INHIBITION AND RECOVERY OF RAT HEPATIC GLUTATHIONE S-TRANSFERASE ZETA
AND ALTERATION OF TYROSINE METABOLISM FOLLOWING DICHLOROACETATE
EXPOSURE AND WITHDRAWAL

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ABSTRACT:

Dichloroacetate (DCA) is an investigational drug for certain metabolic disorders, a by-product of water chlorination and a metabolite of certain industrial solvents and drugs. DCA is biotransformed to glyoxylate by glutathione S-transferase zeta (GSTz1-1), which is identical to maleylacetate isomerase, an enzyme of tyrosine catabolism. Clinically relevant doses of DCA (mg/kg/day) decrease the activity and expression of GSTz1-1, which alters tyrosine metabolism and may cause hepatic and neurological toxicity. The effect of environmental DCA doses (µg/kg/day) on tyrosine metabolism and GSTz1-1 is unknown, as is the time course of recovery from perturbation following subchronic DCA administration. Male Sprague-Dawley rats (200 g) were exposed to 0 µg, 2.5 µg, 250 µg, or 50 mg DCA/kg/day in drinking water for up to 12 weeks. Recovery was followed after the 8-week exposure. GSTz specific activity and protein expression (Western immunoblotting) were decreased in a dose-dependent manner by 12 weeks of exposure. Enzyme activity and expression decreased 95% after a 1-week administration of high-dose DCA. Eight weeks after cessation of high-dose DCA, GSTz activity had returned to control levels. At the 2.5 or 250 µg/kg/day doses, enzyme activity also decreased after 8 weeks’ exposure and returned to control levels 1 week after DCA was withdrawn. Urinary excretion of the tyrosine catabolite maleylacetone increased from undetectable amounts in control rats to 60 to 75 µg/kg/24 h in animals exposed to 50 mg/kg/day DCA. The liver/body weight ratio increased in the high-dose group after 8 weeks of DCA. These studies demonstrate that short-term administration of DCA inhibits rat liver GSTz across the wide concentration range to which humans are exposed.

Dichloroacetate (DCA) is an unusual xenobiotic that has had an impact on both clinical therapeutics and environmental toxicology. It has been used as an investigational drug for treating several acquired and congenital metabolic and cardiovascular diseases at acute or chronic doses that typically range between 25 mg/kg/day and 100 mg/kg/day. These concentrations are thousands of times greater than those to which most humans are usually exposed (Stacpoole et al., 1998).

Many haloacetates, including DCA and its mono- and tri-chloro analogs, are distributed ubiquitously in the biosphere, including lakes, groundwater, drinking water, glacial ice, fog, rain, precipitation, air, and soil (reviewed in Stacpoole et al., 1998 and Amminiti and Stacpoole, 2003). DCA is one of the most prevalent haloacetates found in samples from these sources and has been implicated as a toxin to plants and trees at atmospheric exposure levels (Hoekstra et al., 1999; Rompp et al., 2001). DCA is also a by-product of drinking water disinfection (Krasner et al., 1989; Mughal, 1992) and a metabolite of two widely used chlorinated industrial solvents, trichloroethylene and tetrachloroethylene (Coleman et al., 1976; Westrick et al., 1984; Elcombe et al., 1985; Odum et al., 1988) and of certain drugs (Stacpoole et al., 1998). Humans may be exposed to DCA by chlorination of municipal drinking water or by groundwater contamination at certain Superfund sites. The World Health Organization has set a target safety level for DCA in the drinking water of 50 µg/l, whereas the United States Environmental Protection Agency limit is 60 µg/l for the total of five halogenated acetic acids in drinking water (WHO, 1996). Consequently, drinking water is considered the major route of exposure to this compound by humans and may approximate 2 to 4 µg/kg/day (Stacpoole et al., 1998).

Toxicological studies of DCA in animals have usually used chronic exposure levels in the mg/kg/day dose range that greatly exceed levels typical of human environmental exposure. These studies have identi-

ABBREVIATIONS: DCA, dichloroacetate; GSTz1-1 or GSTz, glutathione S-transferase zeta; MA, maleylacetone.
fied liver, kidney, nervous system, testis, and eye as susceptible target tissues in rodents and dogs (reviewed in Stacpoole et al., 1998 and Ammini et al., 2003). DCA is carcinogenic in mice (Herren-Freund et al., 1987; Bull et al., 1990; DeAngelo et al., 1991, 1996; Anna et al., 1994; Pereira 1996) and rats (Richmond et al., 1995). Clinical case reports have also indicated that DCA is a reversible peripheral neurotoxin and hepatotoxin (Moore et al., 1979; Mather et al., 1990; DeAngelo et al., 1991, 1996; Stacpoole et al., 1998; Spruijt et al., 2001).

Glutathione S-transferase zeta (GSTz1-1) catalyzes the glutathione-dependent conversion of DCA to glyoxylate (James et al., 1997; Tong et al., 1998; Anderson et al., 1999; Tzeng et al., 2000) (Fig. 1). GSTz1-1 is identical to maleylacetoacetate isomerase (Blackburn et al., 1998), which catalyzes the penultimate reaction in the catabolism of tyrosine and phenylalanine (Fig. 1). Liver expresses the highest amount of total GSTz1-1 protein per milligram of cytosolic protein, followed by brain and lung. DCA is a mechanism-based inhibitor of GSTz1-1 (Anderson et al., 1999, 2002; Tzeng et al., 2000) at clinically relevant doses and increases the urinary excretion of maleylacetone (MA), an endogenous substrate for the isomerase (Cornett et al., 1999; Ammini et al., 2003).

Human GSTz1-1 is reported to undergo covalent modification of the active site cysteine-16 in vitro by DCA (Anderson et al., 1999, 2002). Inactivation of GSTz1-1 is considered to be irreversible, since human GSTz1a-1a inactivated in vitro by DCA could not be restored by dialysis of the inactivated protein against 0.1 M potassium phosphate buffer (Tzeng et al., 2000). In vivo studies also suggest that inactivation of GSTz1-1 by DCA is irreversible, since immunoreactive GSTz expression decreased in livers of dosed Fischer 344 rats and Sprague-Dawley rats (Anderson et al., 1999; Ammini et al., 2003). Recovery of protein expression apparently requires protein synthesis (Schultz et al., 2002) and thus should lead to a restoration of enzyme expression and activity. Anderson et al. (1999) reported recovery of GSTz expression 8 to 12 days after a single administration of 45 mg/kg/day DCA. Recovery after withdrawal of subchronic exposure to DCA at any exposure level has not been investigated.

Here we report results obtained in rats administered DCA at exposure levels that span the environmental to therapeutic concentration range. Recovery was examined after 8 weeks’ exposure to the compound. We determined the effect of DCA on drinking water consumption, body weight, liver weight, and the time course of inactivation and recovery of hepaticGSTz and expression. We also measured the urinary excretion of DCA and MA.

Materials and Methods

Chemicals and Antibodies. Dichloroacetic acid-1-14C (specific activity 52 mCi/mmol) was obtained from American Radiolabeled Chemicals (St. Louis, MO), and converted to its sodium salt by addition of NaHCO3. Unlabeled clinical grade sodium DCA was purchased from TCI America (Portland, OR). Polyclonal chicken anti-mouse GSTz1-1 antibodies were kindly supplied by Dr. Markus Grompe (Oregon Health & Science University, Portland, OR). ECL Western Blotting Detection Reagents was purchased from GE Healthcare (Little Chalfont, Buckinghamshire, UK). In this study, mineral water (Zephyrhills brand) purchased from a local grocer was used as drinking water to avoid contamination by DCA present in municipal water. The maleylacetone was synthesized as described previously (Cornett et al., 1999). BF3-methanol (reagent grade) was obtained from Aldrich Chemical Co. (Milwaukee, WI) and methylene chloride (pesticide grade) was obtained from Fisher Scientific Co. (Pittsburgh, PA). All other chemicals used in this study were of high purity and were bought from commercial suppliers.

Animals and Administration. Male Sprague-Dawley rats (200 g, 2 months old) were purchased from Harlan (Indianapolis, IN). Animals were housed individually under constant conditions of temperature and humidity, and maintained on a 12-h light/dark cycle. They were placed in separate cages with free access to unchlorinated spring water (to minimize uncontrolled exposure to DCA) and food. Animals were randomly assigned to 22 treatment groups.
Male Sprague-Dawley rats (200 g, 7 weeks old) were randomly assigned to one of the 22 administration groups (A to V, n = 6/group). After receiving bottled water for 1 week, the rats (8 weeks old) were given low-dose (2.5 μg and 250 μg/kg/day) DCA or high-dose (50 mg/kg/day) DCA in bottled water (free from chlorination by-products including DCA). Rats of series 1 were administered DCA for up to 8 weeks (1 week for groups A and B, 4 weeks for groups C to F, 8 weeks for groups G to R) and after that, they were given drinking water with no DCA for up to 8 weeks (1 week for groups K to N and 8 weeks for group O to R) for recovery studies. Rats of series 2 received DCA for 12 weeks (groups S to V). Animals were sacrificed on the last day of the indicated week time point.

### Table 1

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<th>Age (wk)</th>
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### Immunoblotting

Cytosol samples or human GSTz1-1 were heated in SDS-polyacrylamide gel electrophoresis sample buffer at 95°C for 5 min, and 40 μg of total protein from each of the cytosol samples and 0.5 μg of protein from the pure human GSTz1-1 were resolved by SDS-polyacrylamide gel electrophoresis on a polyacrylamide gel (12%; Bio-Rad Laboratories). The proteins were then transferred to a 0.45-μm nitrocellulose membrane (Bio-Rad Laboratories). After blocking for 1 h in 5% fat free milk in Tris-buffered saline-Tween 20 buffer (Bio-Rad Laboratories), the membrane was incubated with chicken anti-mouse GSTz1-1 polyclonal antibody (primary antibody) for 1 h, followed by donkey anti-chicken IgG secondary antibody (Research Diagnostics, Flanders, NJ) for 2 h. Detection was performed using ECL Western Blotting Detection Reagents (GE Healthcare), and bands were quantitated by image analysis software (ScanAnalysis).

### Determination of Urinary DCA and MA

Urine samples and the internal standard 2-oxohexanoic acid were derivatized to their methyl esters by reacting with 12% BF3-methanol complex. DCA and MA were extracted with methylene chloride. The concentrations of DCA and MA were measured by gas chromatography-mass spectrometry (Yan et al., 1997; Shroads et al., 2004), using a Hewlett Packard (Palo Alto, CA) 5890 Series II plus gas chromatograph and a 5972A Series mass selective detector.

### Statistical Analysis

Means, standard deviations, and statistical significance for the various experiments were carried out using Excel software (Microsoft, Redmond, WA). For comparing mean values of two treatment groups, one-tailed Student’s t test was used to determine significance of data. A p value of ≤0.05 was considered to be statistically significant.

### Results

#### Age-Dependent Changes in Hepatic GSTz

In untreated rats, the activity and expression of GSTz changed with age and body weight of the rats (Table 2). Twenty-four-week-old rats weighed 20% more than 12-week-old rats from the same source, purchased at the same time. However, hepatic GSTz activity was higher in the 12-week-old rats than in any of the older age groups studied (p < 0.005). Hepatic GSTz protein expression, analyzed by immunoblotting, followed the same trend, but because of higher experimental variability between replicates in the Western blots, the difference was not statistically significant. The experimental error in GSTz activity measurement was less than 2%, whereas the experimental error in measuring GSTz protein expression by immunoblotting was up to 15%, which meant that differences in protein expression less than this could not be determined reliably.

#### Inactivation and Restoration of Hepatic GSTz Activity and Protein Expression

Enzyme activity with DCA as substrate was reduced 95% by 1 week of dosing with 50 mg/kg DCA (p < 0.0001) and remained low over the 8-week exposure period (Fig. 2). GSTz protein expression, shown in Fig. 3, was barely detectable after 1...
week’s treatment, falling over 95% \( (p < 0.0001) \), and remained suppressed over the 8-week exposure. After withdrawal of DCA, GSTz activity returned to 59% of control by 1 week and returned completely to control levels by 8 weeks. After DCA withdrawal, GSTz protein expression increased gradually, although mean expression remained below that of control animals by 8 weeks.

The lower doses of DCA, 2.5 \( \mu g/kg/day \) or 250 \( \mu g/kg/day \), had no significant effect on enzyme activity after 4 weeks of treatment, but activity had decreased significantly by 8 weeks’ exposure to DCA \( (p < 0.05) \). Similar trends were found for GSTz protein expression but did not attain statistical significance. One week after withdrawal of these doses, GSTz activity had returned to the control levels.

With the particular aim of confirming the reduction in GSTz activity following low-dose exposure to DCA, four additional groups (S to V, Table 1) were treated with DCA for 12 weeks. The GSTz activity and protein expression in these groups showed a clear dose-dependent reduction (Fig. 4).

**Drinking Water Consumption, Body Weight, and Liver Weight.** Consumption of drinking water was measured in each rat group that was studied over 1 (A and B) or 16 (O, P, W, and R) weeks (Table 1). The highest water consumption (133.5 ml/kg/day) occurred in the 50 mg DCA/kg/day group by 1 week of exposure and was 20% greater \( (p < 0.05) \) than the amount consumed by control animals (111.1 ml/kg/day). Otherwise, water consumption among the groups was similar.

The body weight of each rat was monitored three times a week. There was no effect of any dose level of DCA on body weight during the 16-week study. DCA did not alter liver weight in animals exposed to the 2.5 \( \mu g/kg/day \) or 250 \( \mu g/kg/day \) dose. The 50 mg/kg/day DCA dose increased the liver/body weight ratio 21% \( (p < 0.005) \) above control by 8 weeks of exposure, but this ratio returned to control levels 8 weeks after discontinuing DCA.

**Urinary DCA and MA.** Rats excreted DCA in urine at a rate of 4.5 mg/kg/24 h after 1 week of the 50 mg/kg/day dose, and the amount increased to 5.5 mg/kg/24 h after 8 weeks of exposure. Only trace amounts of DCA (less than 10 \( \mu g/kg/24 h \)) were excreted 1 week after discontinuing DCA administration. No DCA was measured in urine samples (limit of detection, 1 \( \mu g/ml \)) from animals receiving either the 2.5 \( \mu g/kg/day \) or 250 \( \mu g/kg/day \) doses.

Rats that received 50 mg/kg/day DCA excreted MA at a rate of 60 to 75 \( \mu g/kg/24 h \) during the 8-week dosing period. MA excretion decreased below detectable limits (0.2 \( \mu g/ml \)) 1 week after DCA withdrawal. No MA was detected in the urine of rats receiving either the 2.5 \( \mu g/kg/day \) or 250 \( \mu g/kg/day \) doses.

**Discussion**

At the 50 mg/kg/day DCA dose, both GSTz enzyme activity and protein expression decreased significantly \( (p < 0.0001) \) and remained low during the entire 8-week exposure (Figs. 2 and 3). After withdrawal of DCA, GSTz enzyme activity and expression gradually increased. High doses of DCA are known to alter rat hepatic GSTz activity and protein expression (James et al., 1997; Anderson et al., 1999; Cornett et al., 1999; Ammini et al., 2003), but the time required for restoration of enzyme activity and expression after subchronic treatment has not been determined previously. The results of this study suggest that recovery of protein expression lagged behind enzyme activity in the high-dose animals, for reasons that are unclear. Possibly, liver cytosol of control animals contains enzymatically
inactive GSTz protein that is still recognized by the antibody, and this is not the case in rats recovering from high-dose DCA. It is noteworthy that, in the present study, even the lowest dose of DCA (2.5 g/kg/day) reduced the hepatic GSTz activity after 8 to 12 weeks’ exposure. This suggests that DCA levels found in some municipal water sources may be sufficient to alter the hepatic GSTz activity and, hence, amino acid metabolism in humans.

The hepatic specific activity of GSTz in control animals decreased with age, falling 20% between 12 and 24 weeks of age during the period of study (p < 0.05; Table 2). This is consistent with our previous observation that the plasma elimination half-life of 50 mg/kg/day DCA was 5.4 h in young adult rats (3–4 months; 180–265 g) versus 9.7 h in old rats (16 months; 580–690 g) (James et al., 1998). Similarly, Schultz et al. (2002) showed that 60-week-old mice had lower GSTz expression and reduced capacity to metabolize DCA compared with 8-week-old animals. Together, these data indicate that hepatic GSTz levels decline with age in rodents and reinforce the need for age and weight-matched controls in studies of the biotransformation and pharmacological effects of DCA.

Few studies have examined the effect of DCA on water consumption in animals. However, DeAngelo et al. (1989) and Mather et al. (1990) demonstrated that DCA caused a dose-dependent decrease in water consumption in exposed rats. We found that high-dose DCA actually resulted in an initially higher water consumption than that measured in control animals.

Mather et al. (1990) reported that high-dose (500 and 5000 ppm) DCA for 90 days in drinking water blunted weight gain in rats, and similar results were obtained by Pereira et al. (1997) in mice. The mechanism was considered to be due to the consequences of liver carcinogenesis. In contrast, we found no effect of DCA on body weight at any of the exposure levels used in this study. DCA may cause hepatic hypertrophy or hyperplasia in rats (Anderson et al., 1975; Mariash and Schwartz, 1986; Mather et al., 1990; Smith et al., 1992). Although high-dose DCA (50 mg/kg/day) also increased liver weight and the liver to body weight ratio in this study, these indices returned to baseline levels after DCA was withdrawn.

Events leading to the loss of GSTz1 from the liver following administration of DCA are incompletely understood. There is evidence from in vitro studies that in the presence of GSH, DCA itself can bind covalently to GSTz1, and it has been postulated that this leads to destruction of the enzyme (Anderson et al., 2002). Other candidates for forming adducts with GSTz1, which may lead to its destruction, are MA and maleylacetoacetate. These are the physiologically important substrates for GSTz1, and both are electrophilic molecules. MA inhibits hepatic GSTz in vitro in a dose-dependent manner (Cornett et al., 1999) that is only partly reversible following removal of MA by dialysis (Ammini et al., 2003; Lantum et al., 2003). The present study was not designed to resolve the mechanism by which GSTz1 is lost from the liver following DCA administration but, rather, to explore the lowest exposure levels associated with this loss.

Figure 3. Inhibition of hepatic GSTz protein expression by DCA and its recovery after withdrawal of DCA. Rats were given 0 µg, 2.5 µg (a), 250 µg (b), or 50 mg (c) of DCA/kg/day in drinking water for up to 8 weeks, followed by no DCA for up to 8 weeks. At each time point, one group of rats served as controls (no DCA exposure). Data are shown as mean percentage of control ± S.D. (n = 6 for 1, 4, or 16 weeks’ administration and n = 12 for 8 or 9 weeks’ administration) from the matched control group. An asterisk indicates a significant change relative to the matched controls (p < 0.05). Also shown is a representative Western immunoblot for hepatic GSTz. Lanes 1, 8, and 12 contain 0.5 µg of recombinant hGSTz (mol. wt. 26,000). Lanes 2 to 7 contain 40 µg of cytosolic protein from 8-week controls (group G), and lanes 9 to 11 contain 40 µg of cytosolic protein from rats treated for 8 weeks with 2.5 µg/kg (group H).
sufficient enzyme to metabolize the levels of maleylacetoacetate and MA normally produced from tyrosine catabolism. It should be noted, however, that both MA and maleylacetoacetate are unstable molecules, and the limit of chemical detection by our present analytical method may not correspond to the limit of toxicity from these substances.

In conclusion, the results of this investigation may have important implications about the effects of chronic DCA exposure on human health. Daily, oral administration of DCA at a concentration typical of that present in municipal drinking water supplies (reviewed in Stacpoole et al., 1998) significantly decreased the activity and expression of hepatic GSTz. Thus, exposure of humans and other species to so-called “environmental” levels of this compound may perturb certain fundamental pathways of intermediary metabolism in ways that may lead to cumulative toxicity.

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