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DECELERATED TRANSPORT AND ITS MECHANISM OF 2,5-HEXANEDIONE ON MIDDLE-MOLECULAR-WEIGHT NEUROFILAMENT IN RAT DORSAL ROOT GANGLIA CELLS

X.-Y. HAN, [a,b] **D. CHENG,** [c] **F.-Y. SONG,** [a] **T. ZENG,** [a] **L.-H. AN** [a] **AND K.-Q. XIE** [a]*

[a] *Institute of Toxicology, Shandong University, Jinan 250012, China*

[b] *College of Life Sciences, Shandong Normal University, Jinan 250014, China*

[c] *Department of Toxicology, Shandong Center for Disease Control and Prevention, Jinan 250014, China*

Abstract—Chronic exposure to *n*-hexane induces peripheral-central axonopathy, mediated by its metabolite 2,5-hexanedione (2,5-HD), in occupational workers and experimental animals, but the underlying mechanism is still unclear. In the current study, we investigated the effects of 2,5-HD on middle-molecular-weight neurofilament (NF-M) axonal transport using live-cell imaging technique in cultured rat dorsal root ganglia (DRG) cells. PA-GFP-NF-M plasmid was transfected into DRG neurons and live-cell imaging was performed to observe the slow axonal transport of NF-M. The levels of cytoskeleton and motor proteins in DRG cells were detected by Western-blot and the concentration of ATP was determined using an ATP Assay Kit. The results showed that 2,5-HD administration resulted in a decrease of NF-M axonal transport and a reduction of three neurofilament subunits levels in DRG cells. Furthermore, 2,5-HD exposure significantly decreased ATP contents and the protein levels of kinesin heavy chain (KHC). These findings indicated that 2,5-HD reduced slow axonal transport, neurofilaments cargoes, motor proteins and ATP energy in rat DRG cells, which may contribute to 2,5-HD-induced neurotoxicity.

Key words: 2,5-hexanedione, slow axonal transport, neurofilaments, motor proteins.

INTRODUCTION

The organic solvent *n*-hexane belongs to one of the most important aliphatic compounds and is widely used in the glue industry, paints, varnishes, printing inks, shoe manufacturing, shoe repair, and the food industry (DeCaprio et al., 2009). Occupational or experimental exposure to *n*-hexane induces a central-peripheral distal axonopathy (Spencer and Schaumburg, 1977; Couri and Milks, 1982). Morphologic manifestations of neuropathologic lesions include internodal swelling and axonal atrophy, retraction of myelin from the nodes of Ranvier and segmental demyelination, and distal axonal Wallerian degeneration (Graham et al., 1995). The metabolism studies have shown that 2,5-hexanedione (2,5-HD) is the toxic metabolite of *n*-hexane, mediating the neuropathy of the parent compound (Perbellini et al., 1982). However, the molecular mechanisms for 2,5-HD-induced neuropathy remains unclear.

The primary morphologic feature of 2,5-HD-induced neuropathy is axon atrophy in peripheral and central nerves (LoPachin and Lehning, 1997; Lehning et al., 2000; LoPachin et al., 2004). Axon atrophy involves disruption of the neurophysiological processes responsible for maintaining axon caliber in neurons. Neurofilaments (NFs) are a major intrinsic determinant of axonal caliber (Hoffman et al., 1984). NFs, together with microtubules and microfilaments, are three main components of the cytoskeleton in most mature neurons. NFs belong to the class IV family of intermediate filaments, which can be classed into three distinct groups according to their molecular masses, i.e. NF-H (heavy chain), NF-M (middle chain) and NF-L (light chain) (Lee et al., 1993). NFs account for up to 85% of the total protein of fully differentiated neurons and play important roles in neuronal structure and functions (Griffin and Watson, 1988). Microtubules are polar polymers composed of α/β tubulin heterodimers. In the axon, the microtubules are organized in a polar array and provide a track for transport. Microfilaments, also known as actin filaments, determine cell shape and enable cell movement and cytoplasmic streaming. Animal studies reveal that the cytoskeletal proteins might be the probable target of 2,5-HD-induced axonopathy as shown by decrease of the cytoskeletal protein levels in both central and peripheral nervous systems (CNS and PNS) after 2,5-HD intoxication (Abou-Donia et al., 1988; LoPachin et al., 2005a,b; Zhang et al., 2005a,b; Song et al., 2007; Wang et al., 2008). Mechanistic studies demonstrate that both decrease of protein synthesis (Zhang et al., 2005b) and increase of protein degradation contribute to 2,5-HD-induced reduction of NFs in the brain (Wang et al., 2011).

*Corresponding author. Address: Institute of Toxicology, School of Public Health, Shandong University, Jinan 250012, China. Tel: +86-531-88382132; fax: +86-531-88382129.
E-mail address: keqinx@sdu.edu.cn (K.-Q. Xie).

Abbreviations: 2,5-HD, 2,5-hexanedione; ALS, amyotrophic lateral sclerosis; BSA, bovine serum albumin; DRG, dorsal root ganglia; HRP, horse radish peroxidase; KHC, kinesin heavy chain; NF, neurofilament; NF-H, heavy-molecular-weight neurofilament; NF-L, light-molecular-weight neurofilament; NF-M, middle-molecular-weight neurofilament; PBS, phosphate-buffered saline; PNS, peripheral nervous system; SDS, sodium dodecyl sulfate.

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Materials in the axon, like cytoskeletal proteins, are synthesized and assembled in the neuronal cell body and transported along the axon toward the distal tip. This directional movement is known as anterograde axonal transport. In contrast, once proteins at the axon terminal reach the end of their lifetime, they must be returned to the cell body for degradation and recycling through a process of retrograde axonal transport (Morfini et al., 2011). According to the kinetic studies, axonal transport is classified into two types: fast and slow. Components of fast axonal transport move rapidly and frequently at rates of up to several hundred millimeters per day; those of slow move in an intermittent manner at average rates of approximately millimeters or tenths of millimeters per day. The way of cytoskeletal proteins transportation belongs to slow axonal transport (Brown, 2003a). Growing evidence supports the idea that deficits in axonal transport contribute to the pathogenesis of multiple neurodegenerative diseases, such as amyotrophic lateral sclerosis (ALS), Huntington's disease, Alzheimer's disease, and so on (Chevalier-Larsen and Holzbaur, 2006). Previous studies reported that 2,5-HD can inhibit fast retrograde axonal transport in animal sciatic nerves (Sickles, 1989; Sabri, 1992). However, these experiments are all performed *in vivo* and focus on all components of axonal transport. Until now, in our knowledge, there is no report on the effects of 2,5-HD on the slow axonal transport of a single component.

In the present study, we investigated the effects of 2,5-HD on slow axonal transport in cultured dorsal root ganglia (DRG) neurons. The live-cell imaging technique was used to observe the transport of a specific protein, NF-M. Furthermore, the cytoskeletal cargoes, motor proteins and energy metabolism were also studied to reveal the underlying mechanism. Our findings would give further evidences for the 2,5-HD-induced neuropathy.

EXPERIMENTAL PROCEDURES

Agents

2,5-HD (purity > 97%) was purchased from Merck biosciences, Inc. (Darmstadt, Germany). PA-GFP-NF-M and DsRed plasmid were kind gifts from Dr. Anthony Brown (Ohio State University, Columbus, USA). Monoclonal antibodies of anti-NF-H (clone NE-14), anti-NF-M (clone NN-18), anti-NF-L (clone NR-4), anti-alpha-tubulin (clone DM 1A), anti-beta-tubulin (clone TUB2.1), anti-beta-actin (clone AC-15), anti-dynein (clone 70.1), and horse radish peroxidase (HRP)-conjugated goat-anti-mouse immunoglobulin G (IgG) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Monoclonal anti-kinesin-1 (heavy chain, clone H2) antibody was purchased from Millipore Corp. Monoclonal anti-dynactin p50 (clone 25) antibody was purchased from BD Biosciences. Leibovitz's L-15 medium was bought from Invitrogen (San Diego, CA, USA). All other chemicals were of the highest quality commercially available.

DRG cells culture

Primary cultures of DRG were prepared as previously described (Brown, 2003b). In brief, 2-day-old neonatal Sprague–Dawley rats (Laboratory Animal Center of Shandong University, China) were decapitated. DRG cells were dissected from all levels of the spinal cord and pooled in Leibovitz's L-15 medium and followed by enzymatic digestion (0.125% collagenase and 0.125% trypsin for 30 min, respectively) at 37 °C. The cells were cultured on glass coverslips coated with poly-D-lysine and Matrigel in Leibovitz's L-15 medium supplemented with glucose, L-glutamine, nerve growth factor, adult rat serum, and hydroxypropylmethylcellulose at 37 °C. The experiments were conducted in accordance with the NIH Guide for Care and Use of Laboratory Animals and the principles in the ''Use of Animals in Toxicology'', and were approved by the Ethics Committee of Shandong University Institute of Preventive Medicine.

Cytotoxicity determination

To investigate the cytotoxicity of 2,5-HD on DRG cells, different concentrations of 2,5-HD (0, 8, 16, 32, 64, 128 mM) (Ogawa et al., 1996) were added into DRG cell dishes. 24 h later, DRG cells were washed with 100 μl Leibovitz's L-15 medium for three times, and followed by the addition of 100 μl working solution (Calcein AM 4 μM, Ethidium homodimer-1 2 μM in 1000 μl L15). Live cells appeared in green color and dead cells in red color. The viability was calculated to determine the potential concentrations of 2,5-HD for the following experiments. The cells were observed under a Nikon microscope (Nikon, Melville, NY, USA).

Transfection of neurons

DRG cells were centrifuged at 100*g* for 5 min at room temperature and resuspended in 100 μl of mouse neuron nucleofection solution (Amaxa Biosystems, Gaithersburg, MD, USA). The cells were co-transfected with 5-μg pPA-GFP-NF-M and 5-μg pDsRed by electroporation using an Amaxa Nucleofector (Amaxa Biosystems), After transfection, cells were maintained in Leibovitz's L-15 medium containing 0.5 mg/ml bovine serum albumin (BSA) at 37 °C. After recovery for 10 min, the DRG cells were plated at a density of two dissociated ganglia per coverslip onto 40-mm round No. 1.5 glass coverslips (Bioptechs, Butler, PA, USA) coated with poly-D-lysine and Matrigel as described previously (Brown, 2003b), and cultured in the medium [Leibovitz's L15 medium, 0.6% (w/v) D-glucose, 2 mM L-glutamine, 100 ng/ml 2.5S nerve growth factor, 10% (v/v) adult rat serum] overnight. Then, the plating medium was replaced with the above medium containing 0.5% (w/v) hydroxypropylmethylcellulose. The cells were maintained in this cultured medium at 37 °C without CO_2 for an additional 2 days.

Observation of NF-M transport by using live-cell imaging technique

At 24 h after the treatment with 0, 4, 8, 16 mM 2,5-HD, live-cell imaging experiment was performed as previously described (Trivedi et al., 2007). The cultured cells were transferred into a Bioptechs FCS2