GLUTATHIONE-DEPENDENT CONVERSION TO GLYOXYLATE, A MAJOR PATHWAY OF DICHLOROACETATE BIOTRANSFORMATION IN HEPATIC CYTOSOL FROM HUMANS AND RATS, IS REDUCED IN DICHLOROACETATE-TREATED RATS

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ABSTRACT:

Although it has been postulated that glyoxylate is an intermediate in the biotransformation of DCA to oxalate, CO₂, and glycine, there has been no positive identification of glyoxylate as a metabolite of DCA. We have demonstrated that a GSH-dependent pathway in dialyzed hepatic cytosol from rats and humans converts a mixture of 1-14C- and 1,2-13C-DCA to isotopically labeled glyoxylate. The reaction does not occur in the presence of NADPH or NADH or in the absence of GSH. The identity of the glyoxylate was demonstrated by HPLC with radiochemical detection and confirmed by GC/MS of the methylated glyoxylate, which showed the 13C-labeled product. The apparent Kₘ for GSH was 0.075 mM in rat hepatic cytosol. Pretreatment of rats with NaDCA, 50 mg/kg, p.o. (by mouth) for 2 days prior to preparation of hepatic cytosol on the third day affected the cytosolic metabolism of DCA. With 0.2 mM DCA as substrate, in the presence of 1 mM GSH, control rats formed 1.45 ± 0.13 nmol glyoxylate/min/mg cytosolic protein, whereas the rate in DCA-treated rats was 0.45 ± 0.10 nmol glyoxy- late/min/mg protein (mean ± SD, N = 4 in each group). The mechanism of this reduction in the rate of DCA biotransformation in DCA-treated rats is unknown but is consistent with in vivo observations that the elimination of DCA from plasma of humans and rats is slowed by prior administration of DCA.

DCA is an investigational drug for the treatment of various cardiovascular and metabolic diseases (1). DCA is also a product of water chlorination and a metabolite of several halogenated solvents and anesthetic agents. Chronic treatment of rodents with high doses of DCA and related organic acids has been shown to cause peroxisome proliferation and liver tumors (2, 3). In part because of its carcinogenicity in rodents, the presence of DCA in the ground water is considered a potential public health hazard. Despite considerable knowledge about the biological activities of DCA, several questions remain concerning its biotransformation. It has been shown that in humans and rats one dose of NaDCA, 25 to 50 mg/kg, causes an increase in the half-life of elimination from plasma of subsequent doses of DCA (4, 5). The mechanism of this effect is unknown, but may be a result in part to slowed DCA metabolism. DCA is known to be converted to oxalate, CO₂, and glycine (5–7). Although it has been proposed that glyoxylate is the common intermediate leading to the formation of these products (6), there is as yet no direct evidence for this in any animal tissue. Lipscomb et al. (8) showed that in the presence of NADPH and GSH, undialyzed hepatic cytosol, but not microsomes from rats metabolized DCA; however, the product formed was not identified. In the present study it was shown that DCA was converted to glyoxylate by a GSH-dependent pathway that is present in hepatic cytosol from rats and humans.

Materials and Methods

Chemicals. 1,4-C-Dichloroacetic acid, >99.9% radiochemical purity, specific activity 55.5 mCi/mmol, was purchased from American Radiolabeled Chemicals (St. Louis, MO) and converted to the sodium salt by equimolar addition of NaOH. HPLC analysis with UV detection at 220 nm showed no UV-absorbing impurities. Sodium 1,2-13C-DCA, 99% pure, was purchased from Cambridge Isotopes Laboratories (Cambridge, MA). Unlabeled NaDCA was purchased from TCI America (Portland, OR). GSH, NADPH, NADH, glyoxylate, oxalate, glycine, and glycollate were from Sigma Chemical Co. (St. Louis, MO), Tetrabutylammonium sulfate (low UV PIC-A) was purchased from TCI America (Portland, OR). 1-Chloro-2,4-dinitrobenzene was purchased from TCI America (Portland, OR). 4-(2-hydroxyethyl)-1piperazineethane-sulfonic acid was purchased from TCI America (Portland, OR). Polyacrylamide gels were purchased from Bio-Rad Laboratories, Inc. (Richmond, CA). All other chemicals used were of the highest available purity.

Animals and Tissue Preparations. Male Harlan Sprague-Dawley rats, 170–260 g were maintained on a 12-hr light/dark cycle and fed Purina rat chow. Four rats were treated with 50 mg/kg NaDCA by oral gavage of 2 ml/kg of a 25 mg/ml solution on days 1 and 2 and were sacrificed on day 3. A control group of 4 rats was treated with an equal volume of water. Other rats used were untreated. The rat livers were removed, rinsed in ice-cold 1.15% KCl, 0.05M potassium phosphate buffer pH 7.4, and cytosolic fractions were prepared by differential centrifugation (9). The human liver cytosol was prepared by the International Institute for the Advancement of Medicine (Exton, PA). Aliquots of all the cytosolic fractions were frozen under nitrogen at −80°C. Before being used in assays, samples of cytosol, 1–3 ml, were dialyzed in 10,000 mol.

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wt. cut-off tubing at 4°C against two changes of 1.15% KCl, 0.05M potassium phosphate buffer, pH 7.4. In most studies, the dialyzed cytosol samples were then incubated with 1 mM NADPH, or an equal volume of water, at 37°C for 15 min prior to redialysis. Protein concentration was measured after dialysis by the method of Lowry et al. (10).

**Assays.** A mixture of 0.2 mM [1-14C and 1,2-13C]-DCA, specific activity 5.55 mCi/mmole, was incubated with 0.5 mg dialyzed cytosolic protein, 1 mM GSH and 0.1M HEPES-NaOH buffer, pH 7.6, in a final volume of 0.25 ml. The dependence of the reaction rate on the concentration of GSH, DCA, protein, and incubation time was determined. In some experiments, 2 mM NADPH or NADH was added in place of GSH. Assay tubes were incubated in a water bath at 37°C for 15 to 60 min, and the reaction was terminated by adding 0.5 ml of ice-cold methanol. After vortex mixing, tubes were placed on ice for 15–20 min, then centrifuged at 1,500 rpm for 20 min to separate the precipitated protein. The supernatant samples were filtered through a 0.45 μm nylon centrifuge filter and analyzed by HPLC. Some samples were also analyzed by GC/MS.

Cytosolic GST activity with 1 mM CDNB and 2 mM GSH as substrates was measured as described by Habig et al. (11).

**HPLC Analysis.** Samples from DCA incubations were routinely analyzed at room temperature (22–24°C) by isocratic, C18 reversed phase HPLC. An Isco model 2350 pump (Isco Inc., Lincoln, NE) with a manual injection port was attached to a 50 × 4.6 mm Beckman octadecylsilane pre-column coupled to a 250 × 4.6 mm Beckman octadecylsilane analytical column (Rainin-Varian, Inc., Woburn, MA). The mobile phase was 0.05M tetrabutylammonium sulfate in 30% aqueous methanol and the flow rate was 1 ml/min. The column eluent passed through a UV detector (Dynamax UV-1, Rainin Inst., Woburn, MA) set at 220 nm and a radiochemical detector (Radiomatic Flex-One Beta, Tampa, FL). Under these conditions, the retention time of DCA was 7.2 min, of glyoxylate 3.8 min, of glycollate 3.5 min, and of oxalate 4.3 min. Sets of DCA, glyoxylate, and oxalate standards were routinely monitored.

**GC/MS Analysis.** In some studies the identities of the metabolites produced from incubation with the isotopically labeled DCA were confirmed by conversion to the methyl esters and GC/MS analysis (12, 13). A supernatant sample, 200 μl, from the assay mixture and an internal standard, 0.5 mM 4-chlorobutyric acid in water, 50 μl, were mixed with 500 μl of 12% BF3-methanol complex in a glass culture tube and the tube was tightly capped with a Teflon-lined (Dupont, Wilmington, DE) cap. The mixture was heated at 100°C for 15 min. After cooling, 1 ml of methylene chloride and 1 ml of water were added to the reaction mixture. The tube was vortex mixed, allowed to stand for 10 min, then centrifuged at 3,000 rpm for 15 min at 10°C in a refrigerated centrifuge (Beckman J-6B, Beckman Instruments, Palo Alto, CA). The methylene chloride layer was transferred to a sample vial for GC/MS analysis. The GC/MS system consisted of a Hewlett-Packard (Palo Alto, CA) 5890 series II plus GC, a 5972A series mass selective detector, a 6890 series auto-injector, a G1521A autosampler controller and a Vectra multimedia VL2 4/66 computer using ChemStation software. The column was HP-wax, 30m × 0.25 mm, 0.15 μm film thickness. The methylene chloride extract, 1 μl, was injected using a splitless mode. The injection port temperature was 150°C and the detector interface was set at 280°C. The GC column oven temperature was held at 40°C for 2 min, raised to 100°C at 5°C/min, then to 240°C at 50°C/min, and held at 240°C for 2 min. At the mass detector an electron impact ionization mode was used with ionization energy of 70 eV. The mass detector was calibrated with perfluorotributylamine. The retention times and mass fragmentation patterns of metabolites produced from incubations were compared with those of methylated authentic standards of DCA, glyoxylate, glycollate, and oxalate.

**Statistical Analysis.** Differences between groups were determined by ANOVA, using Excel software (Microsoft, Redmond, WA).

**Results and Discussion.** These studies showed that dechlorination of DCA in the presence of rat or human hepatic cytosol and GSH yielded glyoxylate. Fig. 1 shows a typical HPLC radiochemical detector trace of the products formed by rat hepatic cytosol in the presence and absence of GSH. Small amounts of [13C]-oxalate were evident in some, but not all, incubations. To confirm the identity of the major product peak at 3.8 min, GC/MS was employed. Fig. 2 shows gas chromatographs of the methylated extracts from 60 min incubations of rat and human hepatic cytosol with 0.2 mM DCA and 1 mM GSH. This lengthy incubation time was selected to ensure that enough product was formed for unambiguous identification. The gas chromatograms showed peaks with the same retention times as methylated glyoxylate (11.1 min) and oxalate (11.5 min), but not glycollate. The mass spectra of the 11.1 min methylated glyoxylate peaks from incubations with human and rat cytosol are shown in fig. 3. These spectra match the spectrum of an authentic methylated glyoxylate, in which the fragment at m/z 75 corresponding to CHO(OCH3)2, is the most abundant (14). The fragments at m/z 75 and 76 arise from 12C and 13C glyoxylate, respectively. The presence of high concentrations of the m/z 76 fragment in incubations from rat and human hepatic cytosols conclusively demonstrated that glyoxylate is a metabolite of 1,2-13C-DCA. The mass spectra of the methylated oxalate peaks, separated by GC from incubations with both rat and human cytosol fractions, matched those of an authentic methylated oxalate. The most abundant fragments in the methylated oxalate mass spectra were at m/z 59 and 60, corresponding to 12COOCH3 and 13COOCH3, arising from 12C- and 13C-oxalate, respectively (data not shown). The ratios of the 13C to 12C fragments were at least 3-fold higher than expected from the natural abundance of 13C, indicating that at least part of the oxalate found in these incubations was derived from DCA.

The rate of GSH-dependent glyoxylate formation from DCA in human hepatic cytosol, 0.15 mmol/min/mg protein, was slower than in rat hepatic cytosol (see table 1 for rat activities). Since the human cytosol samples were frozen for several months before use, GST activity with CDNB was measured as a general indicator of enzymatic activity. The human hepatic cytosol sample used had very low GST activity, 0.10 μmole CDNB conjugate formed/min/mg protein, compared with the average activity found in human hepatic cytosol, 1.1 μmol/min/mg protein (15). Thus, the activity with DCA found in this sample may underestimate normal human activity of the dechlorination pathway. These studies did, however, clearly demonstrate that the pathway for conversion of DCA to glyoxylate was similar in human and rat liver. In vivo studies have shown that the elimination half-lives of DCA in rats and humans are similar (4, 5), Stacpoole et al., submitted), suggesting that the rat is a suitable model for humans.

Further studies to characterize the conditions for formation of
glyoxylate formation from DCA were conducted with rat hepatic cytosol fractions. Glyoxylate formation from 0.2 mM DCA in the presence of 1 mM GSH and 0.5 mg rat hepatic cytosolic protein/ml was linear with time from 5 to 30 min (fig. 4A). During a 15-min. incubation, glyoxylate formation was linear with rat hepatic cytosolic protein over the range 0.5 to 6 mg protein per ml (fig. 4B). Rat hepatic cytosolic activity was stable for at least 6 months if the undialyzed cytosol was stored under N2 at 280°C. The cofactor dependence of the cytosolic formation of glyoxylate was investigated. Although initial experiments, with cytosol that was dialyzed against two changes of buffer, showed that 2 mM NADPH or NADH gave about 1/3 of the rate of glyoxylate formation found with 1 mM GSH (5), further studies showed that the reaction was GSH dependent. Complete removal of GSH and GSSG from cytosol was accomplished by incubating dialyzed cytosol at 37°C with 1 mM NADPH for 15 min to reduce the GSSG and any protein-bound GSH, then dialyzing again. With the NADPH-incubated, dialyzed cytosol as an enzyme source, little or no glyoxylate was produced in subsequent incubations with DCA in the presence of NADPH or NADH, although GSH still supported glyoxylate formation (table 1). It was found that dialyzed cytosol that was sham incubated, with water instead of NADPH, had much lower activity than cytosol dialyzed in the presence of NADPH (table 1). This suggests either that the enzyme responsible for dechlorination of DCA was unstable at 37°C in the absence of reducing agent or that a reduced form of the enzyme was more active than a nonreduced form.

Pretreatment of rats with 2.50 mg NaDCA/kg for 2 days before preparing hepatic cytosol reduced the rate of glyoxylate formation considerably but did not alter the cofactor dependency (table 1). The

| TABLE 1 | Biotransformation of DCA to glyoxylate by hepatic cytosol from control rats and rats treated with NaDCA, 2 \times 50 mg/kg |
| Reducing Agent<sup>2</sup> | Control rats<sup>1</sup> | DCA-pretreated rats<sup>1</sup> |
| Cytoisol Incubated with NADPH<sup>1</sup> | Cytoisol Incubated with Water<sup>1</sup> | Cytoisol Incubated with NADPH<sup>1</sup> | Cytoisol Incubated with Water<sup>1</sup> |
| GSH (1 mM) | 1.43 ± 0.13<sup>a,b</sup> | 0.58 ± 0.16<sup>a</sup> | 0.45 ± 0.10<sup>b</sup> | 0.09 ± 0.06<sup>d</sup> |
| NADPH (2 mM) | 0.02 ± 0.02<sup>c</sup> | <0.01<sup>d</sup> | <0.01<sup>c</sup> | <0.01<sup>c</sup> |
| NADH (2 mM) | 0.14 ± 0.17<sup>d</sup> | <0.01<sup>d</sup> | <0.01<sup>d</sup> | <0.01<sup>d</sup> |

<sup>1</sup> Rats were treated by oral gavage with water (controls) or NaDCA, 50 mg/kg, for 2 days before preparation of hepatic cytosol fractions.

<sup>2</sup> Assay tubes contained 0.2 mM DCA (0.18 mM 1,2-<sup>13</sup>C-DCA with 0.02 mM 1-<sup>14</sup>C-DCA, 55.5 μCi/mmmole), 1 mM GSH and 0.5 mg cytosolic protein/ml, 0.5 mg cytosolic protein and GSH, NADPH or NADH at the indicated concentrations in 0.25 ml 0.1 M HEPES-NaOH buffer pH 7.4. After incubation at 37°C for 15 min, the reaction was stopped by addition of methanol, and product formation was measured by HPLC as described in the Methods section.

<sup>3</sup> Dialysis conditions: Hepatic cytosol samples from four individual rats for each pretreatment (water or DCA) were dialyzed at 4°C against two changes of 1.15% KCl, 0.05 M potassium phosphate buffer pH 7.4, then incubated at 37°C for 15 min with one-tenth volume of NADPH final concentration 1 mM, or an equal volume of water. Incubated cytosol fractions were redialyzed at 4°C against two changes of 1.15% KCl/0.05M potassium phosphate pH 7.4 and used in assays.

<sup>4</sup> Values shown are mean ± SD, N = 4. Mean values with differing superscripts indicate significant differences between groups, p < 0.001.

<sup>5</sup> Not detectable.
enzyme responsible for dechlorination of DCA in cytosol from the DCA-pretreated rats also lost activity at 37°C in the absence of NADPH (table 1). These results fit in vivo observations that the plasma elimination half-life of DCA in rats was slowed by administration of one prior dose of DCA³ (5) and suggest that the initial conversion of DCA to glyoxylate in the liver is a key step controlling DCA elimination from plasma. Further studies are needed to determine whether the reduced rate is a result of down-regulation, reversible inhibition, or irreversible inhibition of the dechlorination enzyme by DCA treatment.

The rate of glyoxylate formation from 0.2 mM DCA was maximal at GSH concentrations above 0.2 mM (fig. 5). The apparent \( K_m \) for GSH was 0.075 mM, a value that is much lower than the usual rat liver GSH concentration of about 5 mM (16). This finding indicates that metabolism of DCA to glyoxylate is unlikely to be highly sensitive to GSH depletion. The low \( K_m \) for GSH may explain why NADPH and NADH appeared to support conversion of DCA to glyoxylate in dialyzed cytosol. If small amounts of GSSG or protein-sulphhydryl-bound GSH remained, incubation with NADPH or NADH could produce enough free GSH to support the reaction. In the presence of 1 mM GSH, the rate of glyoxylate formation was maximal for DCA concentrations in the range 0.075 to 0.5 mM, and preliminary studies showed that the apparent \( K_m \) for DCA, determined from DCA concentrations ranging from 0.0026 to 0.100 mM, was 0.006 to 0.008 mM.

The mechanism of dechlorination of DCA remains unknown. Although the reaction is cytosolic and GSH-dependent, it is unclear whether a GST is involved in the dechlorination steps. Further studies are needed to characterize the enzyme, or enzymes, that catalyze the dechlorination. It is, however, important to our understanding of DCA metabolism to positively identify glyoxylate as a major intermediate in the metabolism of DCA, as the present studies demonstrate. This intermediate is consistent with in vivo findings that the major end products of DCA metabolism in humans and rats are glycine, \( \text{CO}_2 \), and oxalate⁴ (5–7). Glycine can be formed from transamination of glyoxylate (17), \( \text{CO}_2 \) from decarboxylation of glyoxylate (18), and oxalate from oxidation of glyoxylate (19).

In summary, these studies have shown that conversion of NaDCA to glyoxylate proceeds in rat and human hepatic cytosol fractions in the presence of low concentrations of GSH, that the enzyme or enzymes catalyzing this reaction lose activity at 37°C in the absence of NADPH, and that pretreatment of rats with DCA before preparation of cytosol fractions results in lowered activity.

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³ James, M.O., K.U.M.M. Jayanti, G.N. Henderson, et al., in preparation.


References


