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A comparative study of protein carbonylation and mitochondrial dysfunction using the neurotoxicants 1,3-dinitrobenzene, 3-nitropropionic acid, and 3-chloropropanediol

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ABSTRACT

This comparative evaluation of neurotoxicants previously identified as models of chemical-induced mitochondrial dysfunction and energy deprivation demonstrated that subtoxic concentrations of 1,3-dinitrobenzene (1,3-DNB), 3-nitropropionic acid (3-NPA), and 3-chloropropanediol (3-CPD) each led to concentration-dependent loss of the mitochondrial membrane potential ($\Delta\Psi_m$) associated with similar patterns of protein carbonylation. Subtoxic concentrations of each neurotoxicant were determined by measuring DI TNC1 cell viability using the MTS cell proliferation assay. Although exposure 1 μM, 10 μM, and 100 μM concentrations of each toxicant did not result in loss of cell viability after 48 h, exposure to each toxicant at these concentrations led to concentration-dependent loss of tetramethyl rhodamine methyl ester (TMRM) fluorescence over the same exposure period. Preincubation with the antioxidant, deferoxamine, was effective in preventing loss of TMRM flurorescence. Through the combined use of two-dimensional polyacrylamide gel electrophoresis (2D PAGE) and Oxyblot analysis, this study demonstrated that exposure to each toxicant resulted in the formation of distinctly similar patterns of protein carbonylation comprised of specific proteins identified with tandem MS/MS. Our results provide insight as to how exposure to different neurotoxicants that enhance oxidative stress may, in fact, lead to mitochondrial injury and subsequent toxicity through selective, yet shared, pathways of protein modification by oxidative carbonylation.

1. Introduction

The chemical intermediate 1,3-dinitrobenzene (1,3-DNB) serves as a model toxicant for acute energy deprivation syndromes that affect the central nervous system and lead to the development of clinical symptoms dependent on the exposure amount and duration (Cody et al., 1981; Philbert et al., 2000). Exposure to 1,3-DNB causes symmetrical, bilateral gliovascular lesions distinctive of the brainstem regions affected in other chemical-induced mitochondrial encephalopathies, Leigh's syndrome, and Wernicke's encephalopathy (Philbert et al., 1987). The primary cellular target in the initial phase of 1,3-DNB intoxication is type 1 astrocytes found within the affected brainstem nuclei (Philbert et al., 1987; Romero et al., 1996). Although the molecular mechanism that underlies the selective nature of 1,3-DNB neurotoxicity is still undetermined, research findings support a mechanism that includes, but is not limited to, metabolic perturbation through the inhibition of pyruvate dehydrogenase and adenosine deaminase that could contribute to increased reactive oxygen species (ROS) production, loss of the mitochondrial membrane potential ($\Delta\Psi_m$) and induction of the mitochondrial permeability transition (MPT) (Romero et al., 1995; Tjalkens et al., 2000; Miller et al., 2011; Wang et al., 2012). 1,3-DNB-induced oxidative stress and mitochondrial dysfunction has also been linked to the oxidative carbonylation of specific proteins distributed within different intracellular locations (Steiner and Philbert, 2011). Overall, these findings suggest that oxidative stress and altered enzyme function, meditated through structural alteration, early in 1,3-DNB neurotoxicity may be indicative of downstream metabolic derangement and the development of cellular dysfunction.

The accumulation of proteins damaged by oxidative carbonylation is an established hallmark of pathology observed within the nervous system, the detection of which has been routinely used as an indication of increased oxidative stress within the cellular environment (Smith et al., 1998; Aksenov et al., 2001; Castegna

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et al., 2002; Choi et al., 2003; Madian and Regnier, 2011). Oxidative carbonylation alters protein structure and function through the irreversible, selective modification of specific amino acid residues (Dalle-Donne et al., 2006; Nyström, 2005). Oxidative stress overwhelms cellular mechanisms of ROS scavenging which increases the likelihood that susceptible proteins become structurally damaged by oxidative carbonylation. Another factor that determines protein susceptibility to carbonylation is the intracellular localization of a specific protein. Proteins located in close proximity to ROS-generating mechanisms, such as the mitochondrial protein complexes that mediate aerobic respiration, are prone to oxidative damage when metabolic disturbances lead to increased mitochondrial ROS production (Gibson, 2005; Sas et al., 2007). 1,3-DNB exposure stimulates increased superoxide anion production that occurs prior to functional loss of the $\Delta\Psi_m$ (Steiner and Philbert, 2011). This study demonstrated that exposure to 1,3-DNB resulted in the oxidative carbonylation of specific protein targets prior to the development of cellular manifestations of toxicity. The array of proteins oxidized during exposure to 1,3-DNB demonstrate how neurotoxicity may develop through a pathway that includes, but is not solely limited to, oxidation of mitochondrial proteins.

The purpose of the current study is to determine whether the pattern of 1,3-DNB-induced protein carbonylation is unique to the mechanism of 1,3-DNB neurotoxicity. This study was designed as a comparative analysis using other neurotoxicants that disrupt energy production through mechanisms that involve oxidative stress. Identifying whether protein carbonylation exists in other chemical-induced mechanisms of cellular toxicity may help elucidate (1) specific targets of protein carbonylation that drive the development of 1,3-DNB neurotoxicity or (2) if protein carbonylation exists as a non-specific outcome related to increased ROS production. The other neurotoxicants included in this study are 3-chloropropanediol (3-CPD) and 3-nitropropionic acid (3-NPA). 3-CPD is a metabolite of α-chlorohydrin and has been used as a model of chemical-induced energy deprivation syndromes (Cavanagh and Nolan, 1993; Willis et al., 2004; Brown et al., 2011). 3-CPD targets type 1 brainstem astrocytes and likely disrupts cellular function through alterations in redox state that promote increased oxidative stress (Skamarauskas et al., 2007). 3-NPA disrupts mitochondrial function through the suicide inhibition of succinate dehydrogenase and increased formation of ROS, in addition to promoting the formation of protein carbonylation in vivo (La Fontaine et al., 2000; Phelka et al., 2003; Huang et al., 2006; Sandhir et al., 2010).

We identified and implemented the use of subtoxic concentrations of each chemical that did not induce significant reductions in cellular viability, but would result in perturbations of mitochondrial function. By using subtoxic concentrations of each toxicant, our goal was to increase the likelihood of successfully identifying specific molecular targets of oxidative carbonylation that could potentially be masked during the onset of terminal pathological mechanisms that incorporate substantial increases in oxidative stress, leading to widespread protein oxidation (Bizzozero et al., 2006; Dasgupta et al., 2012). Identifying the ideal subtoxic concentrations for use in this study was performed by conducting concentration-dependent analyses over a 48-h exposure period using a MTS-based cellular viability assay. Analysis of mitochondrial function was performed using the fluorescent probe, TMRM. 2D PAGE followed by immunodetection was used to detect protein carbonylation in response to toxicant exposure. Carbonylated proteins were then identified using tandem mass spectrometry. The results of this study demonstrated that exposure to subtoxic concentrations of 1,3-DNB, 3-CPD, and 3-NPA toxicant resulted not only in concentration-dependent mitochondrial dysfunction, but also identified patterns of protein carbonylation that contained targets of oxidative carbonylation conserved among each treatment group along with specific proteins identified only within certain treatment groups.

2. Materials and methods

2.1. Chemicals

Cell culture media and additional cell culture materials were obtained from Gibco (Invitrogen, Carlsbad, CA). The CellTiter 96 MTS assay was obtained from Promega (Madison, WI). The flurorescent probe, TMRM, was obtained from Molecular Probes (Invitrogen Eugene, OR). Oxyblot immunodetection materials were obtained from Chemicon (Millipore, Billerica, MA). Secondary antibodies, 2D Clean-Up kits, and Destreak rehydration buffer were obtained from GE Healthcare (Piscataway, NJ). IPG Readystrips and 12% Tris–HCl Readygels were obtained from Bio-Rad (Hercules, CA). The BCA protein quantification reagents were obtained from Pierce (Thermo Scientific, Rockford, IL). PVDF membranes used for immunoblotting were obtained from Millipore (Billerica, MA). All remaining reagents were of analytical grade and were obtained from Sigma–Aldrich (St. Louis, MO).

2.2. Cell culture

DI TNC1 cells were obtained from the American Type Culture Collection (ATCC). The DI TNC1 cell line was established from cultured, type 1 astrocytes originating from 1-day old rat brain diencephalon tissue (Radany et al., 1992). Cells were maintained in a humidified incubator at 37 °C in an atmosphere of 5% CO_2. DMEM containing 4.5 g/L D-glucose, L-glutamine, 10% fetal bovine serum, and 1% penicillin–streptomycin–glutamine mixture was the media used for maintenance of cell growth. Cell culture passages 5–25 were used for all experiments.

2.3. MTS assay

The CellTiter 96 cell proliferation assay was used to measure concentration-dependent deficits in metabolic activity of DI TNC1 cells exposed to concentrations of 1,3-DNB, 3-NPA, and 3-CPD within a 48-h time period. The CellTiter 96 quantifies cellular proliferation, via colorimetric measurement, using a solution that contains a tetrazolium salt which is converted into formazan by cellular dehydrogenases within viable cells. DI TNC1 cells were cultured at a density of approximately 5000 cells/well in a 96-well tissue culture plate. Cultures were allowed to proliferate for 48 h, without reaching full confluence, before addition of 1 μM, 10 μM, 100 μM, 500 μM, and 1 mM concentrations of 1,3-DNB, 3-NPA, and 3-CPD in DMEM containing 10% FBS and 1% PSG. After each 48 h exposure period, the media from each well was replaced with equivalent volumes of serum-free, phenol-free DMEM containing 20 μL MTS/PMS solution/well. After 1.5 h of incubation, formazan production was measured at an absorbance of 490 nm in a Gemini SpectraMax spectrophotometer (Molecular Devices, Sunnyvale, CA). The mean absorbance measurements from four independent experiments per exposure group were graphed as percentages ± SEM of the DMSO (v/v) control.

2.4. Laser scanning confocal microscopy

Confocal microscopy was used to study the effect exposure to either 1,3-DNB, 3-NPA, and 3-CPD had on DI NTC1 mitochondrial function. DI TNC1 cells were grown on 22 mm round, glass coverslips and treated with 1 μM, 10 μM, and 100 μM concentration of each chemical for 48 h, using DMSO (% 0.05, v/v) as a vehicle control. Subsequent to each exposure period, $\Delta\Psi_m$ was measured