enzyme activity was determined at the time periods DCM was actively transformed to CO and hepatic GSH decreased significantly. The hydroxylation of \( p \)-nitrophenol, a substrate highly specific for CYP 2E1 or the hepatic cytochrome P-450 content was not affected by the GSH depletor at the dose used in this study.

4. Discussion

It has been well established that two major pathways are involved in the metabolic degradation of DCM; an oxidative cytochrome P-450-mediated pathway that yields CO, and a GSH-dependent pathway that produces CO\(_2\) but not CO (Kubic et al., 1974; Ahmed and Anders, 1978; Kubic and Anders, 1978; Gargas et al., 1986). Distribution of DCM metabolism between two pathways seems to be dose-dependent (Andersen et al., 1987). The oxidative pathway is high-affinity with limited capacity, which is saturated at relatively low concentrations (200–500 ppm) in rats (McKenna et al., 1982; Kim and Carlson, 1986). In contrast, the GSH-dependent pathway has low affinity for DCM, but does not appear to be saturated even at extremely high concentration (10 000 ppm) (Gargas et al., 1986). Thus, at low concentrations the cytochrome P-450-mediated pathway accounts for most of the DCM metabolized, but as exposure concentrations are increased, disproportionate increases in the amount of DCM metabolized by the secondary GSH pathway are shown (Andersen et al., 1987).

In the present study, GSH depleters with different mechanisms of action were employed to reduce hepatic GSH. BSO is an irreversible inhibitor of \( \gamma \)-glutamylcysteine synthetase (GCS) that catalyzes a rate-limiting step in GSH synthesis (Griffith and Meister, 1979). DEM and PHO are \( \alpha,\beta \)-unsaturated carbonyl compounds that rapidly react with GSH (Boyland and Chasseaud, 1970). The hepatic GSH levels were markedly decreased by either DEM or PHO treatment but recovery of hepatic GSH also seemed to be rapid. It was reported that both the activity and the protein contents of GCS were increased by either DEM or PHO (Borroz et al., 1994; Kitteringham et al., 2000), which could account for the rapid increase in GSH. In rats treated with BSO the reduction of hepatic GSH level was less, but more prolonged at the dose used in this study. An additional dose of PHO depleted hepatic GSH almost completely.

The COHb elevation induced by DCM was significantly enhanced in rats treated with a GSH
depletor(s). The effects on the COHb generation appeared to have considerable correlation with the magnitude of decrease in hepatic GSH. It is postulated that the inhibition of the cytosolic pathway due to depletion of GSH, shifts the balance between the two major metabolic pathways leading to introduction of a greater portion of this solvent into the oxidative microsomal reaction. However, the increase in supply of DCM to the microsomal pathway could not account for the enhancement of COHb elevation. In rats treated with a 6 mmol/kg dose of DCM, decline in blood COHb was prolonged, but the peak COHb level was not altered in the present study. This confirms earlier observations that the generation of CO from DCM in animals was saturable (McKenna et al., 1982; Kim and Carlson, 1986; Kang and Kim, 1995). It was shown that CYP 2E1 was the major catalyst for the oxidation of DCM to CO (Guengerich et al., 1991). In rats treated with a CYP 2E1 inducer, such as ethanol, pyrazole, benzene, toluene or m-xylene, the peak COHb level was elevated, and on the other hand, disulfiram, an inhibitor of CYP 2E1, blocked this elevation completely (Pankow et al., 1991; Kim and Kim, 1996; Wirkner et al., 1997). The GSH depletor(s) used in this study did not affect the CYP 2E1 activity as determined by p-nitrophenol hydroxylation. Therefore, the increase in the peak COHb level in rats pretreated with a GSH depletor(s) could not be explained by an induction of the enzyme activity.

It was observed that the radioactivity derived from [14C]DCM was covalently bound to both microsomal proteins and lipids (Anders et al., 1977). The similar characteristics of metabolism to CO and covalent binding suggested that a common intermediate, perhaps formyl chloride, might be involved. Several studies have provided evidence that formyl chloride is generated in the metabolism of DCM to CO (Kubic and Anders, 1978; Gargas et al., 1986; Andersen et al., 1994). The formyl chloride intermediate from DCM would act as an acylating agent for tissue nucleophiles or decompose to CO. It has been well known that GSH serves as an antioxidant by detoxifying electrophiles and scavenging free radicals (Meister and Anderson, 1983; Kaplowitz et al., 1985). Formation of formyl chloride would be limited by the activity of CYP 2E1 in liver as suggested by the failure of increasing DCM dose to increase the peak COHb level. However, the decrease in GSH availability induced by a GSH depletor would enhance non-enzymatic decomposition of this reactive metabolite to CO rather than generation of CO2. This view is in agreement with the earlier study showing that in rats treated with 2,3-epoxypropane the maximum COHb levels induced by inhaled dihalomethanes including DCM and bromochloromethane were increased (Gargas et al., 1986).

It has been reported that the primary route of elimination of DCM is through the breath (Di-Vincenzo and Halmilton, 1975; McKenna and Zempel, 1981). Greater than 90% of DCM (511 mg/kg, i.p.) was exhaled unchanged and only 7% was eliminated as CO, CO2 and uncharacterized metabolite in rats (DiVincenzo and Halmilton, 1975). Previously, we observed that both the COHb elevation and the DCM half-life in blood were significantly altered in rats treated with either a CYP 2E1 inducer or an inhibitor (Kim and Carlson, 1986; Kim and Kim, 1996). The present study indicates that the availability of hepatic GSH also has significant influences on the metabolic fate of DCM. The reduction in hepatic GSH levels markedly elevated the COHb generation and depressed the rate of DCM disappearance from blood, suggesting that the GSH-dependent cytosolic reactions, as well as the cytochrome P-450-dependent pathway, play a quantitatively important role in the overall elimination and metabolic disposition of DCM.

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References