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Copper accumulation and lipid oxidation precede inflammation and myelin lesions in *N*,*N*-diethyldithiocarbamate peripheral myelinopathy

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Abstract

Dithiocarbamates have a wide spectrum of applications in industry, agriculture and medicine with new applications being actively investigated. One adverse effect of dithiocarbamates is the neurotoxicity observed in humans and experimental animals. Results from previous studies have suggested that dithiocarbamates elevate copper and promote lipid oxidation within myelin membranes. In the current study, copper levels, lipid oxidation, protein oxidative damage and markers of inflammation were monitored as a function of *N*,*N*-diethyldithiocarbamate (DEDC) exposure duration in an established model for DEDC-mediated myelinopathy in the rat. Intra-abdominal administration of DEDC was performed using osmotic pumps for periods of 2, 4, and 8 weeks. Metals in brain, liver and tibial nerve were measured using ICP-MS and lipid oxidation assessed through HPLC measurement of malondialdehyde in tibial nerve, and GC/MS measurement of F_2 isoprostanes in sciatic nerve. Protein oxidative injury of sciatic nerve proteins was evaluated through quantification of 4-hydroxynonenal protein adducts using immunoassay, and inflammation monitored by quantifying levels of IgGs and activated macrophages using immunoassay and immunohistochemistry methods, respectively. Changes in these parameters were then correlated to the onset of structural lesions, determined by light and electron microscopy, to delineate the temporal relationship of copper accumulation and oxidative stress in peripheral nerve to the onset of myelin lesions. The data provide evidence that DEDC mediates lipid oxidation and elevation of total copper in peripheral nerve well before myelin lesions or activated macrophages are evident. This relationship is consistent with copper-mediated oxidative stress contributing to the myelinopathy.

Keywords: Diethyldithiocarbamate; Lipid oxidation; Myelinopathy; Copper

Introduction

Human exposure to dithiocarbamates results from the numerous uses of these compounds. Indirect or unintentional exposures result from residues on food crops, in the occupational setting through their applications as pesticides, and in various industrial processes. Direct or intentional exposures also occur from therapeutic applications of dithiocarbamates. In addition to the use of disulfiram in alcohol aversion therapy (Eneanya et al., 1981), and *N*,*N*-diethyldithiocarbamate in the treatment of nickel carbonyl intoxication (Sunderman, 1979), a wide range of new medical applications for dithiocarbamates is currently being explored. Investigations have presented support for potential uses of dithiocarbamates in treating cocaine addiction (Sofuoglu and Kosten, 2005), inflammation (Fang et al., 2005), viral infections (Krenn et al., 2005; Si et al., 2005), and as adjuncts for chemotherapy (Bach et al., 2000). *In vitro* mechanistic studies have demonstrated the ability of dithiocarbamates to modulate numerous biological processes including apoptosis, oxidative stress, and transcription, providing the molecular basis for these proposed medical applications (Kang et al., 2001; Kimoto-Kinoshita et al., 2004).

One obstacle to the development of new dithiocarbamate-based therapeutic agents is their potential toxicity. Neurotoxicity

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has been observed in humans and in experimental animals; and at least two independent neuropathies, an axonopathy and a myelinopathy (Johnson et al., 1998; Tonkin et al., 2000) have been reported. Depending upon the chemical structure of the dithiocarbamate and route of exposure, some dithiocarbamates release sufficient CS_2 *in vivo* to produce identical protein cross-linking and morphological changes to those observed in CS_2 neurotoxicity following inhalation exposure (Johnson et al., 1998) supporting CS_2 as the proximate toxic species responsible for the dithiocarbamate-mediated axonopathy. However, the molecular processes underlying the myelinopathy are not well defined. Previous studies have reported increased levels of copper and lipid oxidation to be associated with dithiocarbamate-induced neurotoxicity (Calviello et al., 2005; Delmaestro and Trombetta, 1995; Tonkin et al., 2004) and correlative data have shown a relationship between copper levels in peripheral nerve and the severity of myelin injury produced by pyrrolidine dithiocarbamate and *N*,*N*-diethyldithiocarbamate (DEDC) in rodent models (Valentine et al., 2006; Valentine et al., 2007). Additionally, proteomic analysis of sciatic nerve proteins using this animal model has demonstrated significant elevations in expression levels of glutathione transferase isozymes within Schwann cells consistent with activation of the antioxidant response element pathways subsequent to DEDC exposure (Viquez et al., 2007). These observations coupled with the affinity of dithiocarbamates for copper have led to the hypothesis that dithiocarbamates bind copper and generate a lipophilic complex that accumulates and promotes lipid oxidation within myelin membranes leading to a subsequent myelinopathy. But because analyses have typically been performed at time points associated with advanced injury, i.e., myelin lesions identifiable by light microscopy, it is not clear whether the observed markers of oxidative stress result from a direct effect of dithiocarbamates and represent processes contributing to the development of myelin injury or alternatively are a result of the injury and associated inflammatory responses that occur with demyelinating injuries in general.

If copper accumulation and increased oxidative stress contribute to the myelin lesions produced by DEDC, then significant changes in these parameters are expected to precede structural changes. In the current study, copper levels, lipid oxidation, protein oxidative damage and markers of inflammation were monitored as a function of DEDC exposure duration in an established model for DEDC-mediated myelinopathy in the rat. Intra-abdominal administration of DEDC was performed using osmotic pumps for periods of 2, 4, and 8 weeks. At the end of each exposure period, tissues including peripheral nerve, liver and brain were collected. Metals in brain, liver and tibial nerve were measured using inductively coupled plasma-mass spectrometry (ICP-MS); and lipid oxidation was assessed through HPLC measurement of malondialdehyde (MDA) in tibial nerve and GC/MS measurement of F_2 isoprostanes in sciatic nerve. Protein oxidative injury of sciatic nerve proteins was evaluated through quantification of 4-hydroxynonenal (HNE) protein adducts using immunoassay, and inflammation monitored by quantifying levels of IgGs and activated macrophages using immunoassay and immunohistochemistry methods, respectively. Changes in these parameters were then correlated to the onset of structural lesions, as ascertained by light and electron microscopy, to delineate the temporal relationship of copper accumulation and oxidative stress in peripheral nerve to the onset of myelin lesions.

Materials and methods

Chemicals. 2ML4 Alzet® osmotic pumps were obtained from Braintree Scientific (Braintree, MA). Sodium *N*,*N*-diethyldithiocarbamate (DEDC) was obtained from Alfa Aesar (Ward Hill, MA). Glutaraldehyde and paraformaldehyde were obtained from Electron Microscopy Sciences (Ft. Washington, PA). Fetal bovine serum was obtained from Mediatech Inc. (Herdon, VA). Proteinase K and bovine serum albumin (BSA) were obtained from Sigma-Aldrich (St. Louis, MO). Dulbecco's phosphate buffered saline (1X PBS) was purchased from MP Biomedicals (Solon, OH). All solvents used were HPLC grade and purchased from Fisher Scientific (Pittsburgh, PA).

Animals and exposures. All treatments and procedures using animals were conducted in accordance with the National Institutes of Health *Guide for Care and Use of Laboratory Animals* and approved by the Institutional Animal Care and Use Committee of Vanderbilt University. Forty-four adult male Sprague-Dawley rats were obtained from Harlan Bioproducts (Indianapolis, IN) and caged at Vanderbilt University animal facilities in a temperature controlled room (21–22 °C) with a 12 h light–dark cycle, supplied with Purina Lab Diet 5001 and water *ad libitum*. After a 10–14 day acclimatization period, animals were exposed to DEDC at 0.3 mmol/Kg/day for 2, 4 or 8 weeks using intra-abdominal 2 mL 4-week Alzet osmotic pumps surgically implanted under anesthesia (100 mg/Kg ketamine with 8 mg/Kg xylazine ip). DEDC was delivered as an aqueous solution, the concentration of which was determined from the UV absorbance at 282 nm (ε=13,000 M^{-1} cm^{-1}). For the DEDC 8-week exposure groups, the osmotic pumps were replaced after 4 weeks to extend the exposure period to 8 weeks. Two exposure models were used. The first consisted of one control group and 2-, 4-, and 8-week exposure groups with n=4 for each group except for the 8-week DEDC exposure group, where n=5. The average starting weight of the 17 animals in the first exposure model was 362.8±9.7 g (SEM). Samples collected from the experimental animals in the first exposure model were used to analyze globin, F_2-isoprostanes, metals and peripheral nerve lesions. For the second exposure model 27 rats were assigned to either a control or exposure group of 2, 4, or 8 weeks, n=4 per group, except for the 8-week time point where n=5 (control), and n=6 (DEDC-exposed). The average starting weight of the animals for the second exposure model was 319.4±1.7 g (SEM). Samples collected from the animals in the second exposure model were used to analyze globins, HNE protein adducts, MDA, immunoglobulins (IgG and IgG2a), and macrophages. Sham pump implantation surgery was not performed on control animals.

Collection and analysis of tissue. At the end of each exposure period, control and exposed animals were deeply anesthetized with pentobarbital (100 mg/Kg body weight, ip), and exsanguinated by cardiac puncture. Whole blood (1 mL) was placed into heparinized vials for globin isolation. The animals were then perfused through the left ventricle of the heart with PBS (pH 7.4). For rats in the first exposure model, the left sciatic nerve was removed and immediately frozen in liquid nitrogen and stored at −80 °C for isoprostane analysis. The right sciatic nerve was immersed in 4% glutaraldehyde in 0.1 M PBS and used for histopathology. The liver, brain, and both posterior tibial nerves were immersed in 4% glutaraldehyde in 0.1 M PBS (pH 7.4) and stored at 4 °C for metal analysis. Metal analysis was performed by ICP-MS at the Diagnostic Center for Population and Animal Health at Michigan State University (East Lansing, MI). Copper, zinc, arsenic, selenium, manganese, molybdenum, iron, cadmium, and mercury were determined. For the rats in the second exposure model, the left sciatic nerve, left half of the brain cut sagitally, the left posterior tibial nerve and one half of the right posterior tibial nerve were removed and immediately frozen in liquid nitrogen and stored at −80 °C until further analysis. A liver section, the right half of the brain and the other half of the right posterior tibial nerve were immersed in 4% glutaraldehyde in 0.1 M PBS (pH 7.4) and stored at 4 °C. The right sciatic nerve was immersed in 4% paraformaldehyde and stored at 4 °C.