NeuroToxicology 40 (2014) 65–74

Contents lists available at ScienceDirect

NeuroToxicology

# Nicotinamide adenine dinucleotide prevents neuroaxonal degeneration induced by manganese in cochlear organotypic cultures

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ARTICLE INFO

*Article history:*
Received 9 September 2013
Accepted 25 November 2013
Available online 3 December 2013

*Keywords:*
Manganese
Ototoxicity
NAD
Spiral ganglion neurons
Hair cells
Apoptosis

ABSTRACT

Manganese (Mn) is an essential trace mineral for normal growth and development. Persistent exposures to high atmospheric levels of Mn have deleterious effects on CNS and peripheral nerves including those associated with the auditory system. Nicotinamide adenine dinucleotide (NAD) is a coenzyme which functions in the electron transfer system within the mitochondria. One of the most notable protective functions of NAD is to delay axonal degenerations caused by various neurodegenerative injuries. We hypothesized that NAD might also protect auditory nerve fibers (ANF) and SGN from Mn injury. To test this hypothesis, cochlear organotypic cultures were treated with different doses of Mn (0.5–3.0 mM) alone or combined with 20 mM NAD. Results demonstrate that the percentage of hair cells, ANF and SGN decreased with increasing Mn concentration. The addition of 20 mM NAD did not significantly reduce hair cells loss in the presence of Mn, whereas the density of ANF and SGN increased significantly in the presence of NAD. NAD suppressed Mn-induced TUNEL staining and caspase activation suggesting it prevents apoptotic cell death. These results suggest that excess Mn has ototoxic and neurotoxic effects on the auditory system and that NAD may prevent Mn-induced axonal degeneration and avoid or delay hearing loss caused by excess Mn exposure.



## 1. Introduction

Manganese (Mn) is an essential trace metal required for normal growth and development and cellular homeostasis. Mn serves as a discrete cofactor for many enzymes including glutamine synthetase, superoxide dismutase, and pyruvate carboxylase (Rivera-Mancia et al., 2011). However, excessive exposure to Mn is linked with a severe and debilitating disorder known as manganism (Olanow et al., 1996; Pal et al., 1999). Manganism usually results from extended occupational exposure to Mn (e.g., Mn miners, welders, and individuals living near ferroalloy plants and battery factories) and decreased Mn excretion in diseased liver (Bouchard et al., 2007; Bowler et al., 2007; Lucchini et al., 2000, 2007). Symptoms of manganism are characterized by extrapyramidal disturbances similar to those seen in Parkinson's disease (Dobson et al., 2004); however, manganism deviates from Parkinsonism in its early clinical stages including differences in behavioral and cognitive function, symmetrical effects, milder tremors at rest, little or no response to L-DOPA, and prominent histological damage to the globus pallidus rather than substantia nigra pars compacta (Calne et al., 1994; Olanow, 2004; Pal et al., 1999). Recent studies have further suggested that Mn may have deleterious effects on hearing. Studies in our laboratory using cochlear organotypic cultures isolated from rats showed that Mn produced significant damage to peripheral auditory nerve fibers, the sensory hair cells (more toxic to inner hair cells than outer hair cells), and SGN (Ding et al., 2011). Sensory hair cells were slightly more resistant to Mn toxicity than the auditory nerve fibers.

Management of Mn-induced ototoxicity has yet to be established; however by understanding the mechanisms of damage may facilitate the development of new therapeutic strategies. It is generally accepted that Mn promotes cell death via degeneration of mitochondria leading to apoptosis. Mitochondrial dysfunction is brought about by excess accumulation of $Ca^{2+}$ produced by Mn inhibition of sodium-dependent and sodium-independent $Ca^{2+}$ exporters within the mitochondria (Gavin et al., 1992, 1999; Gunter et al., 2006). This subsequently results in uncoupling of the respiratory chain and activation of the permeability transition pore (PTP) allowing diffusion of low-molecular weight solutes into the mitochondria resulting in its swelling and the subsequent loss of cytochrome c. Ultimately, this leads to the generation of reactive oxygen species (ROS) which subsequently activates many of the

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0161-813X/$ – see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.neuro.2013.11.007

classical signaling pathways associated with programmed cell death including increased TUNEL staining, internucleosomal DNA cleavage, activation of the JNK and p38 pathways (stress activated protein kinase) and activation of caspase-3 (Chun et al., 2001; Desole et al., 1996, 1997; Hirata et al., 1998; Latchoumycandane et al., 2005; Roth et al., 2000; Schrantz et al., 1999). In addition, Mn also interferes with oxidative phosphorylation by inhibiting both mitochondrial F1-ATPase (Gavin et al., 1992, 1999) and complex I (Galvani et al., 1995) leading to the depletion of ATP (Chen and Liao, 2002; Roth et al., 2000). Consistent with the fact that Mn-induced cell death involves oxidative stress is the observation that treatment with antioxidants can prevent Mn toxicity (Migheli et al., 1999).

Oxidized and reduced forms of NAD serve as coenzyme in all living cells and can be synthesized in the human body from tryptophan and aspartic acid or supplemented in vivo via intake of niacin, which is commonly used as a dietary supplement and is easily accessible to the general public (Belenky et al., 2007). Besides involvement in redox reactions within the mitochondria, NAD is also utilized in metabolic events including ADP-ribosylation, poly (ADP-ribose) polymerization, cADP-ribose synthesis, and sirtuins, a group of enzymes that function primarily in reversing acetyl modifications of lysine on histones and other proteins (Sauve, 2008). Several studies have found that NAD can delay axonal degenerations caused by various diseases, including traumatic brain injury, ischemia damage, autoimmune encephalomyelitis, p53-induced neuron apoptosis, and radiation-induced immunosuppression (Belenky et al., 2007; Sauve, 2008). Although Mn has been found to damage the neurons and sensory cells in the inner ear, no studies have been conducted to explore the possibility that NAD may protect against Mn ototoxicity. Since NAD can attenuate mitochondrial oxidative damage in rat brain, we hypothesized that it would prevent ototoxicity induced by excessive exposure to Mn. Thus, using the Mn ototoxicity model of rat cochlear organotypic cultures as reported in our previous study (Ding et al., 2011), we explored the in vitro protective effects of NAD on sensory hair cells (HC), auditory nerve fibers (ANF), and SGN.

## 2. Materials and methods

### 2.1. Animal subjects

Postnatal day 3 SASCO Sprague-Dawley rats purchased from Charles River Laboratories were used for this study. The experiments were carried out according to the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and approved by the University at Buffalo Animal Care and Use Committee.

### 2.2. Cochlear organotypic cultures, $MnCl_2$ and NAD

Rat cochlear organotypic cultures were prepared as described previously (Ding et al., 2002; McFadden et al., 2003). The cochleae were carefully dissected out and the whole basilar membrane containing the organ of Corti, auditory nerve fibers and SGN were transferred onto rat tail collagen gel in the culture dish (Type 1 collagen, 3.76 mg/ml in 0.02 N acetic acid, 10× basal medium Eagle, 2% sodium carbonate, 9:1:1 ration). Serum-free culture medium (1.3 ml, 0.01 g/ml bovine serum albumin [Sigma A-4919], 1% Serum-Free Supplement [Sigma I-1884], 2.4% of 20% glucose, 0.2% Penicillin G, 1% 200 mM glutamine, 95.4% 1× basal medium Eagle [Sigma B-1522]) was added to each culture dish. The whole basilar membrane prepared as a flattened preparation was placed on the surface of the collagen gel and then maintained in an incubator (37 °C, 5% $CO_2$) overnight. On the second day, the culture medium was removed and exchanged with fresh medium plus the desired compounds specific for each experimental protocol. The cultures were exposed to various concentrations of $MnCl_2$ in the presence or absence of 20 mM NAD and incubated for various times at 37 °C in 5% $CO_2$. The concentrations of Mn used in these experiments were selected to be comparable to that employed in our laboratory (Ding et al., 2011; Lin et al., 1993) and others (Giordano et al., 2009; Hernandez et al., 2011; Rovetta et al., 2007) using a variety of cell cultures systems. The concentration of NAD employed in this paper are similar to that used previously to examine protection against neuronal degeneration (Wang et al., 2005) and cell death (Alano et al., 2004; Ding et al., 2012; Ying et al., 2003).

### 2.3. Staining: auditory nerve and cochlear hair cells

Cells were removed from the culture dishes at the end of each treatment, fixed for 2 h in 10% formalin at room temperature, and rinsed three times in 0.1 M phosphate buffered saline (PBS). As described in our previous publications (Ding et al., 2002; McFadden et al., 2003, #3192), specimens were immersed in a monoclonal primary antibody against neuronal class III β-tubulin (Covance, MMS-435P) and diluted in 1% Triton X-100 and 5% goat serum in 0.1 M PBS for 48 h at 4 °C. Afterward, specimens were rinsed three times with 0.1 M PBS and incubated for 2 h at room temperature in a second antibody conjugated with Cy3 (goat anti-mouse IgG, Jackson ImmunoResearch). Specimens were subsequently rinsed three times with 0.1 M PBS and labeled with phalloidin conjugated with Alexa Fluor488 (Invitrogen A12379, diluted in 1% Triton X-100, 5% goat serum in 0.1 M PBS, 1 h at room temperature). After rinsing three times with 0.1 M PBS, specimens were mounted on glass slides in glycerin and coverslipped. To quantify the neurotoxic effect of Mn on SGN and the protection by NAD, the number of β-tubulin labeled SGN were counted in a 141.4 μm × 141.4 μm × 5 μm volume from 8 cochlear cultures (3 locations in each cochlear culture).

### 2.4. Cochleograms

Cochleograms were prepared from cochlea cultures as described previously (Wei et al., 2010). Cochlear hair cells (HC) stained with Alexa Fluor488 phalloidin were examined under a fluorescent microscope (Zeiss Axioskop, 400×) with the appropriate filter. Using previously established methods, the numbers of missing hair cells were counted over 0.24 mm intervals along the entire length of the organ of Corti from apex to base (Deng et al., 2013; Wei et al., 2010). Using laboratory norms from control animals (Ding et al., 2007) and custom software, cochleograms were used to show the percent viable of inner hair cells (IHC) and outer hair cells (OHC) as a function of the distance from the apex of the cochlea for each treatment condition as described above (Ding et al., 2007). Results from eight samples were averaged to obtain the mean cochleogram from each experimental group.

### 2.5. TUNEL staining

To evaluate DNA strand breaks for cells undergoing apoptosis, specimens were labeled for TUNEL (terminal deoxynucleotidyl transferase biotin-dUPT nick end labeling) using the APO-BrdU TUNEL Assay Kit (Invitrogen A23210) following the manufacturer's protocol. Nine hours following treatment with 1.0 mM Mn in the presence or absence of 20 mM NAD, the specimens were fixed with formalin as described above. Specimens were transferred to ice-cold 70% ethanol overnight in a −20 °C freezer and subsequently rinsed twice in washing buffer. Afterwards, specimens were immerged in 100 μl DNA-labeling solution (20 μl reaction buffer,