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Proteomic Identification of Carbonylated Proteins in 1,3-Dinitrobenzene Neurotoxicity

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Abstract

This study demonstrated that 1,3-dinitrobenzene-induced (1,3-DNB) oxidative stress led to the oxidative carbonylation of specific protein targets in DI TNC1 cells. 1,3-DNB-induced mitochondrial dysfunction, as indicated by loss of tetramethyl rhodamine methyl ester (TMRM) fluorescence, was initially observed at 5 h and coincided with peak reactive oxygen species (ROS) production. ROS production was inhibited in cells pre-treated with the mitochondrial permeability transition (MPT) inhibitor, bongkrekic acid (BKA). Pre-incubation with the antioxidant deferoxamine inhibited loss of TMRM fluorescence until 24 h after initial exposure to 1,3-DNB. Two-dimensional polyacrylamide gel electrophoresis (2D PAGE) and subsequent Oxyblot analysis were used to determine if 1,3-DNB exposure led to the formation of protein carbonyls. Exposing DI TNC1 cells to 1,3-DNB led to marked protein carbonylation 45 min following initial exposure. Pre-treatment with deferoxamine or Trolox reduced the intensity of protein carbonylation in DI TNC1 cells exposed to 1mM 1,3-DNB. Tandem MS/MS performed on protein samples isolated from 1,3-DNB-treated cells revealed that specific proteins within the mitochondria, endoplasmic reticulum (ER), and cytosol are targets of protein carbonylation. The results presented in this study are the first to suggest that the molecular mechanism of 1,3-DNB neurotoxicity may occur through selective carbonylation of protein targets found within certain intracellular compartments of susceptible cells.

Keywords

1, 3-dinitrobenzene; carbonylation; mitochondria; mitochondrial permeability transition; oxidative stress

1. Introduction

1,3-DNB is a chemical intermediate used to manufacture various industrial compounds including dyes, plastics and explosives. Exposure to 1,3-DNB can result in cyanotic levels of methemoglobin, decreased sperm count, as well as clinical signs of neurotoxicity including headache, vertigo, and ataxia (Philbert et al., 2000, Romero et al., 1995,

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Conflict of Interest

None declared

Tengowski et al., 2006). *In vivo* analysis of 1,3-DNB exposure demonstrated a symmetrical, bilateral lesion similar to the most sensitive regions of the central nervous system affected in a group of neurological disorders known as acute energy deprivation syndromes which include chemical-induced mitochondrial encephalopathy, Wernicke's encephalopathy, and Leigh's syndrome (Philbert et al., 1987, Tjalkens et al., 2000). The primary cellular targets of 1,3-DNB are Type 1 brainstem astrocytes, with secondary involvement of oligodendrocytes and neurons (Philbert et al., 1987). 1,3-DNB induced significant perturbations in metabolic function and increased ROS production within susceptible brainstem astrocytes (Romero et al., 1995, Tjalkens et al., 2000, Reeve et al., 2002, Romero et al., 1996). In *in vitro* models of neurotoxicity, 1,3-DNB exposure led to significantly increased levels of ROS, loss of the mitochondrial membrane potential ($\Delta\Psi_m$), ATP depletion, and onset of the MPT which preceded cell death. Decreased reduced glutathione content and increased production of superoxide anion in primary rat astrocyte cultures were early indications of disturbances in cellular redox potential that lead to progressive metabolic imbalance, a cellular state which also included increased glucose consumption and lactic acid production (Romero et al., 1995). Pre-treatment with the antioxidants α -tocopherol and deferoxamine significantly reduced 1,3-DNB-induced loss of the $\Delta\Psi_m$ in rat C6 glioma and SY5Y neuroblastoma cell lines (Tjalkens et al., 2000). The exact biochemical and molecular mechanism by which 1,3-DNB produces oxidative stress and mitochondrial dysfunction and how early increases in oxidative stress are causally linked to cellular damage in susceptible astrocyte populations is unclear. Whether ROS overproduction is the cause or result of energy deprivation syndromes has yet to be defined.

Oxidative stress is associated with a variety of pathophysiological outcomes resulting from toxicant exposure to age-related neurodegenerative diseases (Romero et al., 1995, Bulteau et al., 2007, Aksenov et al., 2001, Smith et al., 1998). Abnormal increases in the cellular production of ROS can overwhelm cellular antioxidant defense mechanisms and result in oxidative damage to highly susceptible macromolecules. One example of how oxidative stress can affect protein structure and lead to cellular damage is through the formation of protein carbonyls. Protein carbonylation is an irreversible, non-enzymatic form of post-translational protein modification that is commonly used as a marker of oxidative stress (Castegna et al., 2002, Dalle-Donne et al., 2006). Carbonylation alters protein function through selective modification of amino acid residues including histidine, proline, arginine and lysine. Several proteins that participate in cellular quality control and metabolism are known targets of oxidative carbonylation, the susceptibility of these proteins having been demonstrated in various research models that link oxidative stress to neurological disease and exposure to chemotherapeutic agents (England K et al., 2004, Andreazza et al., 2010, Sultana and Butterfield 2009, Joshi et al., 2010). Acute energy deprivation syndromes and certain neurodegenerative diseases, such as Alzheimer's disease, are similar with respect to how oxidative stress and mitochondrial dysfunction are implicated in the development of both forms of neuropathology (Romero et al., 1996, Ansari and Scheff 2010). Though the similarities likely end there, the oxidative carbonylation of key proteins in one form of neurological disease provides a rationale as to how oxidative stress may underlie the molecular mechanism in other disorders of the central nervous system through the irreversible alteration of protein structure which may lead to subsequent functional impairment of a particular neural cell type. The resemblance between the lesions that result from exposure to 1,3-DNB and the regional distribution of lesions that develop in other acute energy deprivation syndromes suggests a common cellular pathogenic mechanism, at least in the early stages, that may result from oxidative stress-induced protein modification. Like other nitroaromatic chemicals, 1,3-DNB undergoes a nitroreductive metabolism that, in the presence of molecular oxygen, produces superoxide anion (Reeve et al., 2002, Tay et al., 2005). Additional intracellular sources of ROS, such as mitochondria, could conceivably increase the oxidative burden of a cell thus increasing the likelihood that protein damage

may result from an oxidative mechanism. Increased protein carbonylation is known to occur in conjunction with increased mitochondrial ROS production (Nyström 2005). The involvement of oxidative stress and mitochondrial dysfunction in mitochondrial encephalopathy is also well-established (Tjalkens et al., 2000, Reeve et al., 2002, Romero et al., 1996).

1,3-DNB is currently used as a chemical model for studying the development of acute energy deprivation syndromes. This in vitro study was designed to elucidate how 1,3-DNB-induced oxidative stress and mitochondrial dysfunction may involve the oxidative modification of specific proteins through protein carbonylation. Confocal microscopy was employed in conjunction with a proteomic analysis of samples isolated from an immortalized cell line derived from Type 1 astrocytes to determine whether 1,3-DNB exposure results in the carbonylation of specific protein targets. Recent studies were successful in employing the use of 2D-PAGE followed by mass spectrometry in a proteomic approach to identify proteins carbonylated as the result of an oxidative insult (Castegna et al., 2002, England et al., 2004, Maisonneuve et al., 2009, Lii CK et al., 2010). Analysis of mitochondrial function and ROS production was performed using the fluorescent dyes TMRM and MitoSOX Red. 2D PAGE, followed by immunodetection, was used to isolate and identify the presence of carbonylated proteins. The proteins identified as targets of protein carbonylation were then, in turn, identified using tandem mass spectrometry. The results of this study revealed that 1,3-DNB exposure lead to the formation of carbonyl groups on specific proteins and that these structurally altered proteins may contribute to the development of 1,3-DNB neurotoxicity.

2. Methods

2.1. Chemicals

Tissue culture media and materials were obtained from Gibco (Invitrogen, Carlsbad, CA). CellTiter 96 MTS assay solution was obtained from Promega (Madison, WI). Fluorescent probes were obtained from Molecular Probes (Invitrogen Eugene, OR). The Oxyblot immunodetection kit was obtained from Chemicon (Millipore, Billerica, MA). Secondary antibodies for immunoblot visualization, 2D Clean-Up kits, and Destreak rehydration buffer were obtained from GE Healthcare (Piscataway, NJ). IPG Readystrips, 12% Tris-HCl Readygels, Protean IEF cell, and Mini-Protean III electrophoresis units were obtained from Bio-Rad (Hercules, CA). BCA protein quantification reagents were obtained from Pierce (Thermo Scientific, Rockford, IL). PVDF membranes were purchased from Millipore (Billerica, MA). All other reagents were of analytical grade and were obtained from Sigma-Aldrich (St. Louis, MO).

2.2. Cell Culture

DI TNC1 cells, obtained from the American Type Culture Collection, were maintained in a humidified incubator at 37°C in an atmosphere of 5% CO₂. DI TNC1 cells are an immortalized astrocyte cell line phenotypically characteristic of Type 1 astrocytes, from which they were derived (Radany et al., 1992). Cells were grown in DMEM containing 4.5 g/L D-glucose, L-glutamine, 10% fetal bovine serum, and 1% penicillin-streptomycin-glutamine mixture. Cells grown between passages 5 through 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 1,3-DNB within a 24 hr period. The CellTiter 96 solution contains a tetrazolium salt that is converted into formazan by

cellular dehydrogenases within metabolically active cells. DI TNC1 cells were sub-cultured at a density of 5000 cells/well in a 96-well tissue culture plate. Cultures were allowed to proliferate for 24 hrs before addition of 1 μ M, 10 μ M, 100 μ M, 500 μ M, and 1 mM concentrations of 1,3-DNB in DMEM containing 10% FBS and 1% PSG. After 24 hrs of exposure, the dosing media from each well was removed and replaced with serum-free, phenol-free DMEM containing 20 μ L MTS/PMS solution per well. After 1.5 hrs of incubation, MTS reduction to the formazan product was measured at an absorbance of 490 nm in a Gemini SpectraMax spectrophotometer (Molecular Devices, Sunnyvale, CA). Mean absorbance values from 4 independent experiments for each exposure group were graphed as percentages $\pm$ SEM of the 0.1% DMSO (v/v) vehicle control.

2.4. Laser Scanning Confocal Microscopy

Confocal microscopy was used to study the effect of 1,3-DNB on mitochondrial function in DI TNC1 cells. Cells were plated on 22 mm round, glass coverslips and treated with 1,3-DNB for 45 min, 5 h, 12 h, and 24 h using 0.1% DMSO (v/v) as a vehicle control. Following each exposure, $\Delta\Psi_m$ was measured by incubating the cells in 20 mM HBSS/HEPES buffer containing 500 nM TMRM for 15 min at 37°C. To determine whether limiting oxidative stress could inhibit 1,3-DNB-induced loss of $\Delta\Psi_m$, cells were pretreated with 600 μ M deferoxamine or 100 μ M Trolox for 20 min before incubation with 1 mM 1,3-DNB. The presence of ROS was determined using the fluorescent indicator, MitoSOX Red. Cells were plated on 22 mm round, glass coverslips and treated with 1,3-DNB for 45 min, 3 h, 5 h, 8 h, 12 h, and 24 h using 0.1% DMSO (v/v) as a vehicle control. 5 μ M BkA, which inhibits ADP-ATP translocase activity within mitochondria, was used to determine if blocking the permeability transition pore would inhibit production of ROS. Following each exposure, cells were loaded with 5 μ M MitoSOX Red in 20 mM HBSS/HEPES buffer for 10 min at 37°C. After individual groups of treated cells were incubated in buffer solution containing each respective dye, the coverslips were placed in a heated stage for live-cell imaging analysis. Confocal analysis was conducted using an Olympus Fluoview FV300 combined with an inverted Olympus IX-70 microscope. The confocal microscope was equipped with a system of Argon and HeNe lasers (Melles Griot, Carlsbad, CA) with emission parameters for TMRM and MitoSOX Red, each set at 543 nm for single wavelength capture. Fluorescent images were obtained using a 60X oil immersion lens at an exposure rate of 2.41 s/scan, in order to limit photobleaching of the dyes. About 2% of the maximum laser intensity was used for analyzing TMRM fluorescence, while about 10% of the maximum laser intensity was used for analyzing MitoSOX Red fluorescence. Mean fluorescence intensities from 3–4 independent experiments, ~7–10 cells per experiment, were measured using Adobe Photoshop and graphed using Microsoft Excel.

2.5. Protein Sample Preparation

DI TNC1 cells were grown to >90% confluence in 75 cm² flasks and exposed to 1 μ M and 1 mM concentrations of 1,3-DNB for 45 minutes using 0.1% DMSO (v/v) as a vehicle control. To determine whether limiting oxidative stress could inhibit the formation of protein carbonyls, cells were pretreated with 600 μ M deferoxamine or 100 μ M Trolox for 20 min before incubation with 1 mM 1,3-DNB. After exposure, mitochondria-enriched protein samples were isolated from each exposure group using a differential centrifugation protocol based upon the MITOISO1 kit for soft tissue. Cells from 6–7 flasks per exposure group were homogenized using a Teflon tissue grinder and glass pestle in 10 volumes of extraction buffer containing 2 mg/ml bovine serum albumin. The extraction buffer's composition consists of 220 mM mannitol, 70 mM sucrose, 0.5 mM EGTA, and 2 mM HEPES at a final pH of 7.4. The homogenate was transferred to a 15 ml conical tube and centrifuged at 500 $\times$ g for 5 min. The resulting supernatant was then removed, centrifuged at 11000 $\times$ g, and the pellet re-suspended in extraction buffer. This step was repeated two more times in order to

thoroughly wash the pellet. The protein pellet was re-suspended in storage buffer containing 10 mM HEPES, 250 mM sucrose, 1 mM ATP, 0.08 mM ADP, 5 mM sodium succinate, 2 mM K_2HPO_4 , and 1 mM DTT. A protease inhibitor cocktail was added to each sample before protein concentrations were quantified or the samples placed in storage at $-80^{\circ}C$. Protein concentrations were quantified using the BCA Protein Assay kit.

2.6. 2D PAGE

Aliquots of the protein sample containing 70 μg of protein were prepared for SYPRO ruby staining and detection of protein carbonyls through Oxyblot analysis. Protein samples designated for Oxyblot analysis were derivatized for approximately 15 min at room temperature in a 1X dinitrophenylhydrazine (DNPH) solution provided for in an Oxyblot immunodetection kit. This reaction derivatizes carbonyl groups located on protein side chains to 2,4-dinitrophenylhydrazones that can be detected using Western blot analysis. Upon completion of the derivatization reaction, excess salts and other impurities were removed from each set of samples using a 2-D Clean-Up kit. Precipitates were incubated in wash buffer for at least an hour before centrifugation at $12,000 \times g$ for 5 min. After the wash buffer was removed, 200 μl of Destreak rehydration buffer containing 2.5 $\mu l/ml$ IPG buffer was added to the protein pellet. A final centrifugation at $12,000 \times g$ was required in order to remove any insoluble material that might hinder isoelectric focusing. Each sample was then added to a ReadyStrip IPG Strip 7 cm pH 3–10. The IPG strips were actively rehydrated overnight in the IEF focusing tray of the Protean IEF cell using a current of 50 $\mu A/IPG$ strip at $20^{\circ}C$. Isoelectric focusing was performed overnight at a voltage of 4000 V for a minimum of 20,000 Vh at $20^{\circ}C$. Prior to separation in the second dimension, the IPG strips were equilibrated for 20 min in 0.375 M Tris-HCl (pH 8.8) containing 6 M urea, 2% SDS, 20% glycerol, and 2% (w/v) dithiothreitol. The IPG strips were then equilibrated for 20 min in 0.375 M Tris-HCl (pH 8.8) containing 6 M urea, 2% SDS, 20% glycerol, and 2.5% (w/v) iodoacetamide. The IPG strips were then placed on ReadyGels 12% Tris-HCl in Mini-Protean III systems for second dimension electrophoresis. For the second dimension separation, the gels were run at a constant voltage of 100 V for approximately 1.45 h.

2.7. SYPRO Ruby staining

The gels were fixed in a 50% methanol/ 7% acetic acid solution in two consecutive steps, with each step lasting for 30 min. Approximately 60 ml of SYPRO Ruby stain was added after the fixative was removed and the gels were stained overnight at room temperature. The gels were then washed in 10% methanol and 7% acetic acid and visualized in a Fujifilm FLA-5000 image acquisition system using Image Reader software.

2.8. Western Blot Analysis

Following separation in the second dimension, 2D gels designated for Oxyblot analysis were transferred to PVDF membranes. The nitrocellulose membranes were exposed to blocking buffer for 45 min and then incubated overnight at $4^{\circ}C$ in rabbit primary antibody specific to 2,4-dinitrophenylhydrazine (2,4-DNP, 1:150). An anti-rabbit, alkaline phosphatase-labeled secondary (1:10000), used for ECF-based immunodetection, was added following removal of the primary antibody and washing of the PVDF membranes. Images of carbonylated proteins were visualized using the Fujifilm FLA-5000 imaging system and Image Reader software. Densitometry measurements were conducted using Multi Gauge V2.2.

2.9. Tandem Mass Spectrometry

LC/MS/MS analysis and protocol were provided by the Michigan Proteome Consortium (www.proteomeconsortium.org). Images of both the Coomassie-stained 2D gels and 2D Oxyblots were overlaid on one another using bovine serum albumin (2 mg/ml) as a

reference marker (Spot number 8/Figure 7 and Table 1). Carbonylated proteins identified through 2D Oxyblot analysis were matched with protein spots from corresponding 2D gels, were then excised and digested with trypsin using a Micromass MassPrep robot. Target peptides were extracted from the gel plugs with 30 μ l of 2% acetonitrile (ACN) and 0.1% trifluoroacetic acid (TFA). 5 μ l of MALDI matrix was added to each extract and the extracts were dried using a Speed Vac. The peptides were dissolved in 5 μ l of 60% ACN and 0.1% TFA. An aliquot of each sample was spotted onto a MALDI target for MS/MS analysis. Protein identification was accomplished by matching peptide sequences using the International Protein Index (IPI) database.

2.10. Statistical Analysis

Spectrophotometric results from the MTS assay were graphed as % control with regard to tetrazolium reduction and were compared to the vehicle control using one-way ANOVA followed by a post-hoc Dunnett's test, with $p < 0.05$ representing a significant difference between the means. Results from the confocal microscopy analysis were expressed as the mean of the observed fluorescence intensity $\pm$ SEM and each fluorescent image was compared to the vehicle control using one-way ANOVA followed by a post-hoc Dunnett's test, with $p < 0.05$ representing a significant difference between the means. Carbonylation index ratios representing percent change in carbonylation status were calculated using densitometric analysis of carbonylated protein targets normalized to the amount of protein detected in SYPRO Ruby-stained 2D gels. Changes between the index ratios of vehicle control and treatment groups were compared using one-way ANOVA followed by a post-hoc Dunnett's test, with $p < 0.05$ representing a significant difference between the means.

3. Results

3.1. Metabolic activity in DI TNC1 cells exposed to 1,3-DNB

The concentration-response data depicting 1,3-DNB-induced deficits in the metabolic activity of DI TNC1 cells are shown in Figure 1. The reductive capacity of DI TNC1 cells exposed to 1,3-DNB diminished at 1 μ M 1,3-DNB, but the difference was determined to be statistically insignificant. Metabolic activity in the 10 μ M exposure group increased, but still remained below the level of activity demonstrated by the DMSO control group. Decreased MTS reduction followed a concentration-dependent pattern, with the first statistically significant drop in metabolic activity (~45%) occurring in the 1 mM 1,3-DNB exposure group.

3.2. ROS production and mitochondrial function in DI TNC1 cells exposed to 1,3-DNB

The fluorescent indicator probe MitoSOX Red was used to ascertain ROS production over a 24-hour period resultant from exposure to 1,3-DNB. The MitoSOX Red probe selectively migrates to the mitochondrial matrix where oxidation of the fluorophore by superoxide anion triggers fluorescence. Exposure to 1 mM 1,3-DNB led to increased production of ROS in a time-dependent and multiphasic manner (Figure 2 and Figure 3). ROS production reached a 25-fold peak (compared to VC) level at 5 h, which occurred in conjunction with the first indication of $\Delta\Psi_m$ impairment as observed by loss of TMRM fluorescence at 5 h (Figure 4 and Figure 5). MitoSOX Red fluorescence decreased after 5 h, but remained higher than the DMSO control. Pre-treatment with the MPT inhibitor, BkA, did not have a significant attenuating effect on ROS production when compared to the DMSO control. However, the levels of ROS in cell cultures pretreated with BkA prior to 1 mM 1,3-DNB exposure were perceptibly lower during the 45 min, 5 h, and 24 h exposure periods as compared to cells treated solely with 1,3-DNB.

Maintenance of the $\Delta\Psi_m$ was assessed using TMRM, a cationic fluorescent probe that selectively migrates to mitochondria sustaining membrane potentials. Robust TMRM fluorescence in healthy cells indicates the presence of an intact $\Delta\Psi_m$. Previous *in vitro* studies demonstrated that the application of antioxidants protected cells from loss of the $\Delta\Psi_m$ at 40 min and onset of MPT following exposure to 1,3-DNB at concentrations as low as 50 μ M (Tjalkens et al., 2000). Significant loss of TMRM fluorescence was initially observed in DI TNC1 cells exposed to 1,3-DNB for 5 h (Figure 4). Little TMRM fluorescence was observed after 24 h of continuous exposure to 1 mM 1,3-DNB, suggesting that 1,3-DNB exposure induces a time-dependent loss of the $\Delta\Psi_m$. Exposure to DMSO alone had no effect on TMRM fluorescence. DI TNC1 cells pretreated with 600 μ M deferoxamine exhibited a slight drop in TMRM fluorescence after being exposed to 1,3-DNB for 45 min, while pre-treatment of cells with 100 μ M Trolox delayed loss of the $\Delta\Psi_m$ until the 5 h mark. Trolox pre-treatment appeared to offer little, if any, protection against 1,3-DNB-induced loss of the $\Delta\Psi_m$. Both Trolox-treated cells and cells treated solely with 1,3-DNB demonstrated similar time-dependent patterns of TMRM fluorescence loss beginning at 5 h post initial exposure. With respect to the overall efficacy of the two antioxidants, deferoxamine was more effective at inhibiting loss of the $\Delta\Psi_m$ in that the first substantial decrease of TMRM fluorescence was not observed in deferoxamine-treated cells until after 24 h of continuous 1,3-DNB exposure.

3.3. Immunodetection of protein carbonyls

Confocal microscopic analysis of DI TNC1 cells exposed to 1 mM 1,3-DNB revealed that loss of $\Delta\Psi_m$ at 5 h occurred subsequent to and concomitant with significant production of ROS at 45 min and 5 h, respectively. 2D Oxyblots were used to determine whether ROS production prior to loss of the $\Delta\Psi_m$ altered the carbonylation status of proteins, specifically 45 min after initial exposure to 1,3-DNB. The oxidation of specific proteins in the presence of increased ROS production during the early stages of toxicant exposure may contribute to impaired mitochondrial function downstream, as exhibited by significant loss of the $\Delta\Psi_m$ at 5 h. Carbonylation index ratios were measured by normalizing the intensities of anti-2,4-DNP immunoreactivity observed in the 2D Oxyblots to the protein signatures in the 2D gels stained with Sypro Ruby in order to reduce the possibility that any changes in carbonylation intensity would be due to changes in protein concentration.

The 2D Oxyblot analysis revealed that several proteins were sensitive to 1,3-DNB-induced carbonylation at 45 min, as indicated by an intense pattern of anti-DNPH immunoreactivity (Figure 6). Carbonylation intensity is distinctly less in 2D Oxyblots represented in protein isolated from DI TNC1 cells exposed to only the DMSO control. Pre-incubation of DI TNC1 cells with the antioxidants, Trolox or deferoxamine, attenuated the intensity of protein carbonylation as compared to treatment with 1 mM 1,3-DNB alone. The negative control demonstrated that proteins isolated from DI TNC1 cells exposed to 1,3-DNB would not react with primary antibody unless the protein samples were derivatized with DNPH, thus emphasizing the specificity between the primary antibody and the 2,4-DNP moiety. In addition, comparing an Oxyblot to its corresponding 2D gel revealed that not all protein spots within a 2D gel exhibit carbonyl immunoreactivity (Figure 7). In order to determine if the number of proteins carbonylated is determined by a potential concentration-dependent relationship, DI TNC1 cells were incubated with 1 μ M 1,3-DNB for 45 min. Exposing the cells to a lower concentration of 1,3-DNB resulted in an overall decrease in number of detectable carbonylated proteins (Figure 8). The selectivity of proteins that appear to be targets of 1,3-DNB induced carbonylation, as demonstrated by the pattern of carbonylation, remained consistent throughout each treatment group.

3.4. Tandem mass spectrometric identification of carbonylated proteins

Targets of 1,3-DNB induced protein carbonylation were identified using tandem mass spectrometry. 2D Oxyblot images were matched with corresponding Coomassie-stained 2D gels containing protein isolated from DI TNC1 cells treated with 1mM 1,3-DNB for 45 min. Since the use of dinitrophenylhydrazine to derivatize protein carbonyls for immunodetection may interfere with mass spectrometric identification of proteins (Castegna et al., 2002), images of non-DNPH exposed proteins separated on 2D gels were overlaid and matched with proteins identified as carbonylated proteins using the Oxyblot analysis (Figure 7). Proteins not exposed to DNPH were subsequently excised from these 2D gels and designated for further identification. Several proteins demonstrated increased carbonyl immunoreactivity as indicated by the 2D Oxyblot analysis. Peptide sequences were generated using LC/MS/MS and then matched using the Mascot search engine and the IPI database.

The proteins identified as targets of oxidative carbonylation by LC/MS/MS analysis are listed in Table 1. Identification revealed a diverse assortment of proteins/enzymes that not only vary with respect to cellular function, but also with respect to intracellular localization (i.e. mitochondria, cytosol, and ER). The majority of these susceptible proteins have molecular mass values within the 50 kDa and 70 kDa range, while the pI values of these proteins are between 4 and 7. The predicted mass and pI values of the identified proteins are in agreement with the observed position of these proteins in the 2D gels. The total ion score of each protein listed in Table 1 was identified with greater than 95% confidence.

LC/MS/MS analysis was further applied to protein samples isolated from DI TNC1 cells exposed to 1 μ M 1,3-DNB for 45 min (Figure 8). There were fewer carbonylated proteins observed during the 2D Oxyblot analysis of proteins isolated from cells treated with 1 μ M 1,3-DNB for the same amount of time. Carbonylated mitochondrial proteins identified in both the 1 mM and 1 μ M 1,3-DNB treatment groups include the F1-ATP synthase beta-chain and mtHsp70. The carbonylation signal intensity ratios obtained for the mitochondrial proteins identified in both exposure groups are listed in Table 2. A comparison of the index ratios representing carbonylation intensity of the F1-ATP synthase beta-subunit between the different treatment groups demonstrated that this mitochondrial protein was particularly susceptible to 1,3-DNB-induced carbonylation and that antioxidant pre-treatment protected this protein from being carbonylated. Each treatment group designated for LC/MS/MS analysis was repeated three times in order to confirm protein identification.

4. Discussion

This study demonstrated the capability of 1,3-DNB to alter protein structure by oxidative carbonylation. DI TNC1 cells exposed to 1,3-DNB exhibited time-dependent increases in both ROS production and loss of the $\Delta\Psi_m$, in addition to a concentration-dependent increase in the number of proteins carbonylated. Antioxidants were effective in delaying 1,3-DNB-induced loss of the $\Delta\Psi_m$ and the MPT pore inhibitor, BkA, attenuated ROS production at 45 min, 5 h, and 24 h. Proteins were carbonylated within the first hour of 1,3-DNB exposure and pre-treatment with antioxidants prior to 1,3-DNB exposure inhibited the formation of protein carbonyls. Protein carbonylation occurred before loss of the $\Delta\Psi_m$, but during the production of ROS within the first 45 min of 1,3-DNB exposure. Fewer proteins were carbonylated when the cells were exposed to a lower concentration of 1,3-DNB, suggesting that a potential relationship between toxicant concentration and number of proteins prone to oxidative carbonylation may exist. This aforementioned relationship may be a function of the sensitivity individual proteins exhibit to oxidation. These findings not only confirm that the MPT adds to the oxidative burden of cells exposed to 1,3-DNB, but also further implicate oxidative stress as an important precursor event to the onset of the MPT and that

these alterations in oxidative status occur hours before mitochondrial dysfunction is observed. Whether or not these oxidative changes are required and/or are sufficient for induction of the MPT remains to be determined. Nonetheless, the detection of a small and conserved number of proteins acutely sensitive to oxidation leads to the tantalizing conclusion that modification of these proteins may regulate the function and fate of mitochondria in oxidative environments.

Exposure to 1,3-DNB can induce an early burst of ROS that is associated with downstream loss of the $\Delta\Psi_m$, onset of the MPT, ATP depletion, and cell death in susceptible brainstem astrocytes and immortalized cell lines (Romero et al., 1995, Romero et al., 1996, Tjalkens et al., 2000). In cultures of primary astrocytes exposed to increasing concentrations of 1,3-DNB that went as high as 2 mM, cell death was partially prevented by the addition of deferoxamine to the cell cultures, which indicated that 1,3-DNB-induced oxidative damage can contribute to cell injury (Romero et al., 1996). In addition, antioxidant pre-treatment is able to maintain mitochondrial calcium homeostasis by inhibiting 1,3-DNB-induced loss of mitochondrial calcium that occurred concomitantly with onset of MPT (Tjalkens et al., 2000). There is evidence to suggest that 1,3-DNB-induced cell death in susceptible cell types results from a cascade of events initiated by increased ROS production leading to mitochondrial injury and, ultimately, activation of mitochondrial cell death pathways through collapse of the $\Delta\Psi_m$, onset of the MPT, and altered expression of pro- and anti-apoptotic members of the Bcl-2 family of proteins (Tjalkens et al., 2000, Tjalkens et al., 2003, Phelka et al., 2006).

Metabolic function of DI TNC1 cells was initially analyzed by measuring cellular dehydrogenase activity over a 24 h-exposure period using different concentrations of 1,3-DNB. As indicated in the results, 1,3-DNB exposure led to a concentration-dependent decrease in metabolic activity over 24 h, though the first statistically significant drop in metabolic function did not occur until DI TNC1 cells were exposed to a relatively high concentration of (1 mM) 1,3-DNB. Although it is well-established that astrocytes are susceptible to 1,3-DNB both *in vivo* and *in vitro*, Romero et al., 1995 demonstrated that, in culture, 1,3-DNB concentrations fell to $79 \pm 2\%$ over 24 h and $45 \pm 6\%$ over 4 days (in the presence of astrocytes) and $93 \pm 1\%$ over 24 h and $69 \pm 7\%$ over 4 days (in the absence of astrocytes). The results from the study performed by Romero et al., 1995 indicated that 1,3-DNB is both actively and passively lost from culture even though the precise mechanism of passive 1,3-DNB depletion is unclear. This finding suggests that any time-dependent molecular or cellular disturbances observed in culture may result from 1,3-DNB concentrations that are actually less than the concentrations initially applied to the cells due to non-cellular, passive depletion of 1,3-DNB.

The results of this study demonstrated that 1,3-DNB-induced ROS production occurred in phases and followed a time-dependent mechanism. Exposure to 1 mM 1,3-DNB led to a significant increase in ROS at the earliest time point (45 min), followed by a substantial drop in ROS levels at 3 h. ROS production reached an apparent maximum at 5 h, which was followed by another transient drop in ROS levels at 8 h. From the 12 h time point and on, ROS production remained elevated above the vehicle control. At 24 h, ROS production reached a third and final peak level. Pre-treatment with the MPT inhibitor, BkA, attenuated ROS production during the 45 min, 5 h, and 24 h 1,3-DNB exposure periods. Although attenuated, ROS levels still remained elevated above the vehicle control. The robust levels of ROS at the 45 min, 5 h, and 24 h time points decreased dramatically as a result of pre-treatment with BkA, indicating that onset of the MPT had a greater impact on ROS production than exposure to 1,3-DNB alone and that the MPT may contribute to the non-uniform pattern of temporal ROS production. Previous studies that link superoxide production with the onset of MPT demonstrated that net increases in ROS production are

sensitive to MPT inhibitors and that threshold levels of oxidative stress are capable of initiating onset of the MPT which can lead to amplified, though transient, increases in ROS production (Tay et al., 2005, Zorov et al., 2006). However, two observations from this study suggest that MPT is likely not the only source of elevated ROS levels. The first observation is that even after pre-treatment with BkA, 1,3-DNB exposure led to elevated, if not significant, ROS production at each designated time point. The second observation is that at 12 h, elevated ROS production was not sensitive to BkA pre-treatment which suggests that significant ROS production may also occur through an as of yet undetermined, alternative mechanism.

The possibility that the initial burst of ROS observed at the 45 min mark plays an important role in disrupting mitochondrial function cannot be excluded. Though significant levels of ROS were detected prior to loss of the $\Delta\Psi_m$, exposure to 1 mM 1,3-DNB produced a time-dependent decay of the $\Delta\Psi_m$ that started approximately 5 h after initial exposure and coincided with peak production of ROS. Deferoxamine appeared to be the more cytoprotective of the two antioxidants used by delaying loss of TMRM fluorescence until the 24 h time point, when overall mitochondrial TMRM fluorescence was diminished due to almost global $\Delta\Psi_m$ collapse. The production of ROS before complete loss of $\Delta\Psi_m$ suggests that while mitochondrial dysfunction may contribute significantly to overall oxidative stress, induction of the MPT may not be required for initiation of ROS production and may not be the precipitating event for oxidation of cytosolic, ER, and mitochondrial proteins.

1,3-DNB exposure promotes an oxidative environment within a cell that increases the likelihood of oxidative carbonylation damaging susceptible proteins in different cellular compartments which, if antioxidant mechanisms are overwhelmed, can lead to an overall impairment of cellular homeostasis. Mitochondria contain an abundance of transition metals packaged within various macromolecules, such as iron-sulfur clusters, which could degrade during the onset of cellular pathology and result in mitochondrial proteins being exposed to conditions favoring metal-catalyzed oxidations (Kohen and Nyska 2002, Tangeras et al., 1980, Stadtman 1990). On the other hand, mitochondria are also equipped with a variety of antioxidant defense mechanisms, including superoxide dismutase (Kohen and Nyska 2002). Therefore, it is logical to ask whether or not in the face of a competent antioxidant system mitochondrial proteins that are acutely sensitive to carbonyl formation conspire to trigger irreversible formation of the MPT. This study confirmed results from previous research that demonstrated how pre-treatment with the iron-chelating antioxidant, deferoxamine, protected cells from 1,3-DNB induced loss of $\Delta\Psi_m$ as determined by progressive decay of TMRM fluorescence (Tjalkens et al., 2000). Since deferoxamine was more effective at inhibiting loss of the $\Delta\Psi_m$ than Trolox over the course of a 24 h period, it is possible that protecting against metal-catalyzed oxidation reactions is a particularly important deterrent against the formation of oxidative stress that may contribute to 1,3-DNB-induced mitochondrial dysfunction and triggering the MPT.

The results presented in this study are the first to show that altered protein structure through protein carbonylation accompanies ROS production and mitochondrial dysfunction in development of 1,3-DNB neurotoxicity. Tandem mass spectrometry permitted identification of two carbonylated mitochondrial proteins present in samples isolated from cells exposed to both low and high concentrations of 1,3-DNB: mtHsp70 and F1-ATP synthase beta-chain. Comparison of the carbonylation signal intensity ratios in Table 2 demonstrated that both concentrations of 1,3-DNB induced significant increases in carbonylation intensity of the F1-ATP synthase beta-chain as compared to the vehicle control. 1,3-DNB-induced carbonylation of the F1-ATP synthase beta-chain was reduced when cells were pretreated with deferoxamine and Trolox. In contrast, basal levels of carbonylated mtHsp70 appeared to be high in control cells and were not significantly increased as a function of exposure to

1,3-DNB. However, the true changes in the carbonylation status of mtHsp70 are likely masked because of its close proximity to the spot identified as bovine serum albumin, a known target of protein carbonylation (Stadtman, 1990). On one hand, the susceptibility of the F1-ATP synthase beta-chain to carbonylation compared to other mitochondrial proteins that are not carbonylated lends credence to the idea that structural differences or differences in the intracellular location between specific proteins may dictate the susceptibility of a protein to oxidative modification. On the other hand, this analysis illustrates some of difficulties in identifying all individually damaged proteins using 2D Oxyblot analysis in that lack of appropriate protein separation may hinder proteomic identification of certain peptide sequences, as is the case with mtHsp70 and bovine serum albumin.

Although the relationship between 1,3-DNB concentration and protein carbonylation resembles a concentration-dependent increase with respect to the number of proteins carbonylated, the same cannot be stated about the individual carbonylation status of either the F1-ATP synthase beta-chain or mtHsp70. The index ratios of both proteins described in Table 2 demonstrate that 1,3-DNB induced a higher intensity of protein carbonylation, for each specific protein, in DI TNC1 cells exposed to the lower concentration (1 μ M) than those exposed to the higher concentration (1 mM). Results such as these are counterintuitive to what might be observed in a typical concentration-response pattern. One possible explanation is that though it is qualitatively evident that the F1-ATP synthase beta-chain is prone to oxidative carbonylation, acquiring semi-quantitative measurements of protein carbonylation for specific proteins using 2D Oxyblot analysis followed by densitometric analysis may be sensitive to the variable changes that affect protein concentration in individual gels. For example, the concentration of specific proteins separated on a gel may fluctuate slightly caused by events such as protein loss during the initial 2D clean-up step of the original sample or transfer of the separated protein to the PVDF membrane prior to immunodetection.

Another explanation for the difference in carbonylation status between the 1 μ M and 1 mM 1,3-DNB exposure groups may involve a potential interaction between 1,3-DNB and intracellular quality control mechanisms. Carbonylated proteins exhibit an increased susceptibility to proteolytic degradation, when compared to non-oxidized proteins, and it has been suggested that carbonylated proteins may not require ubiquitinylation as a signal for proteasome-mediated degradation (Nyström 2005). In addition, Tengowski et al., 2007 reported that 1,3-DNB exposure led to altered expression of specific genes within the ubiquitin-proteasome pathway in a model of 1,3-DNB-induced testicular toxicity. The findings from these aforementioned studies, along with the results presented in this study, provide some rationale for suggesting that proteins carbonylated as a result of 1,3-DNB exposure become targets of proteolytic degradation mechanisms, the activity of which may be sensitive to 1,3-DNB. Increasing concentrations of 1,3-DNB may lead to increased proteolytic activity and subsequent degradation of carbonylated proteins, which could account for why the index ratios depicted in Table 2 do not follow a typical concentration-response mechanism of increasing carbonylation intensity. Inhibiting proteasome function may, in fact, lead to an increase in the amount of individual proteins damaged by oxidative carbonylation, thus resulting in measurable increases in carbonylation intensity. Future research analyzing proteasome activity as a function of exposure to 1,3-DNB may provide further clarification as to why proteins isolated from cells treated with a higher concentration of 1,3-DNB demonstrated less carbonylation intensity than proteins isolated from cells treated with lower concentrations of 1,3-DNB.

A third consideration with regard to oxidation status is the expression level of proteins that are targets of protein carbonylation. The concern surrounding carbonylated proteins isolated using the Oxyblot method is whether selective carbonylation is directly related to increased

protein expression. Several of the proteins listed in Table 1 are known to be upregulated in response to increased oxidative stress (Stacchiotti et al., 2009, Park et al., 2010). A quantitative analysis of 1,3-DNB-related protein expression for each protein in Table 1 employing methods for studying transcription (i.e. RT-PCR) and translation (i.e. Western Blot/densitometry) would be required to thoroughly address this particular concern, though it is a focus of current and future research in our laboratory. However, it is possible to make a qualitative assessment concerning the selectivity of protein carbonylation from the present study. A comparison of the 2D Oxyblots with the corresponding 2D gels represented in Figures 7 and 8 reveals a contrast between the numerous proteins that are expressed at various levels contained within the 2D gel and the small number of proteins that are actually carbonylated. In addition, the number of carbonylated proteins decreases when exposing the DI TNC1 cells to a lower concentration of 1,3-DNB (Figures 7 and 8), suggesting that both protein expression and/or carbonylation may be dependent on the concentration of 1,3-DNB. The presence of particular proteins identified as targets of carbonylation can provide clues as to the selective nature of protein carbonylation, while taking into account specific patterns of protein expression. For example, the catalytic activity of mitochondrial aconitate hydratase (spot 7 in Figure 7), not its expression levels, is known to be sensitive to changes in redox status (Cabiscol et al., 2000, Bulteau et al., 2003). Aconitate hydratase, as shown in Figures 6 and 7, only appeared carbonylated after exposure to 1 mM 1,3-DNB, not 1 μ M 1,3-DNB or the DMSO control. Though the influence of 1,3-DNB exposure on expression levels has not been determined, it is likely that the detectable presence of carbonylated aconitate hydratase is due to 1,3-DNB-induced increases in oxidative stress rather than an increase in protein expression.

1,3-DNB-induced protein carbonylation in DI TNC1 cells occurred not only in mitochondrial proteins, but also in proteins found within the ER and the cytosol. One cannot dismiss the notion that oxidative carbonylation of certain ER or cytosolic proteins may lead to mitochondrial dysfunction and downstream pathology. Communication between the ER and mitochondria mediated through direct contact via mitochondrial-associated membranes or through cytosolic signaling pathways are known to control normal cellular functions including protein-folding, calcium homeostasis, and apoptosis (Scheffler et al., 2001, Goetz and Nabi 2006, Walter et al., 2005, Kohen and Nyska 2002, Yoneda et al., 2004). Vimentin, protein disulfide isomerase, Grp78 and calreticulin were identified as targets of 1,3-DNB-induced carbonylation in this study and as targets of carbonylation in previous research focused on oxidative stress (Choi et al., 2003, Rabek et al., 2003). Increased carbonylation of Grp78 and the other targeted ER chaperones, PDI and calreticulin, could result in impaired protein assembly that disrupts ER function leading to dysfunctional calcium homeostasis. Carbonylation of calreticulin alone may disrupt the modulation of calcium binding, increasing the intracellular calcium concentrations that could feasibly lead to mitochondrial calcium overload and onset of the MPT. Vimentin is an intermediate filament protein that colocalizes with various organelles, including mitochondria (Katsumoto et al., 1990). Caspase-dependent cleavage of vimentin occurs during the cytoskeletal restructuring phase of apoptosis and is thought to be a precipitating event in the execution of the apoptotic signaling cascade (Byun et al., 2001). Carbonylation of vimentin during 1,3-DNB exposure may predispose susceptible cells to apoptosis by causing premature proteolysis of vimentin filaments and initiation of the apoptotic cascade.

In summary, this study provides important new insights into how oxidative stress may mediate the neurotoxicity of 1,3-DNB. Several previous reports confirm the involvement of oxidative stress during the development of nitrocompound-induced neuropathology. However, this report is the first to identify macromolecular targets of oxidative carbonylation that can potentially lead to a better understanding of the pathogenic mechanism underlying 1,3-DNB neurotoxicity. Whether the specific proteins identified as

targets of oxidative carbonylation in this study are the cause or result of the neurotoxic mechanism requires further analysis into how 1,3-DNB exposure may specifically affect the function of each protein. Future studies will need to address the functional role of these proteins and how the loss of function may predispose susceptible cells to developing acute energy deprivation syndromes as a result of chemical exposure or idiopathic disease.

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References

- Aksenov MY, Aksenova MV, Butterfield DA, Geddes JW, Markesbery WR. Protein oxidation in the brain in Alzheimer's disease. *Neuroscience*. 2001; 103:373–383. [PubMed: 11246152]
- Andreazza AC, Shao L, Wang JF, Young LT. Mitochondrial complex I activity and oxidative damage to mitochondrial proteins in the prefrontal cortex of patients with bipolar disorder. *Arch Gen Psychiatry*. 2010; 67(4):360–368. [PubMed: 20368511]
- Ansari MA, Scheff SW. Oxidative stress in the progression of Alzheimer's disease in the frontal cortex. *J Neuropathol Exp Neurol*. 2010; 69(2):155–167. [PubMed: 20084018]
- Bulteau A, Ikeda-Saito M, Szweda LI. Redox-dependent modulation of aconitase activity in intact mitochondria. *Biochemistry*. 2003; 43:14846–14855. [PubMed: 14674759]
- Bulteau A, Dancis A, Gareil M, Montagne J, Camadro J, Lesuisse E. Oxidative stress and protease dysfunction in the yeast model of Friedreich ataxia. *Free Rad Biol Med*. 2007; 42:1561–1570. [PubMed: 17448903]
- Byun Y, Chen F, Chang R, Trivedi M, Green KJ, Cryns VL. Caspase cleavage of vimentin disrupts intermediate filaments and promotes apoptosis. *Cell Death Differ*. 2001; 8:443–450. [PubMed: 11423904]
- Cabiscol E, Piulats E, Echave P, Herrero E, Ros J. Oxidative stress promotes specific protein damage in *saccharomyces cerevisiae*. *J Biol Chem*. 2000; 275(35):27393–27398. [PubMed: 10852912]
- Castegna A, Aksenov M, Aksenova M, Thongboonkerd V, Klein JB, Pierce WM, Booze R, Markesbery WR, Butterfield DA. Proteomic identification of oxidatively modified proteins in Alzheimer's disease brain. Part I: Creatine kinase BB, glutamine synthase, and ubiquitin carboxy-terminal hydrolase L-1. *Free Rad Bio Med*. 2002; 33:562–571. [PubMed: 12160938]
- Choi J, Malakowsky CA, Talent JM, Conrad CC, Carroll CA, Weintraub ST, Gracy RW. Anti-apoptotic proteins are oxidized by A β _{25–35} in Alzheimer's fibroblasts. *Biochim Biophys Acta*. 2003; 1637:135–141. [PubMed: 12633901]
- Dalle-Donne I, Aldini G, Carini M, Colombo R, Rossi R, Milzani A. Protein carbonylation, cellular dysfunction, and disease progression. *J Cell Mol Med*. 2006; 10:389–406. [PubMed: 16796807]
- England K, O'Driscoll C, Cotter TG. Carbonylation of glycolytic proteins is a key response to drug-induced oxidative stress and apoptosis. *Cell Death Differ*. 2004; 11:252–260. [PubMed: 14631408]
- Goetz JG, Nabi IR. Interaction of the smooth endoplasmic reticulum and mitochondria. *Biochem Soc Trans*. 2006; 34:370–373. [PubMed: 16709164]
- Joshi G, Aluise CD, Cole MP, Sultana R, Pierce WM, Vore M, St Clair DK, Butterfield DA. Alterations in brain antioxidant enzymes and redox proteomic identification of oxidized brain proteins induced by the anti-cancer drug adriamycin: implications for oxidative stress-mediated chemobrain. *Neuroscience*. 2010; 166:796–807. [PubMed: 20096337]
- Katsumoto T, Mitsushima A, Kurimura T. The role of the vimentin intermediate filaments in rat 3Y1 cells elucidated by immunoelectron microscopy and computer graphic reconstruction. *Biol Cell*. 1990; 68:139–146. [PubMed: 2192768]

- Kohen R, Nyska A. Oxidation of biological systems: oxidative stress phenomena, antioxidants, redox reactions, and methods for their quantification. *Toxicol Pathol.* 2002; 30:620–650. [PubMed: 12512863]
- Lii CK, Lin AH, Lee SL, Chen HW, Wang TS. Oxidative modifications of proteins by sodium arsenite in human umbilical vein endothelial cells. *Environ Toxicol.* 2010 Epub ahead of print.
- Maisonneuve E, Ducret A, Khoeiry P, Lignon S, Longhi S, Talla E, Dukan S. Rules governing selective protein carbonylation. *PLoS ONE.* 2009; 4(10):e7269. [PubMed: 19802390]
- Nyström T. Role of oxidative carbonylation in protein quality control and senescence. *Embo J.* 2005; 24:1311–1317. [PubMed: 15775985]
- Park B, Yang J, Yun N, Choe K, Jin BK, Oh YJ. Proteomic analysis of expression and protein interactions in a 6-hydroxydopamine-induced rat brain lesion model. *Neurochem Int.* 2010; 57:16–32. [PubMed: 20403401]
- Phelka AD, Sadoff MA, Martin BP, Philbert MA. BCL-XL expression levels influence differential regional astrocyte susceptibility to 1,3-dinitrobenzene. *Neurotoxicology.* 2006; 27:192–200. [PubMed: 16257055]
- Philbert MA, Nolan CC, Cremer JE, Tucker D, Brown AW. 1,3-dinitrobenzene-induced encephalopathy in rats. *Neuropathol Appl Neurobiol.* 1987; 13:371–389. [PubMed: 3683748]
- Philbert MA, Billingsley ML, Reuhl KR. Mechanisms of injury in the central nervous system. *Toxicol Pathol.* 2000; 28(1):43–53. [PubMed: 10668990]
- Rabek JP, Boylston WH 3rd, Papaconstantinou J. Carbonylation of ER chaperone proteins in aged mouse liver. *Biochem Biophys Res Com.* 2003; 305:566–572. [PubMed: 12763031]
- Radany EH, Brenner M, Besnard F, Bigornia V, Bishop JM, Deschepper CF. Directed establishment of rat brain cell lines with the phenotypic characteristics of type 1 astrocytes. *Proc Natl Acad Sci.* 1992; 89:6467–6471. [PubMed: 1378628]
- Reeve IT, Voss JC, Miller MG. 1,3-dinitrobenzene metabolism and GSH depletion. *Chem Res Toxicol.* 2002; 15:361–366. [PubMed: 11896683]
- Romero IA, Lister T, Richards HK, Seville MP, Wylie SP, Ray DE. Early metabolic changes during *m*-dinitrobenzene neurotoxicity and the possible role of oxidative stress. *Free Radic Biol Med.* 1995; 18:311–319. [PubMed: 7744316]
- Romero IA, Ray DE, Chan MWK, Abbott NJ. An *in vitro* study of *m*-dinitrobenzene toxicity on the cellular components of the blood-brain barrier, astrocytes and endothelial cells. *Toxicol Appl Pharmacol.* 1996; 139:94–101. [PubMed: 8685913]
- Scheffler NK, Miller SW, Carroll AK, Anderson C, Davis RE, Ghosh SS, Gibson BW. Two-dimensional electrophoresis and mass spectrophotometric identification of mitochondrial proteins from an SH-SY5Y neuroblastoma cell line. *Mitochondrion.* 2001; 1:161–179. [PubMed: 16120276]
- Smith MA, Sayre LM, Anderson VE, Harris PLR, Beal MF, Kowall N, Perry G. Cytochemical demonstration of oxidative damage in Alzheimer disease by immunochemical enhancement of the carbonyl reaction with 2,4-Dinitrophenylhydrazine. *J Histochem Cytochem.* 1998; 46:731–735. [PubMed: 9603784]
- Stacchiotti A, Morandini F, Bettoni F, Schena I, Lavazza A, Grigolato PG, Apostoli P, Rezzani R, Aleo MF. Stress proteins and oxidative damage in a renal derived cell line exposed to inorganic mercury and lead. *Toxicology.* 2009; 264:215–224. [PubMed: 19720107]
- Stadtman ER. Metal ion-catalyzed oxidation of proteins: biochemical mechanism and biological consequences. *Free Radic Biol Med.* 1990; 9:315–325. [PubMed: 2283087]
- Sultana R, Butterfield DA. Oxidatively modified, mitochondria-relevant brain proteins in subjects with Alzheimer disease and mild cognitive impairment. *J Bioenerg Biomembr.* 2009; 41:441–446. [PubMed: 19777328]
- Tangeras A, Flatmark T, Backstrom D, Ehrenberg A. Mitochondrial iron not bound in heme and iron-sulfur clusters. Estimation, compartmentation, and redox state. *Biophys Acta.* 1980; 589:162–175.
- Tay VKS, Wang AS, Leow KY, Ong M, Wong KP, Boelsterli UA. Mitochondrial permeability transition as a source of superoxide anion induced by the nitroaromatic drug nimesulide *in vitro*. *Free Rad Biol Med.* 2005; 39:949–959. [PubMed: 16140214]

- Tengowski MW, Feng D, Sutovsky M, Sutovsky P. Differential expression of genes encoding constitutive and inducible 20S proteasomal core subunits in the testis and epididymis of theophylline- or 1,3-dinitrobenzene-exposed rats. *Biol Reprod.* 2007; 76:149–163. [PubMed: 16988215]
- Tjalkens RB, Ewing MM, Philbert MA. Differential cellular regulation of the mitochondrial permeability transition in an *in vitro* model of 1,3-dinitrobenzene-induced encephalopathy. *Brain Res.* 2000; 874:165–177. [PubMed: 10960601]
- Tjalkens RB, Phelka AD, Philbert MA. Regional variation in the activation threshold for 1,3-DNB-induced mitochondrial permeability transition in brainstem and cortical astrocytes. *Neurotoxicology.* 2003; 24:391–401. [PubMed: 12782104]
- Walter L, Hajnóczky G. Mitochondria and endoplasmic reticulum: the lethal interorganelle cross-talk. *J Bioenerg Biomembr.* 2005; 37:191–206. [PubMed: 16167176]
- Yoneda T, Benedetti C, Urano F, Clark SG, Harding HP, Ron D. Compartment-specific perturbation of protein handling activates genes encoding mitochondrial chaperones. *J Cell Sci.* 2004; 117:4055–4066. [PubMed: 15280428]
- Zorov DB, Juhaszova M, Sollott SJ. Mitochondrial ROS-induced ROS release: an update and review. *Biochim Biophys Acta.* 2006; 1757:509–517. [PubMed: 16829228]

Figure 1.

Concentration-dependent decrease in MTS reductive capability of DI TNC1 cells exposed to 1,3-DNB was measured and graphed during a 24 h period. Data is expressed as % DMSO control $\pm$ SEM with statistical significance (*) if $p < 0.05$.

Figure 2.

1,3-DNB-induced production of ROS in DI TNC1 cells measured using laser scanning confocal microscopy. DI TNC1 cells were loaded with the fluorescent probe MitoSOX Red for 10 min after exposure to A) 1 mM 1,3-DNB or B) following pre-treatment with 5 μ M BkA for 20 min prior to 1 mM 1,3-DNB exposure. MitoSOX Red fluorescence was subsequently measured at each selected time point.

Figure 3.

Changes in MitoSOX Red fluorescence were measured and graphed as linear arbitrary units (LAU) in 3 or 4 independent experiments (~7–10 cells per experiment). The figure legend indicates which groups of DI TNC1 cells were treated with 1 mM 1,3-DNB alone and which groups were pre-treated with 5 μM of the MPT inhibitor, BkA, prior to 1 mM 1,3-DNB exposure. Values are expressed as mean ± SEM with statistical significance (*) when $p < 0.05$ compared to DMSO vehicle control (24 h).

Figure 4.

1,3-DNB-induced loss of $\Delta\Psi_m$ in DI TNC1 cells measured using laser scanning confocal microscopy. DI TNC1 cells were loaded with the fluorescent probe TMRM for 15 min after exposure to DMSO vehicle control (A) or 1 mM 1,3-DNB (B). Following pre-treatment with 600 μ M deferoxamine (C) or 100 μ M Trolox (D) for 20 minutes, cells were exposed to 1 mM 1,3-DNB and loss of $\Delta\Psi_m$ was measured.

Figure 5.

Loss of TMRM fluorescence was measured and graphed as LAU in 3 or 4 independent experiments (~7–10 cells per experiment). Values are expressed as mean $\pm$ SEM. Statistical significance is expressed using (*) when $p < 0.05$ compared to DMSO vehicle control (24 h).

Figure 6. 2D Oxyblot analysis of mitochondrial protein isolated from DI TNC1 cells exposed to A) DMSO control B) 1 mM 1,3-DNB C) 600 μ M Deferoxamine prior to 1 mM 1,3-DNB exposure D) 100 μ M Trolox prior to 1 mM 1,3-DNB exposure E) 1 μ M 1,3-DNB and F) negative control. Protein samples designated as negative controls were isolated from cells exposed to 1 mM 1,3-DNB *in vitro* and incubated in primary antibody without prior derivatization using DNPH.

Figure 7.

LC/MS/MS analysis was conducted on carbonylated proteins isolated using the 2D Oxyblot method after DI TNC1 cells were exposed to 1 mM 1,3-DNB for 45 min. Thirteen proteins were identified as targets of protein carbonylation. Protein names that correspond to each spot label and relevant information pertaining to each protein identified using LC/MS/MS are described in Table 1.

2D Oxyblot

2D gel

Spot Label	Protein
1	F1-ATP synthase subunit beta, mitochondrial precursor
2	Vimentin
3	Actin, cytoplasmic 2
4	Serum albumin precursor
5	mtHsp70, mitochondrial precursor
6	Grp78 kDa glucose-regulated protein precursor

Figure 8.

LC/MS/MS analysis was conducted on carbonylated proteins isolated using the 2D Oxyblot method after DI TNC1 cells were exposed to 1 μ M 1,3-DNB for 45 min. In contrast to the data presented in Figure 7, exposure to a lower concentration of 1,3-DNB resulted in fewer proteins identified as targets protein carbonylation. The six proteins identified in this portion of the analysis were also identified after exposure to 1mM 1,3-DNB and are also listed in Table 1, with a double asterisk (**) next to the name of each protein.

Table 1

Carbonylated proteins identified using LC/MS/MS in 1μM and 1mM 1,3-DNB, 45 min exposure.

Spot Label and Protein Name	Accession Number	Protein MW (kDa)	Protein pI	Best Peptide Sequence	Match Error (Da)	Total Ion Score C.I. %	Total Ion Score
1. Calreticulin precursor	IP100191728	48.1	4	IKDPDAAKPEDWDER	0.00	100	317.09
2. ** Vimentin	IP100230941	53.8	5.06	ISLPLPNFSSLNLR	-0.01	100	392.13
3. ** F1 ATP synthase beta-subunit, mitochondrial precursor	IP100551812	56.3	5	FTQAGSEVSALLGR	0.01	100	540.07
4. ** Actin, cytoplasmic	IP100764461	75.8	9	DLYANTVLSGGTTMYPGIADR	0.01	100	506.18
5. Aldehyde dehydrogenase, mitochondrial precursor	IP100197770	57.0	7	TIPIDGDFSYTR	-0.01	100	269.68
6. Aldehyde dehydrogenase, mitochondrial precursor	IP100197770	57.0	7	AAQAAFLGSPWR	0.00	100	255.74
7. Aconitate hydratase, mitochondrial precursor	IP100421539	86.1	8	NAVTFEGVPDPTAR	0.00	100	311.02
8. ** Bovine serum albumin precursor	IP100191737	70.7	6	ECCHGDLLECAADR	0.02	99.98	55.67
9. Protein disulfide-isomerase A3 precursor	IP100324741	57.5	6	EYDDNGEGITIFRPLHLANK	0.00	100	189.56
10. ** Mitochondrial heat shock protein 70, mitochondrial precursor	IP100363265	74.1	5.97	STNGDTFLGGEDEFQALLR	-0.01	100	677.37
11. ** Grp78 kDa, glucose-regulated protein precursor	IP100206624	72.5	5.07	DNHLLGTFDLTGPPAPR	-0.02	100	972.08
12. Tumor rejection antigen gp96	IP100734561	74.4	5	FQSSHSTDTISLDQYVER	0.00	100	382.54
13. Protein disulfide-isomerase precursor	IP100198887	57.3	4.82	VDATEESDLAQYGVGR	-0.01	100	703.34

Double asterisks (**) before protein name indicate carbonylated proteins identified in 1μM 1,3-DNB, 45 min exposure group. Proteins were identified by the Michigan Proteome Consortium, using the IPI database.

Table 2

Carbonylation index ratios of individual mitochondrial proteins identified after exposing DI TNC1 cells to DMSO, 1 μ M 1,3-DNB, 1 mM 1,3-DNB, and pretreatment with the antioxidants, deferoxamine or Trolox, prior to 1 mM 1,3-DNB exposure.

Protein	DMSO	1 μ M 1,3-DNB	1 mM 1,3-DNB	600 μ M Deferoxamine	100 μ M Trolox
F1-ATP synthase beta-subunit	19.74 $\pm$ 0.09	* 54.21 $\pm$ 6.28	* 45.93 $\pm$ 2.41	20.39 $\pm$ 3.71	12.59 $\pm$ 1.67
mtHsp70, mitochondrial precursor	49.81 $\pm$ 6.22	65.77 $\pm$ 2.67	46.28 $\pm$ 1.84	63.68 $\pm$ 0.95	29.41 $\pm$ 0.2

Index ratios represent the percent change in carbonylation status using densitometric analysis of carbonylated protein targets normalized to densitometric intensity of total protein detected in SYPRO Ruby-stained 2D gels. Changes in carbonylation intensity between the index ratios of vehicle control and treatment groups were expressed as percentages $\pm$ SEM (n= 3, 6–10 pooled biological replicates for each group) with p < 0.05 representing a significant difference (*) between the means.