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Do Antioxidant Enzymes and Glutathione Play Roles in the Induction of Hepatic Oxidative Stress in Mice upon Subchronic Exposure to Mixtures of Dichloroacetate and Trichloroacetate?

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Abstract

Dichloroacetate (DCA) and trichloroacetate (TCA) are water chlorination byproducts, and their mixtures were previously found to induce additive to greater than additive effects on hepatic oxidative stress (OS) induction in mice after subchronic exposure. To investigate the roles of antioxidant enzymes and glutathione (GSH) in those effects, livers of B6C3F1 mice treated by gavage with 7.5, 15, or 30 mg DCA/kg/day, 12.5, 25, or 50 mg TCA/kg/day, and mixtures (Mix I, Mix II and Mix III) at DCA:TCA ratios corresponding to 7.5:12.5, 15:25 and 25:50 mg/kg/day, respectively, for 13 weeks. Livers were assayed for superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px), as well as for GSH levels. In general, DCA suppressed SOD and GSH-Px activities and GSH levels but caused no changes in CAT activity; TCA increased SOD and CAT activities, suppressed GSH-Px activity, but did not change GSH levels; mixtures of DCA and TCA increased SOD and CAT activities and suppressed GSH-Px activity and GSH levels. In conclusion, antioxidant enzymes contribute to DCA-, TCA- and mixtures-induced OS, but not to changes from additive to greater than additive effects produced by different mixture compositions of the compounds. GSH on the hand may contribute to these changes.

INTRODUCTION

Chlorination of drinking water is an important disinfection process associated with generation of by-products (Miller and Uden 1983) such as dichloroacetate (DCA) and trichloroacetate (TCA) (Barron and Paull 2004; Miller and Uden 1983). They are also known to be formed as *in vivo* metabolites of the widely used industrial solvent trichloroethylene (Dekant, Metzler, and Henschler 1984; Fang et al. 2013; Lash et al. 2000) which may contaminate surface waters. The compounds have been also detected along with monochloroacetic acid at concentrations ranging from 0.01 up to 9 µg/L in rainwater collected from some areas of Switzerland (Reimann, Grob, and Frank 1996). Because of the presence of DCA and TCA in chlorinated drinking water, their long term toxicity has been in the focus of several studies demonstrating their hepatotoxic and hepatocarcinogenic

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effects in rodents (Bull et al. 1990; Daniel et al. 1992 ; DeAngelo et al. 1989; 1991; DeAngelo, George, and House 1999; Herren-Freund et al. 1987; Pereira 1996).

Long term studies have also demonstrated that DCA and TCA induce oxidative stress (OS), generation of superoxide anion (SA), lipid peroxidation (LP) and DNA damage, and modulate antioxidant enzyme activities and glutathione (GSH) level in livers of mice, **prior to** any hepatocarcinogenic effects, suggesting their possible contribution to the development of those latter effects (Hassoun, Cearfoss, and Spildener 2010; Hassoun and Cearfoss 2011). While long term exposure to DCA and TCA individually had been of interest **to** several investigators, exposure to mixtures of these pollutants was another problem since they may occur together and the toxic outcomes can be different from those of individual exposure. Only few studies have been reported on the toxic outcome of mixtures of DCA and TCA. For example, DCA and TCA were found to promote N-methyl-N-nitrosourea-initiated cancer in the liver of B6C3F1 mice, though proliferative lesions promoted by DCA were different from those promoted by TCA; when the compounds were administered together they resulted in an additive effect on lesions that were similar to those promoted by DCA (Pereira, Li and Kramer 1997). Bull et al. (2002) reported differences in immunoreactivity to a c-Jun antibody of tumors induced by DCA and by TCA, and when they were administered as mixtures, they induced a few tumors that were c-Jun+, many that were c-Jun-, and some with mixed phenotype that increased with the relative dose of DCA. The study also reported additive effects on tumor numbers when low doses of DCA were combined with high doses of TCA, without the use of tumor initiators (Bull et al. 2002).

In a recent subchronic study in our laboratory on the induction of OS in the livers of B6C3F1 mice, additive effects by certain DCA/TCA mixtures, and greater than additive effects by higher mixture concentrations have been observed (Hassoun et al. 2014). However, it was not known why the increase in mixture concentrations changed the effects from additive to greater than additive. This study was designed to investigate the possible roles of the antioxidant enzymes superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-Px), as well as GSH on the observed differences in DCA/TCA mixture-induced effects after subchronic exposure.

METHODS

Chemicals

The chemicals and reagents were purchased from Sigma-Aldrich Chemical Company (St Louis, MO, USA) at the highest grade available.

Animals and Treatments

Parts of the livers from treated B6C3F1 male mice of a previous study (Hassoun et al. 2014) were used. In brief, Mice were purchased from Harlan Teklad (Indianapolis, Indiana, USA) at 6 weeks of age and of an approximate weight of 20 g. After acclimation for 3 days, groups of 6 animals in each, received 7.5, 15, or 30 mg DCA/kg/day and 12.5, 25, or 50 mg TCA /kg/day. Other groups of mice received mixtures (Mix I, II and III) at DCA/TCA ratios corresponding to 7.5:12.5, 15:25, and 30:50 mg/kg/day, in Mix I, II and III, respectively. All

treatments including distilled water administration to controls were done by gavage, daily for 13 weeks. The animals were euthanized 24 hours after the last dose, using carbon dioxide anesthesia followed by cervical dislocation, and portions of the livers were weighed and used for the determination of SOD, CAT and GSH-Px activities, and for GSH level.

Homogenization of the hepatic tissues

Potter-Elvehjem homogenizer (Thermo Fisher Scientific Inc., Waltham, MA, USA) was used to prepare 10% w/v liver homogenates in sucrose buffer containing 0.32 mol L^{-1} Sucrose, 1 mmol L^{-1} EDTA, and 10 mmol L^{-1} Tris-HCl, centrifuged at $900 \times g$ for 30 min, and supernatants were used for the determination of SOD, CAT and GSH-Px activities (Jiang, Xu, and Klaunig 1998). Other homogenates (20% w/v) were prepared in 5% sulfosalicylic acid, centrifuged at $900 \times g$ for 5 min, the supernatants to be used for determination of GSH content (Anderson 1985).

Determination of SOD activity

The assay of hepatic homogenates for SOD activity was conducted according to Marklund and Marklund (1974), with modification. Mixtures of 200 μL of supernatant from sucrose liver homogenates, 750 μL of buffer containing 50 mmol L^{-1} Tris HCl, 50 mmol L^{-1} cacodylic acid, and 1 mmol L^{-1} EDTA, pH 8.2, and 250 μL 0.5 mmol L^{-1} pyrogallol. After mixing, the absorbances were immediately recorded at 420 nm, and then every 30 sec for 3 min using a spectrophotometer (model 20, Spectronic Instruments, Rochester, NY, USA). Slopes of the reactions were calculated and converted to SOD units, where one unit is equivalent to the quantity of SOD needed to entail 50% inhibition of pyrogallol autooxidation.

Determination of CAT activity

CAT activity was determined according to Cohen, Dembiec, and Marcus (1970), with modification. The assay included three reaction tubes, the blank, standard and sample that contained 100 μL sucrose buffer 100 μL deionized water, and 100 μL of supernatant from sucrose liver homogenates, respectively. One ml H_2O_2 (6 mmol L^{-1}) was added to the sample and blank reaction tubes, and 1 mL of deionized water was added to the standard tube. After vortexing, the reaction tubes were incubated in crushed ice for 3 min, followed by addition of 200 μL of 3 mol L^{-1} H_2SO_4 to each tube, 1.4 mL of an aqueous solution of KMnO_4 (2 mmol L^{-1}) was added, and absorbances were immediately recorded at 480 nm using a spectrophotometer (Biotek, Winooski, VT, USA). Absorbance values were converted to units of CAT, where a unit is equal to $k/0.00693$, and the first order reaction rate constant (k) = $\log (S_0/S_2) \times (2.3/t)$. The logarithmic conversion factor was 2.3, and t was equivalent to the incubation time (3 min). S_0 and S_2 were determined by subtracting the blank absorbance from the standard absorbance, and the sample absorbance from the standard absorbance, respectively (Jiang, Xu, and Klaunig 1998).

Determination of glutathione peroxidase (GSH-Px) activity

The assay described by Lawrence and Burk (1976) was employed, with modification. The reaction tubes contained 100 μL of supernatant from sucrose liver homogenates, 100 μL

phosphate buffer saline (PBS) containing 10 units of glutathione reductase, and 700 μL of mixture containing 1 mmol L^{-1} EDTA, 1 mmol L^{-1} NaN_3 , 0.2 mmol L^{-1} NADPH and 1 mmol L^{-1} GSH in PBS, pH 7.2. The tubes were vortexed, and 5 minutes later, the reaction was initiated by adding 100 μL of 0.2 mmol L^{-1} H_2O_2 . Absorbance was recorded at 340 nm, immediately and then every 30 sec over a period of 3 min, using a spectrophotometer (model 20, Spectronic Instruments, Rochester, NY, USA). Changes in the rate of absorbance were converted into nmoles of NADPH oxidized/min, using an extinction coefficient of $6.22 \times 10^3 \text{ L mol}^{-1} \text{ cm}^{-1}$.

Determination of total GSH

Total GSH (reduced-GSH + oxidized- GSSG) was determined as GSH equivalents by the recycling assay described by Anderson (1985). The assay required the following reagents: a stock buffer containing 143 mmol L^{-1} sodium phosphate and 6.3 mmol L^{-1} tetra sodium EDTA, pH 7.5, a daily buffer containing 0.248 mg/ml NADPH in stock buffer, and GSSG reductase solution containing 266 units /ml of stock buffer. Assay mixtures contained 10 μL of supernatant from sulfosalicylic acid liver homogenates, 700 μL daily buffer, 100 μL DTNB solution, and 190 μL deionized water. The mixture tubes were incubated in a water bath at 30°C for 15 min, after which, 10 μL GSSG-reductase solution was added to each tube. Absorbances of the mixtures were determined at 412 nm immediately, and then every 30 sec for 3 min, using a S spectrophotometer (model 20, Spectronic Instruments, Rochester, NY, USA). A standard curve for GSH was prepared following the same procedure for GSH determination in samples, but replacing the 10 μL of sample with 10 μL solutions containing 1–4 nmole of GSH. Results are reported as nmoles GSH/g tissue.

Protein Determination

The method of Lowry et al. (1951) was used for protein determinations in supernatants of the hepatic tissue homogenates with bovine serum albumin as standard.

Statistical Analysis

Data were analyzed using Microsoft Excel data analysis package^(R), and GraphPad Prism®. Data are expressed as means of 6 samples (animals) + S.D. A one-way analysis of variance (ANOVA) was used to determine the statistical differences between groups, followed by Tukey's post-hoc test. A significance level of $p < 0.05$ was used for all comparisons.

RESULTS

In figure 1 the effects of treatment with various doses of DCA, TCA and three mixtures of the compounds on SOD activity are shown. While DCA suppressed SOD at all doses the three mixtures resulted in dose-dependent increases in SOD activity. Increases in SOD activity in response to TCA alone were even greater.

The effects of DCA, TCA and mixtures on CAT activity are demonstrated in Figure 2. DCA did not result in any changes in CAT activity while TCA and the mixtures resulted in increases in enzyme activities which, however, were not significantly different when compared with each other.

In figure 3, the effects of DCA, TCA and mixtures on GSH-Px activity are demonstrated. Suppression in GSH-Px activity in response to TCA was dose-dependent while with DCA and the mixtures they were not. Also, mixtures produced significantly greater suppressions in enzyme activity, as compared with those induced by DCA and TCA.

The results of the effects of various doses of DCA, TCA and mixtures on hepatic GSH levels are presented in figure 4. While the lowest dose of DCA and all of the TCA doses did not result in significant changes in GSH levels, DCA doses of 25–50 mg/kg/day, and the three mixtures resulted in significant suppressions in the levels of this molecule. Figure 4 also indicates that GSH suppressions induced by the two DCA doses were not significantly different when compared with each other, but those observed with the three mixtures were dose-dependent.

DISCUSSION

Previous studies in the same mice treated and used for the current study have reported the following effects on hepatic OS: dose-dependent increases in response to DCA and TCA, additive effects in response to Mix I and II, and greater than additive effect by Mix.III (Hassoun et al. 2014). However, it was not clear why mixtures of different compositions produced different effects on hepatic OS.

SOD is an antioxidant enzyme causes SA dismutation to the more toxic reactive oxygen species (ROS), H_2O_2 , and it acts in concert with two other antioxidant enzymes, CAT and GSH-Px that detoxify H_2O_2 to water (Josephy, Mannervik, and DeMontellano 1997). It had been suggested that both, SA and H_2O_2 contribute to OS induction by DCA, TCA, and their mixtures (Hassoun, Cearfoss, and Spildener 2010; Hassoun et al. 2014). However, contribution of each species to the observed effects could vary depending on the production level of each species. For example, SOD and GSH-Px suppressions in response to DCA treatments with no changes in CAT activity may indicate low rate of SA conversion to H_2O_2 and a significant production and contribution of SA to the DCA effects on hepatic OS. However, increases in SOD and CAT activities, and suppressions of GSH-Px in response to TCA and mixture treatments indicate H_2O_2 overproduction from SA dismutation, partial H_2O_2 detoxification by CAT, and a significant production and contribution of H_2O_2 to their previously reported OS production in liver. In regard to the mixtures, the observed induction of significantly greater suppressions in GSH-Px activities, as compared with those induced by TCA may indicate significantly greater H_2O_2 production than that generated by the compound. Taken this together with the previously reported greater SA production in response to mixtures than in response to DCA (Hassoun et al. 2014), an equally significant contribution of both species to mixtures-induced OS is suggested.

While SA and H_2O_2 are demonstrated to contribute to the mixtures effects, the three mixtures induced similar effects on the three tested antioxidant enzymes. However, Mix I and II were previously shown to result in additive effects, while Mix III produced greater than additive effect on hepatic OS in mice after subchronic exposure (Hassoun et al 2014). This may indicate that ROS-induced modulation of antioxidant enzyme activities, while

contributing to the effects of compounds and mixtures, it does not contribute to differences among the mixtures effects.

GSH is an important antioxidant molecule that acts as a substrate for GSH-Px (Joseph, Mannervik, and DeMontellano 1997). GSH was determined as total rather than ratios of oxidized: reduced; hence treatments-induced GSH suppression indicate GSH depletion, which may have also contributed to the observed reductions in GSH-Px activities with those same treatments. However, the results indicating TCA-induced GSH-Px suppression, that is not associated with GSH depletion may suggest additional contributing mechanisms to suppression of GSH-Px. While DCA-induced GSH suppressions may indicate DCA major contribution to GSH depletion by the mixtures, the observation that mixtures II and III were associated with significantly greater GSH depletion than those observed with DCA suggests some other contributing factors to that. For example, it is likely that toxicokinetics of DCA and TCA were changed when the compounds were administered as mixtures, and those changes may have contributed to those effects. Previous studies proposed a reductive dechlorination pathway of metabolism for DCA (Stacpoole et al. 1990, 1998), and evidence for the existence of that pathway for DCA and TCA that is associated with transient generation of free radicals in mice was also provided (Larson and Bull 1992). DCA was also shown to undergo GSH-dependent metabolic pathway in the hepatic cytosol (Lipscomb et al. 1995; James et al. 1997) utilizing GSTZ1-1 (Tong, Board and Anders 1998) and acting as a mechanism-based inhibitor of GSTZ after repeated exposure (Gonzalez-Leon et al. 1999; Cornett et al. 1999). Hence, a greater inhibition by DCA of its own metabolism is expected to occur in response to Mix III, resulting in greater DCA accumulation than that occurring in response to the lower mixture concentrations. Accumulated DCA may then favor a reductive dehalogenation pathway of metabolism that is associated with free radicals and ROS overproduction, disturbing the oxidant-antioxidant balance and inducing ROS and/or free radicals production in response to TCA in mixtures.

In addition to the function of GSH as a substrate for GSH-Px that plays a major role in detoxifying H_2O_2 , it is also known to detoxify free radicals (Reed 1994). Therefore, the proposed changes in compounds toxicokinetics in mixtures and their contribution to differences in the production levels of ROS and free radical are suggested here to contribute to the observed dose dependency of mixtures-induced GSH depletion, as well as to the significantly greater mixtures-induced GSH depletions than that induced by DCA. Further studies on the toxicokinetics of DCA and TCA mixtures are required to assess that.

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Figure 1. SOD activity (units/ mg protein) determined in livers of mice treated subchronically with 7.5, 15 and 30 mg DCA/kg/day, 12.5, 25 and 50 mg TCA/kg/day, and mixture I, II and III of DCA/TCA ratios that correspond to 7.5/12.5, 15/25 and 30/50 mg/kg/day, respectively . Columns with non- identical superscript are significantly different ($p < 0.05$).

Figure 2.

CAT activity (units/ mg protein) determined in livers of mice treated subchronically with 7.5, 15 and 30 mg DCA/kg/day, 12.5, 25 and 50 mg TCA/kg/day, and mixture I, II and III of DCA/TCA ratios that correspond to 7.5/12.5, 15/25 and 30/50 mg/kg/day, respectively . Columns with non- identical superscript are significantly different ($p < 0.05$).

Figure 3.

GSH-Px activity determined as nmoles of NADPH oxidized/min/ mg protein in livers of mice treated subchronically with 7.5, 15 and 30 mg DCA/kg/day, 12.5, 25 and 50 mg TCA/kg/day, and mixture I, II and III of DCA/TCA ratios that correspond to 7.5/12.5, 15/25 and 30/50 mg/kg/day, respectively . Columns with non- identical superscript are significantly different ($p<0.05$).

Figure 4.

Amounts of total GSH (nmol/ g tissue) determined in livers of mice treated subchronically with 7.5, 15 and 30 mg DCA/kg/day, 12.5, 25 and 50 mg TCA/kg/day, and mixture I, II and III of DCA/TCA ratios that correspond to 7.5/12.5, 15/25 and 30/50 mg/kg/day, respectively . Columns with non- identical superscript are significantly different ($p < 0.05$).