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Vitamin E Restriction in the Diet Enhances Phagocytic Activation by Dichloroacetate and Trichloroacetate in Mice

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Abstract

The effects of a Vitamin E-restricted diet on the induction of phagocytic activation by dichloroacetate (DCA) and trichloroacetate (TCA) was investigated. Groups of B₆C₃F₁ male mice were either kept on standard diet (Std diet group) or diet that had the vitamin provided only by its natural ingredients (Low-E diet group). The animals in each diet group were administered 77 mg of DCA or TCA/ kg/day, or 5 ml/kg water (controls), by gavage, for 13 weeks. Thereafter, peritoneal lavage cells (PLC) were assayed for superoxide anion (SA), tumor necrosis factor (TNF)- α , and myeloperoxidase (MPO), as well as for the activities of the anti-oxidant enzymes superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-Px). SA and TNF α production, as well as MPO, SOD, CAT and GSH-Px activities were significantly increased in the cells from the Low-E diet group treated with the compounds as compared with cells from hosts in the Std-diet group that received the corresponding treatments. The results indicate that consumption of a Vitamin E-restricted diet enhances the induction of phagocytic activation by DCA and TCA, a mechanism that was previously suggested to be an initial adaptive/protective response against the compounds long-term effects.

Keywords

Chloroacetates; Peritoneal lavage cells; TNF-alpha; Myeloperoxidase; Vitamin E

Introduction

The haloacetates are toxic by-products formed during water chlorination. Dichloroacetate (DCA) and trichloroacetate (TCA) are among the most important ones since they are also formed *in vivo* as metabolites of trichloroethylene, a widely used industrial solvent and water contaminant (Hathway, 1980; Miller and Uden, 1983; Uden and Miller, 1983; Dekant et al, 1984; Green and Prout, 1985; Krasner et al., 1989). Concentrations of DCA and TCA in municipal water supplies have been measured at 30–160 μ g/L (Uden and Miller, 1983; Jolly, 1985), and concerns about potential risks from long-term exposure have arisen. Subsequent long-term toxicology studies showed these agents to be hepatotoxic and hepatocarcinogenic in rodents (Herren-Freund et al., 1987; Bull et al., 1990; DeAngelo et al., 1991; Daniel et al., 1992). In addition, induction of various biomarkers of oxidative stress, including production of reactive oxygen species (ROS), lipid peroxidation, and DNA

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damage, as well as a modulation of the activities of various anti-oxidant enzymes (e.g., superoxide dismutase [SOD], catalase [CAT], and glutathione peroxidase [GSH-Px]) in livers of mice were seen to be associated with acute and long-term exposure to DCA or TCA (Nelson et al., 1989; Larson et al., 1992; Parrish et al., 1996; Hassoun and Dey, 2008; Hassoun et al., 2010a; Hassoun and Cearfoss, 2011). These changes were now recognized as earlier hepatotoxic/ hepatocarcinogenic responses (Hassoun et al., 2010a; Hassoun and Cearfoss, 2011).

Phagocytic activation is associated with the production of ROS, including superoxide anion ($\bullet\text{O}_2^-$) (SA) and hydrogen peroxide (H_2O_2), activation of myeloperoxidase (MPO), and the production of tumor necrosis factor (TNF)- α (see reviews by Josephy, 1996; Klebanoff, 2005; Valledor et al., 2010). While the products of phagocytic activation can mediate tissue damage under certain circumstances, especially during the processes of inflammation and ischemic injury, they are also known to be major contributors to the host defense against bacterial infections and tumors (Klebanoff, 2005; Valledor et al., 2010).

DCA and TCA were found to induce SA over-production in the peritoneal lavage cells (PLC) of $\text{B}_6\text{C}_3\text{F}_1$ male mice within several hours of host exposure to acute doses (Hassoun and Dey, 2008). Both compounds also modulated the levels of various biomarkers of phagocytic activation in the PLC of mice and this activation was suggested to be an early adaptive/protective mechanism against their long term hepatotoxic/ hepatocarcinogenic effects (Hassoun et al., 2010b).

Vitamin E (α -tocopherol) is a major lipid-soluble anti-oxidant providing protection against oxidative stress induction in response to xenobiotics/disease states (see recent review by Wojcik et al., 2010). This role is mainly attributed to the vitamin's ability to inhibit the autooxidation of unsaturated fatty acids by scavenging the lipid free radicals (initiated primarily by ROS) through the phenolic group found on the chroman ring of the molecule. This, together with its lipid solubility render the vitamin useful in preventing oxidation within the plasma membranes (Boxer, 1986). The vitamin also plays an important role in immune system maintenance (de La Fuente et al., 1998, 2000; reviewed in Feki et al. [2001], Han et al. [2004], and Castellani et al. [2010]). In an attempt to investigate the role of this anti-oxidant in the induction of phagocytic activation by DCA and TCA (that was previously suggested to play a role in their associated hepatotoxicity; Hassoun et al., 2010b), the extent of activation induced by each agent was determined in the PLC from $\text{B}_6\text{C}_3\text{F}_1$ mice fed (for 13 weeks) a standard chow diet (Std-diet group) or a diet not supplemented with the vitamin, but with the vitamin present as a component of other natural ingredients in the feed (Low-E diet group).

Materials and Methods

Chemicals

Except for the Quantikine Mouse Immunoassay kit obtained from R&D Systems (Minneapolis, MN), all chemicals and reagents used here were purchased from Sigma-Aldrich (St. Louis, MO) and were of the highest grades available.

Animals and treatments

Based on previous studies that found them to be sensitive to the acute and long-term effects of DCA and TCA, $\text{B}_6\text{C}_3\text{F}_1$ male mice were purchased from Harlan Teklad (Indianapolis, IN) for use in this study. They were caged at 21°C with a 12 hr light/dark cycle and allowed to acclimate for 3 d prior to use in the experiments. Mice were 6 wk-of-age and weighing $\approx$ 20 g when they received the first dose of either compound. All experiments were conducted

according to a protocol approved by the University of Toledo Institutional Animal Care and Use Committee (IACUC).

For the experiments, the mice were divided into two groups. One group was maintained on a standard laboratory chow (Harlan) (Std diet); the other group was maintained on Custom Research (NIH-31 Mod) diet (Harlan) that was not supplemented, but instead had Vitamin E present as a component of the natural ingredients in the diet (Low-E diet). Vitamin E content in the Low E-diet was ≈ 20 – 30 IU/kg and ≈ 90 IU/kg in the Std E-diet. The mice in both groups were allowed free access to their respective feeds and to water.

While Vitamin E supplementation of the Std-diet is believed to provide optimal immunopotentiality, growth, and reproduction in mice (Yasunaga et al., 1982; Bensoussan et al., 1998), and only severe Vitamin E deficiency is known to induce myopathy (Gabriel et al., 1984; Thomas et al., 1993; Nier et al., 2006), the low E-diet was expected to place these mice into marginal Vitamin E deficiency with no appreciable health problems (Personal communication, Harlan Teklad). This was confirmed by the observations regarding behavior, weight gain, and other general health effects that were monitored on a daily basis during the 13-wk treatment regimen, i.e., there were no significant untoward effects noted in the Low E-diet group when compared with the Std-diet group.

DCA and TCA were dissolved in distilled water; the pH of each solution was adjusted to 7.0 by drop-wise addition of 1 M sodium hydroxide solution. Animals in each diet group were sub-divided into three sub-groups that were in turn treated daily (for 13 wk) by gavage with 77 mg DCA/kg, 77 mg TCA/kg, or 5.0 ml pH 7.0 distilled water/kg (control). These doses were based on previous studies demonstrating the hepatotoxic/-carcinogenic effects in B₆C₃F₁ male mice provided drinking water containing the compounds at doses ranging between 0.05–5 g/L for 60–75 wk (Herren-Freund et al., 1987; Bull et al., 1990; DeAngelo et al., 1991).

Based on the calculated time-weighted mean daily dose by DeAngelo et al. (1991), 0.5 g DCA/L in the drinking water was equivalent to 77 mg/kg/d, the threshold carcinogenic dose of this compound in B₆C₃F₁ mice. Previous sub-chronic studies on B₆C₃F₁ mice found this dose to also induce maximal levels of SA, TNF α , and MPO activity in their PLC (Hassoun et al., 2010b), but no apparent hepatomegaly (Hassoun et al., 2010a).

Collection of peritoneal lavage cells (PLC)

All mice were euthanized at the end of the treatment period using CO₂ anesthesia followed by cervical dislocation. A cut was made in the abdominal skin of the euthanized animals to expose the peritoneum and was inspected for signs of inflammations, such as sepsis, but no fluids accumulation/ sepsis were observed in any of the treatment groups. Three ml of a buffer (pH 7.3) containing 140 mM NaCl, 5 mM KCl, 10 mM glucose, 20 mM HEPES, and 2 mM CaCl₂ was then injected into the peritoneal cavity and the cavity was then massaged for 30 seconds to release the PLC which is commonly known to include the three types of phagocytic cells; the macrophages, monocytes and neutrophils. This procedure was done once on each mouse, and the fluid was with-drawn from the peritoneal cavity of each animal with a syringe and centrifuged at 1700 $\times$ g for 10 min.; after which the supernatant was removed and pellets were left on the bottom of the tubes. Because the rate of recovery of the injected fluid among different animals varied between 70–95%, it was not possible to rely on the weight of the pellets as a measure of comparison between different groups. The cell pellet was re-suspended in 2 ml of DMEM containing L-methionine, 200 mM L-glutamine, penicillin-streptomycin solution (1[0,000 U penicillin + 10,000 μ g streptomycin]/ ml, 1 M HEPES buffer, 10 mM MEM-NEAA, 100 mM sodium pyruvate solution, and 10% fetal bovine serum (FBS). The viability of the PLC was confirmed using Trypan blue exclusion

and a hemacytometer; all cells regardless of host treatment were uniformly found to be 98–99% viable. Cell suspensions were then used for the determination of tumor necrosis factor (TNF)- α and superoxide anion (SA) production, as well as of myeloperoxidase (MPO) and antioxidant enzyme activities, as described below.

Determination of TNF α

Aliquots (200 μ l) of each cell suspension were plated into 96-well plates and incubated 24 hr at 37°C in a humidified atmosphere containing 5% CO₂. After this period, aliquots of the culture supernatant in each well were removed and TNF α levels determined in the cell-free media using a Quantikine mouse TNF α immunoassay kit. The assay was conducted following manufacturer's protocols and the plates were analyzed at 450 nm in a μ Quant™ microplate reader (BioTek, Winooski, Vermont). TNF α levels in each sample were estimated by extrapolation from a standard curve prepared in parallel using serial dilutions of kit-provided recombinant murine TNF α .

Determination of superoxide anion (SA)

The cytochrome *c* reduction assay of Babior et al. (1973), with modifications, was used for the determination of SA production in harvested PLC. Briefly, each PLC suspension (50 μ l) was combined with 1.95 ml of a solution of 0.05 mM cytochrome *c* oxidase in phosphate-buffered saline (PBS, pH 7.2). The reaction tube was incubated for 15 min at 37°C, and the reaction then terminated by inserting the tube in crushed ice. The tube was then centrifuged at 3000 rpm (15 min); the supernatant fraction was collected and its absorbance recorded at 550 nm in a Spectronic 20 spectrophotometer (Spectronic Instrument, Rochester, NY). Absorbance values were converted to nmole cytochrome *c* reduced/min, using an extinction coefficient (ϵ) of $2.1 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$.

Determination of MPO activity

Cellular suspensions (0.5 ml) were freeze-thawed thrice, then centrifuged at 4,000 $\times$ g for 15 min, and the supernatants assayed according to the method of Bradley et al. (1982). The assay mixture contained 0.1 ml supernatant mixed and 2.9 ml of 50 mM phosphate buffer (pH 6.0) containing 0.167 mg *o*-dianisidine dihydrochloride/ml and 0.0005% (w/v) hydrogen peroxide. The change in the absorbance over 5 min was recorded at 460 nm in the Spectronic spectrophotometer. All absorbance values were then converted into MPO activity units, with one unit defined as the amount of enzyme degrading 1 μ mole H₂O₂/min at 25°C. An ϵ of 11,300 for oxidized *o*-dianisidine was used in these calculations.

Determination of anti-oxidant enzyme activities

Cellular suspensions were centrifuged at 3000 $\times$ g for 10 min and the pellets were resuspended in 2 ml sucrose buffer (0.32 M sucrose, 1 mM EDTA, and 10 mM Tris-HCl). The resuspensions were freeze-thawed, centrifuged at 4000 $\times$ g for 15 min, and the final supernatants used for determination of various anti-oxidant enzyme activities.

SOD activity was determined according to the method of Marklund and Marklund (1974) with modifications. Fifty μ l of supernatant was diluted by 150 μ l of sucrose buffer and mixed with 750 μ l Tris-cacodylic buffer (50 mM Tris-HCl, 50 mM cacodylic acid, 1 mM EDTA [pH 8.2]) followed by addition of 250 μ l of 0.5 mM pyrogallol. The absorbance of the mixtures was recorded at 420 nm for 3 min in the Spectronic spectrophotometer. Changes in rate of absorbance were calculated and converted into SOD activity units, with one unit equivalent to the quantity of SOD required to produce 50% inhibition of pyrogallol auto-oxidation.

CAT activity was determined according to the method of Cohen et al. (1970). In brief, the assay included blank, standard, and sample tubes that contained 100 μ l of sucrose buffer, 100 μ l of deionized water, or 100 μ l of supernatant, respectively. One ml of 6 mM H_2O_2 was added to each sample and blank tube, and 1 ml deionized water was added to the standard tube. The tubes were vortexed and then inserted in crushed ice for 3 min (t), after which 200 μ l of 6 N H_2SO_4 was added to each to stop the reaction. KMnO_4 (1.4 ml, 2 mM solution) was then added to each tube and their absorbance immediately measured at 480 nm in a Spectronic-20 spectrophotometer. Absorbance values were converted into CAT units with one unit equal to $k/0.00693$, where $k = \log (S_0/S_2) \times (2.3/t)$. S_0 and S_2 were determined by subtracting the blank and sample absorbance, respectively, from the standard absorbance (Jiang et al., 1998).

GSH-Px activity was determined in sucrose cell suspension according to the method of Lawrence and Burk (1976). The assay mixtures contained 100 μ l aliquot of supernatant, 700 μ l of reaction mixture (1 mM EDTA, 1 mM NaN_3 , 0.2 mM NADPH and 1 mM GSH in a PBS [pH 7.2] and 10 U glutathione reductase). The tubes were vortexed and incubated for 5 min at room temperature. At this point, 100 μ l of 0.2 mM H_2O_2 was added to initiate the reaction and the tube absorbance was recorded at 340 nm for 3 min using the Spectronic-20 spectrophotometer. The change in rate of absorbance was converted into nmole NADPH oxidized/min/mg protein, using an $\epsilon = 6.22 \times 10^3 \text{ L mol}^{-1} \text{ cm}^{-1}$.

Determination of protein

Protein contents were determined in the cell suspensions according to the method of Lowry et al. (1951), using bovine serum albumin as a standard. Accordingly, all data for each of the various biomarkers and enzymes assayed herein were expressed per mg protein.

Statistical Methods

Data are expressed as means of 7 samples (animals)/treatment $\pm$ SD. An Analysis of Variance (ANOVA) was used to determine the statistical differences between different groups with Scheffes' S-method used as a *post-hoc* test. Significance was accepted at a p-value of < 0.05 .

Results

The effects of DCA and TCA on SA production, MPO activity, and $\text{TNF}\alpha$ levels in the PLC of the Std and Low-E diet mice are shown in Figures 1, 2, and 3, respectively. DCA and TCA treatment resulted in significant increases in each of these parameters in the PLC of both diet groups as compared with in the PLC from corresponding non-treated counterparts (controls) of each diet group. The respective figures also show that compound-induced increases in SA production, MPO activity, and $\text{TNF}\alpha$ release by the cells from the Low-E diet mice were significantly greater than those produced by cells from counterparts in the Std diet group.

The effects of DCA and TCA treatments on different anti-oxidant enzyme activities in the PLC are demonstrated in Figures 4, 5, and 6. Treatment of the Std and Low-E diet mice with DCA or TCA resulted in significant increases in SOD activity, when compared to that in the PLC from the corresponding non-treated controls (Figure 4). The figure also indicates significantly greater increases in enzyme activity by each compound in the Low-E diet group, as compared against the effects seen in the cells from the treated Std diet group.

While DCA and TCA did not result in significant changes in GSH-Px and CAT activities in the PLC of animals in the Std diet group, they did cause significant increases in these

activities in the PLC of mice in the Low-E group (when compared with activities in PLC from corresponding non-treated controls for each diet group; Figures 5 and 6, respectively).

Discussion

Previous studies have shown that subchronic exposure of male B₆C₃F₁ mice to 77 mg DCA or TCA/kg/d resulted in maximal (compared with that from other tested doses) increases in various biomarkers of phagocytic activation, including SA, TNF α , and MPO activity, as well as in SOD activity (Hassoun et al., 2010b). Therefore this dose was chosen for the study, and the observed increases in the levels of those same biomarkers in the Std diet group are consistent with the previously reported ones.

Vitamin E is a major anti-oxidant known for its scavenging ability against free radicals (Wojcik et al., 2010). We surmise here that this ability helps to explain the significantly greater increases in DCA- and TCA-induced SA production observed in the PLC from mice in the Low-E diet group (compared with that by cells from Std diet mice). SA is produced during the respiratory burst of the polymorphonuclear (PMN) cells by the action of NADPH oxidase that catalyzes a one electron reduction of O₂ (Rossi, 1986; Boxer, 1986), and SA can be converted to H₂O₂ through SOD-mediated SA dismutation. Vitamin E can regulate mitochondrial H₂O₂ generation, and studies on the effects of Vitamin E deficiency on rat PMN cells function have demonstrated a greater metabolic activity in the tocopherol-deficient cells (Chow et al., 1999). This outcome was attributed to the membrane-associated NADPH oxidase now being in an abnormal lipid milieu that 'enhanced' its activation (resulting in formation of toxic concentrations of H₂O₂) (Harris et al., 1980; Boxer, 1986). These earlier observations, taken together with those here of significant increases in SOD activity in the PLC of the Low-E diet mice treated with the compounds, suggest greater H₂O₂ production in that group, as compared with cells from the Std diet group. CAT and GSH-Px act in concert with SOD and catalyze conversion of H₂O₂ to water. As such, the increases in anti-oxidant enzyme activities noted in the cells from Low-E diet mice treated with the compounds are suggestive of an anti-oxidant compensation for the partial loss of protection by the vitamin (though even still, these increases may not be sufficient to detoxify all the H₂O₂ produced in these cells).

MPO reacts with H₂O₂ in the presence of physiological concentrations of Cl⁻, resulting in formation of the powerful oxidant hypochlorous acid/hypochlorite (HOCl/OCl⁻) (Kettle and Winterbourn, 1989; Josephy, 1996). Components of the peroxidase-H₂O₂-halide system, including MPO, contribute to the killing of certain tumor cells (Clark et al., 1975; Klebanoff, 2005). The greater increases in MPO activity in the PLC of the Low-E diet mice treated with DCA or TCA may indicate production of sufficient H₂O₂ to keep up with the observed increases in MPO activity, and reflect the purported increases in H₂O₂ production in response to DCA and TCA in those host, as compared among counterparts in the Std diet group. While the increases in MPO and anti-oxidant enzyme activities could be due to up-regulation of the enzymes in response to lower Vitamin E concentration in the diet (coupled with each compound's induced oxidative stress), future determination of the enzymes levels themselves are required to confirm this.

MPO can act as immunoregulator, inducing the release of ROS, TNF α , and interferon (INF)- α/β , both *in vitro* and *in vivo* (Lefkowitz et al., 1992; 1993). This may support our results showing correlated DCA-/TCA-induced increases in MPO activity and TNF α in the PLC from both diet groups. However, TNF α can be a "double-dealer", i.e., it can be either a tumor promotor or a cancer cell killer (Wang and Lin, 2008), and MPO is known to contribute to the killing of certain types of tumor cells. These facts, together with those from pervious studies suggesting phagocytic activation by DCA and TCA in mice serves as an

early adaptive/protective mechanism against long-term hepatotoxic effects of these compounds (Hassoun et al., 2010b) may indicate there could be better protection of hosts against such long-term effects if they are maintained on a diet that is not supplemented with Vitamin. This is especially important when considering the peritoneal phagocytic cells as surrogates for Kupffer cells and can be further supported by preliminary studies in our lab demonstrating the induction by DCA and TCA of less significant oxidative stress, indicated by SA, lipid peroxidation and DNA damage in the livers of mice kept on a low-E diet, as compared with the corresponding treatment of a group kept on Std-E diet (Cearfoss and Hassoun, 2011).

While 77 mg DCA or TCA /kg/day was found to produce maximal induction of the biomarkers of phagocytic activation, doses corresponded to the hepatotoxic/ hepatocarcinogenic ones produced declines in those biomarkers (Hassoun et al., 2010 B). Accordingly, this study was designed with the expectation that consumption of vitamin E-restricted diet by the animals would result in declines in the biomarkers of phagocytic activation by a dose that was previously shown to induce maximal induction, but the results contradicted that expectation. Therefore, these findings may suggest further investigation of vitamin E restriction in diet on the compounds'-induced phagocytic activation associated with higher than the threshold dose for the hepatotoxic/ hepatocarcinogenic effects. Furthermore, Fuente et al. (2000) have demonstrated changes in phagocytosis and chemotaxis associated with phagocytic activations of peritoneal macrophages and lymphocytes of guinea pigs exposed to different levels of vitamin E in their diet. These studies warrant investigating such changes, as well as possible changes in the number/type of phagocytic cells in the PLC of mice fed low-E diet and exposed to DCA or TCA.

In summary, the results of these studies in B₆C₃F₁ mice indicated that non-supplementation of a diet with Vitamin E results in induction of significantly greater levels of phagocytic activation by subchronic exposure to DCA and TCA. Since induction of phagocytic activation was previously suggested to be an adaptive response to protect against DCA and TCA long term hepatotoxic effects, the reported changes in this study may in turn suggest better protection to be provided if the animals are fed a diet that is not supplemented with the vitamin. However, this conclusion should be interpreted carefully and not be generalized as a beneficial effect of withholding vitamin supplementation from the diet. This is especially so when weighing this very specific effect against DCA/TCA toxicity vs. the beneficial effects associated with the scavenging abilities of Vitamin E against various free radicals produced in response to various insults in cells of the body.

Highlights

- Dichloroacetate (DCA) and trichloroacetate (TCA) induce phagocytic activation
- Phagocytic activation was suggested to protect against their hepatotoxic effects.
- Vitamin E-restricted diet increases the compounds-induced phagocytic activation.
- Vitamin E-restricted diet may provide better protection against their hepatotoxicity.

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Figure 1. SA production in PLC of mice maintained on Std or Low-E diet, and treated subchronically with DCA or TCA

Values shown are in terms of cytochrome c reduced/min/mg protein present. Columns that are labeled with non-identical superscript are significantly different ($p < 0.05$).

Figure 2. MPO activity in PLC of mice maintained on Std or Low-E diet, and treated sub-chronically with DCA or TCA
Values are in terms of units/mg protein present at 25°C. Columns that are labeled with non-identical superscript are significantly different ($p < 0.05$).

Figure 3. TNFα production in PLC of mice maintained on Std or Low-E diet, and treated sub-chronically with DCA or TCA

Values shown are in terms of pg TNFα/mg protein present. Columns that are labeled with non-identical superscript are significantly different ($p < 0.05$).

Figure 4. SOD activity in PLC of mice maintained on Std or Low-E diet, and treated sub-chronically with DCA or TCA

Values are in terms of units/mg protein. Columns that are labeled with non-identical superscript are significantly different ($p < 0.05$).

Figure 5. GSH-Px activity in PLC of mice maintained on Std or Low-E diet, and treated sub-chronically with DCA or TCA
Values are in terms of units/mg protein. Columns that are labeled with non-identical super-script are significantly different ($p < 0.05$).

Figure 6. CAT activity in PLC of mice maintained on Std or Low-E diet, and treated sub-chronically with DCA or TCA

Values are in terms of units/mg protein. Columns that are labeled with non-identical superscript are significantly different ($p < 0.05$).