Neurobiology of Disease 97 (2017) 60–69

Contents lists available at ScienceDirect

Neurobiology of Disease

journal homepage: www.elsevier.com/locate/ynbdi

Cisplatin induces mitochondrial deficits in *Drosophila* larval segmental nerve

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ARTICLE INFO

Article history:
Received 15 February 2016
Revised 4 October 2016
Accepted 16 October 2016
Available online 17 October 2016

Keywords:
Mitochondria
Cisplatin
Motor neuron
Drosophila
Axonal trafficking
Membrane potential
Apoptosis

ABSTRACT

Cisplatin is an effective chemotherapy drug that induces peripheral neuropathy in cancer patients. In rodent dorsal root ganglion neurons, cisplatin binds nuclear and mitochondrial DNA (mtDNA) inducing DNA damage and apoptosis. Platinum-mtDNA adducts inhibit mtDNA replication and transcription leading to mitochondrial degradation. Cisplatin also induces climbing deficiencies associated with neuronal apoptosis in adult *Drosophila melanogaster*. Here we used *Drosophila* larvae that express green fluorescent protein in the mitochondria of motor neurons to observe the effects of cisplatin on mitochondrial dynamics and function. Larvae treated with 10 μg/ml cisplatin had normal survival with deficiencies in righting and heat sensing behavior. Behavior was abrogated by, the pan caspase inhibitor, p35. However, active caspase 3 was not detected by immunostaining. There was a 27% decrease in mitochondrial membrane potential and a 42% increase in reactive oxygen species (ROS) in mitochondria along the axon. Examination of mitochondrial axonal trafficking showed no changes in velocity, flux or mitochondrial length. However, cisplatin treatment resulted in a greater number of stationary organelles caused by extended pausing during axonal motility. These results demonstrate that cisplatin induces behavior deficiencies in *Drosophila* larvae, decreased mitochondrial activity, increased ROS production and mitochondrial pausing without killing the larvae. Thus, we identified particular aspects of mitochondrial dynamics and function that are affected in cisplatin-induced peripheral neuropathy and may represent key therapeutic targets.

1. Introduction

Cisplatin-induced peripheral neuropathy is a dose-limiting side effect occurring in 30–40% of cancer patients (Johnson et al., 2015; Windebank and Grisold, 2008). Symptoms are mainly sensory including tingling, numbness, paresthesia and pain (Cavaletti and Marmiroli, 2010; O'Reilly et al., 2014). Some patients experience a worsening of the sensory symptoms for 2–6 months after completion of their treatment, which is a phenomenon known as "coasting" (Miltenburg and Boogerd, 2014; Reinstein et al., 1980). Long-term neuropathy lowers quality of life for many cancer survivors and currently there are no preventive therapies for cisplatin-induced peripheral neuropathy. Chronic neurotoxicity is also caused by the other commonly used platinum compounds, carboplatin and oxaliplatin. Understanding the underlying mechanisms of chemotherapy-induced peripheral neuropathy (CIPN) is critical for the development of effective therapeutic approaches (Albers et al., 2011).

Cisplatin neurotoxicity is attributed to the formation of platinum-DNA (Pt-DNA) adducts leading to cellular stress and apoptosis (Eastman, 1987; McDonald et al., 2005). Cisplatin binds both nuclear DNA (nDNA) and mitochondrial DNA (mtDNA) inducing DNA damage and apoptosis in rat dorsal root ganglion (DRG) neurons, in vitro (McDonald et al., 2005; McDonald and Windebank, 2002; Podratz et al., 2011a; Ta et al., 2006; Ta et al., 2009). Cisplatin binds nDNA leading to upregulation of p53, cell cycle re-entry and translocation of BCL2-associated X (bax) protein to the mitochondria (Fischer et al., 2001; Gill and Windebank, 1998; McDonald and Windebank, 2002). Cisplatin also binds mtDNA inhibiting replication and transcription leading to mitochondrial degradation (Podratz et al., 2011a). Accumulation of Pt-DNA adducts is higher in DRG neurons although the rate of repair is similar to other cell types (McDonald et al., 2005). Pt-DNA adducts are removed by nucleotide excision repair in the nucleus. However, this mechanism does not exist in mitochondria (Croteau et al., 1999; Larsen et al., 2005). Pt-mtDNA adducts may play an important role in the mechanism of cisplatin-induced neurotoxicity.

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Available online on ScienceDirect (www.sciencedirect.com).

http://dx.doi.org/10.1016/j.nbd.2016.10.003
0969-9961/

Differentiating the effects of Pt-nDNA and Pt-mtDNA adducts on DRG neurons in culture is challenging. nDNA damage can have profound effects on mitochondria through p53 and bax translocation and mitochondrial stress can independently initiate apoptosis (Savitskaya and Onishchenko, 2015). Our previous studies showed that cisplatin induced a neuron specific climbing deficiency in adult *Drosophila melanogaster* (Podratz et al., 2011b). Adult flies fed 50 μg/ml cisplatin for three days formed DNA adducts at the same rate as rat DRG neurons in vitro and induced climbing deficiencies associated with neuronal apoptosis. Imaging of motor neuron mitochondria dynamics and function can be done in live *Drosophila* larvae (Shidara and Hollenbeck, 2010). This allows for examination of mitochondrial dynamics and neuronal apoptosis in relation to larval behavior and survival. Our studies utilized a genetically modified fly with a motor neuron driver, *P[GawB]D42*, (Yeh et al., 1995) and a GFP responder targeted to the mitochondria $P[w^{+mC} = UAS\text{-}mitoGFP.AP]3$ (Pilling et al., 2006) on the third chromosome. These flies expressed GFP specifically in mitochondria of motor neurons and were used to observe the direct effects of cisplatin on mitochondria in the intact nervous system of live larvae. Motor neuron segmental nerves of the *Drosophila* larvae represent an ideal model system to study mitochondrial function and axonal transport in vivo.

2. Materials and methods

2.1. Flies and reagents

Cisplatin was obtained from APP (Lake Zurich, IL) as an aqueous stock solution of 1 mg/ml in saline. The fly strain (w; +/+; D42-GAL4 P{UAS-mitoGFP} e/TM6B, Tb Hu e) was a gift from Dr. P. Hollenbeck (Purdue University, IN). w[*]; P{w[+mW·hs] = GawB}D42 flies were purchased from Bloomington Drosophila Stock Center (Bloomington, IN). UAS-p35 flies were obtained from Bruce Hay's Laboratory (California Institute of Technology). Instant fly food was purchased from Carolina Biologicals (Burlington, NC).

2.2. Drosophila larval drug treatment

Drosophila larvae were age synchronized and treated with or without cisplatin for 3 days (Fig. 1A). On day 1, 150–200 adult flies (w; +/+; D42-GAL4 P{UAS-mitoGFP} e/TM6B, Tb Hu e) were added to an embryo collection chambers containing grape agar plates and yeast paste. Chambers were placed into a 25 °C environmental chamber for 18–24 h. On day 2, adults were removed and the embryos were placed back into a 25 °C environmental chamber for another 24 h. On day 3, first instar larvae were removed from the grape agar plates and placed into the chamber containing instant food overnight at 25 °C. On day 4, larvae were collected and treated with cisplatin for all experiments. Second instar larvae were collected using a sieve and twenty larvae per each experimental group were placed into chambers with instant food rehydrated with and without cisplatin.

2.3. Larval dissection

Drosophila larvae were dissected to expose the segmental nerves for live imaging (Fig. 1B). Wandering third instar larvae treated with and without cisplatin were removed from the wells and washed in water to remove food. The larva was transferred to a Sylgard plate and pinned ventral side down. An insect pin was first placed at the posterior end of the larva between tracheal tubes followed by a second pin at the anterior end near the "beak". Using fine scissors, a small incision at the posterior end of the larva was made following with a longitudinal cut from the posterior to anterior. 400 μl of HL6 buffer (see Supplemental data) was added on top of the larva. Using forceps, tracheal tubes and fat bodies were removed starting at the posterior end. The buffer was removed and 400 μl of fresh HL6 buffer was added to the larva to remove debris. Small lateral incisions were made on the posterior and anterior ends perpendicular to the longitudinal cut to allow the body wall to open and the larvae to lie flat for imaging. The larva was mounted onto a special chamber slide with HL6 buffer containing 10 mM glutamate to desensitize glutamate receptors and decrease muscular movements.

2.4. Larval survival assay

Drosophila larvae were drug treated as described above and observed for survival to the pupae and adult developmental stages. Twenty larvae per condition were placed into vials containing 0, 1, 5, 10, 25, and 50 μg/ml cisplatin in instant food. All treatments were conducted in duplicates. Larvae were observed for another seven to nine days. Pupae were counted on day 10 of the assay, and the number of adults that hatched from those pupae was counted on day 12–14. Survival rates were determined from 100 to 180 larvae as the number of pupae or adults verses the total number of larvae added to the vial.

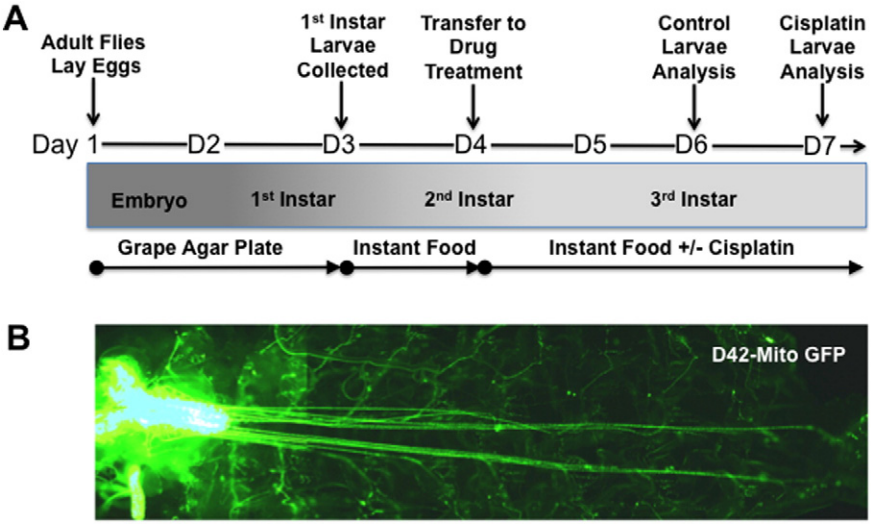

Fig. 1. Experimental model. (A) Schematic diagram of synchronization of larvae developmental stages and drug treatment. (B) Image of dissected larva with exposed motor neurons and segmental nerves. Larvae image was acquired using a Zeiss Axioscope (Carl Zeiss, Thornwood, NY) with a 40× lens.