NeuroToxicology 34 (2013) 175–188

Contents lists available at SciVerse ScienceDirect

NeuroToxicology

Disrupted cytoskeletal homeostasis, astrogliosis and apoptotic cell death in the cerebellum of preweaning rats injected with diphenyl ditelluride

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ARTICLE INFO

Article history:
Received 14 August 2012
Accepted 25 October 2012
Available online 24 November 2012

Keywords:
Diphenyl ditelluride
Cytoskeleton
Apoptosis
Astrogliosis
Cell signaling
Cerebellum

ABSTRACT

In the present report 15 day-old rats were injected with 0.3 μmol of diphenyl ditelluride $(PhTe)_2$/kg body weight and parameters of neurodegeneration were analyzed in slices from cerebellum 3 and 6 days afterwards. The earlier responses, at day 3 after injection, included hyperphosphorylation of intermediate filament (IF) proteins from astrocyte (glial fibrillary acidic protein – GFAP – and vimentin) and neuron (low-, medium- and high molecular weight neurofilament subunits: NF-L, NF-M and NF-H); increased mitogen-activated protein kinase (MAPK) (Erk and p38MAPK) and cAMP-dependent protein kinase (PKA) activities. Also, reactive astrogliosis takes part of the early responses to the insult with $(PhTe)_2$, evidenced by upregulated GFAP in Western blot, PCR and immunofluorescence analysis. Six days after $(PhTe)_2$ injection we found persistent astrogliosis, increased propidium iodide (PI) positive cells in NeuN positive population evidenced by flow cytometry and reduced immunofluorescence for NeuN, suggesting that the *in vivo* exposure to $(PhTe)_2$ progressed to neuronal death. Moreover, activated caspase 3 suggested apoptotic neuronal death. Neurodegeneration was related with decreased [^{3}H]glutamate uptake and decreased Akt immunoreactivity, however phospho-GSK-3-β (Ser9) was not altered in $(PhTe)_2$ injected rat. Therefore, the present results show that the earlier cerebellar responses to $(PhTe)_2$ include disruption of cytoskeletal homeostasis that could be related with MAPK and PKA activation and reactive astrogliosis. Akt inhibition observed at this time could also play a role in the neuronal death evidenced afterwards. The later events of the neurodegenerative process are characterized by persistent astrogliosis and activation of apoptotic neuronal death through caspase 3 mediated mechanisms, which could be related with glutamate excitotoxicity. The progression of these responses are therefore likely to be critical for the outcome of the neurodegeneration provoked by $(PhTe)_2$ in rat cerebellum.

1. Introduction

The cerebellar functions include the control of attention and other cognitive functions, emotions and mood, and social behavior. The cerebellum is considered particularly vulnerable in the newborn human as well as in the developing animal because of its very rapid growth at that time (Biran et al., 2012) and the importance of cerebellar physiology has been confirmed by the frequency of neuropsychiatric disorders in individuals with cerebellar abnormalities (Villanueva, 2012).

Cell death and neuronal loss are the key pathological hallmarks of neurodegeneration in all the neurodegenerative disorders, with apoptosis and necrosis being central to both acute and chronic degenerative processes.

Astrogliosis is a hallmark of diseased CNS tissue (Pekny and Nilsson, 2005). This term refers to progressive changes in gene expression and cellular morphology, often including proliferation. The activation of astrocytes is characterized by changes in their molecular and morphological features. It is believed that progressive changes in astrocytes as they become reactive are finely regulated by complex intercellular and intracellular signaling mechanisms. The most commonly used maker of activated astrocytes is the upregulation of the cytoskeletal protein glial fibrillary acidic protein (GFAP), vimentin, and to some extent

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http://dx.doi.org/10.1016/j.neuro.2012.10.015

nestin, coincident with cellular hypertrophy (Sofroniew and Vinters, 2010).

Neurodegeneration has already been related with disruption of the cytoskeletal homeostasis of neural cells (Perrot and Eyer, 2009). Neurofilaments (NFs) are the most abundant cytoskeletal components of large myelinated axons from adult central and peripheral nervous system. They are constituted of the association of three NF subunits of low, medium and high molecular weight (NF-L, MF-M and NF-H). The assembly, axonal transport and functions of NF are responsible for the normal physiology of the nervous system. Conversely, misregulation of the cytoskeletal homeostasis might be responsible for the toxicity leading to pathological situations and to neuronal death. Following their synthesis and assembly in the cell body, NFs are transported along the axon. This process is finely regulated *via* phosphorylation of the carboxyl-terminal part of the two high molecular weight subunits of NF. The correct formation of an axonal network of NF is crucial for the establishment and maintenance of axonal calibre and consequently for the optimisation of conduction velocity. Accordingly, the frequent disorganisation of NF network is observed in several neuropathologies (Perrot et al., 2008).

Neurotoxicity of tellurium has been reported in the literature. In this context, inorganic tellurium treatment was found to cause significant impairment in retention of the spatial learning task (Widy-Tyszkiewicz et al., 2002). But to date, no telluroproteins have been identified in animal cells. Nowadays, two cases of toxicity in young children from ingestion of metal-oxidizing solutions that contained substantial concentrations of Te were reported in the literature (Yarema and Curry, 2005). Clinical features of acute Te toxicity include a metallic taste, nausea, blackened oral mucosa and skin and garlic odor of the breath (Muller et al., 1989; Taylor, 1996).

Previous data from the literature have indicated that the organic compound of tellurium, diphenyl ditelluride $(PhTe)_2$ is neurotoxic to rodents and exposure to low doses of this compound can cause cognitive impairment (Stangherlin et al., 2009). Furthermore, $(PhTe)_2$ can also have neurotoxic effects *in vitro*, including cytotoxic effect in astrocytes (Roy and Hardej, 2011) and changes in the phosphorylation of intermediate filaments (IFs) in slices obtained from different brain structures of young rats (Heimfarth et al., 2011, 2012a). Most interestingly, we have recently described that young rats injected with $(PhTe)_2$ presented disruption of cytoskeletal homeostasis in the striatum, which was related with neuronal damage and astrogliosis in this brain structure 6 days after injection of the neurotoxicant (Heimfarth et al., 2012b).

One of the prominent findings associated with the $(PhTe)_2$-induced injury in the striatum was the hyperphosphorylation of astrocyte IF protein-GFAP – as well as the three NF subunits – NF-L, NF-M and NF-H. This effect was mediated by the mitogen-activated protein kinases (Erk, JNK and p38MAPK) and protein kinase A (PKA) activities. Also, the $(PhTe)_2$ injection induced reactive astrogliosis, characterized by dramatically increased GFAP expression in the striatum simultaneously with neuronal damage. Also, increased caspase 3 suggested apoptotic cell death in the striatal slices 6 days after $(PhTe)_2$ exposure. These findings showed that $(PhTe)_2$ is able to cause neural dysfunction associated with cytoskeletal disruption in the striatum of injected rats *in vivo*. It is largely described that alterations of protein phosphorylation lead to brain cytoskeletal misregulation and neural cell death. Moreover, these alterations are associated with metabolic and neurochemical dysfunctions that may ultimately disrupt normal cell function and viability, characterizing a neurotoxic condition (Pessoa-Pureur and Wajner, 2007).

Therefore, in an attempt to better identify the signaling mechanisms leading to IF disruption and their consequences on neural cell function under the toxicity of $(PhTe)_2$, we investigated the damage caused by this neurotoxicant in the cerebellum of young rats. This choice was supported by the evidence that cerebellum is a highly vulnerable brain structure greatly implicated in the toxicity mechanisms during the first postnatal weeks (Biran et al., 2012). To access the toxicity of $(PhTe)_2$ in the cerebellum, young rats were acutely exposed to the same concentration of the neurotoxicant able to provoke cell damage in the rat striatum (Heimfarth et al., 2012b). Thus, in the present report we describe the progressive disruption of the cytoskeletal homeostasis, reactive astrogliosis and apoptotic neuronal death in the cerebellum of young rats 3 and 6 days after $(PhTe)_2$ injection. Also, we aimed to investigate some mechanisms related with the neurodegeneration elicited by the neurotoxicant.

2. Material and methods

2.1. Radiochemical and compounds

$[^{32}P]Na_2HPO_4$ was purchased from CNEN, São Paulo, Brazil. Benzamidine, leupeptin, antipain, pepstatin, chymostatin, acrylamide and bis-acrylamide, anti-GSK3β, anti-phosphoGSK3β, anti-PKAcα, anti-PKCaMII, anti-active caspase 3, anti-AKT, anti-phosphoAKT, anti-GFAP (St. Louis, MO, USA; DAKO), anti-vimentin, anti-NF-L, anti-NF-M, anti-NF-H antibodies and propidium iodide were obtained from Sigma (St. Louis, MO, USA). The chemiluminescence ECL kit peroxidase and the conjugated anti-rabbit IgG were obtained from Amersham (Oakville, Ontario, Canada). Anti-ERK, anti-phosphoERK, anti-SAP/JNK, anti-phosphoSAP/JNK, anti-p38, anti-phosphop38 and anti-KSP repeats, were obtained from Cell Signaling Technology (USA). Anti-phosphoSer55NF-L, anti-phosphoSer57NF-L and anti-NeuN antibodies were obtained from Millipore. Anti-rabbit Alexa 488 and anti-mouse Alexa 568 were from Molecular Probes. Fluor Save™ was from Merck. The organochalcogenide $(PhTe)_2$ was synthesized using the method described by Petragnani (1994). Analysis of the 1H NMR and 13C NMR spectra showed that the compound obtained presented analytical and spectroscopic data in full agreement with their assigned structures. The purity of the compounds were assayed by high resonance mass spectroscopy (HRMS) and was higher that 99.9%. Diphenylditelluride was dissolved in dimethylsulfoxide (DMSO) just before use. The final concentration of DMSO was adjusted to 0.1%. Solvent controls attested that at this concentration DMSO did not interfere with the phosphorylation measurement. Platinum Taq DNA polymerase and SuperScript-II RT pre-amplication system were from Invitrogen. All other chemicals were of analytical grade and were purchased from standard commercial supplier.

2.2. Animals

Fifteen day-old male and female Wistar rats were obtained from our breeding stock. Rats were maintained on a 12-h light/12-h dark cycle in a constant temperature (22 °C) colony room. On the day of birth the litter size was culled to seven pups. Litters smaller than seven pups were not included in the experiments. Water and a 20% (w/w) protein commercial chow were provided *ad libitum*. The experimental protocol followed the "Principles of Laboratory Animal Care" (NIH publication 85-23, revised 1985) and was approved by the Ethics Committee for Animal Research of the Federal University of Rio Grande do Sul.

2.3. Drug administration, preparation and labeling of slices

The *in vivo* toxicity was induced by a single subcutaneous (s.c.) injection of $(PhTe)_2$ 0.3 μmol/kg body weight or canola oil (vehicle) (2.8 ml/kg body weight) into male and female 15-day-old Wistar rats. The experiments were performed 3 or 6 days after injection. The rats were killed by decapitation, the cerebellum was dissected