Toxicology 379 (2017) 1–11

Contents lists available at ScienceDirect

Toxicology

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

Astrocyte-neuron interaction in diphenyl ditelluride toxicity directed to the cytoskeleton

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ARTICLE INFO

Article history:
Received 28 November 2016
Received in revised form 5 January 2017
Accepted 23 January 2017
Available online 27 January 2017

Keywords:
Diphenyl ditelluride
Cytoskeleton
Cell signaling
Neurotoxicity
Astrocyte
Neuron

ABSTRACT

Diphenylditelluride $(PhTe)_2$ is a neurotoxin that disrupts cytoskeletal homeostasis. We are showing that different concentrations of $(PhTe)_2$ caused hypophosphorylation of glial fibrillary acidic protein (GFAP), vimentin and neurofilament subunits (NFL, NFM and NFH) and altered actin organization in co-cultured astrocytes and neurons from cerebral cortex of rats. These mechanisms were mediated by N-methyl-D-aspartate (NMDA) receptors without participation of either L-type voltage-dependent calcium channels (L-VDCC) or metabotropic glutamate receptors. Upregulated Ca^{2+} influx downstream of NMDA receptors activated Ca^{2+}-dependent protein phosphatase 2B (PP2B) causing hypophosphorylation of astrocyte and neuron IFs. Immunocytochemistry showed that hypophosphorylated intermediate filaments (IF) failed to disrupt their organization into the cytoskeleton. However, phalloidin-actin-FITC stained cytoskeleton **e**videnced misregulation of actin distribution, cell spreading and increased stress fibers in astrocytes. βIII tubulin staining showed that neurite meshworks are not altered by $(PhTe)_2$, suggesting greater susceptibility of astrocytes than neurons to $(PheTe)_2$ toxicity. These findings indicate that signals leading to IF hypophosphorylation fail to disrupt the cytoskeletal IF meshwork of interacting astrocytes and neurons *in vitro* however astrocyte actin network seems more susceptible. Our findings support that intracellular Ca^{2+} is one of the crucial signals that modulate the action of $(PhTe)_2$ in co-cultured astrocytes and neurons and highlights the cytoskeleton as an end-point of the neurotoxicity of this compound. Cytoskeletal misregulation is associated with cell dysfunction, therefore, the understanding of the molecular mechanisms mediating the neurotoxicity of this compound is a matter of increasing interest since tellurium compounds are increasingly released in the environment.

1. Introduction

Tellurium is a chemical element with no essential physiological roles known in cell biology (Larner, 1995). Tellurium has been extensively used in the electronic industry (Hardman, 2006; Klaine et al., 2008; Ogra, 2009) and although only few cases of human exposure to tellurium have been described until now (Blackadder and Manderson, 1975; Orian and Toppo, 2014; Yarema and Curry, 2005) the increasing release of electronic material constituents in the environment represents a real menace to the human health in the near future. Furthermore, the use of tellurium in organic synthesis or medicine can also be a source of occupational exposure to different forms of the element.

Diphenyl ditelluride $(PhTe)_2$ is the simplest of the diaryl tellurium compounds used in the organic synthesis and a potential prototype for the development of novel biologically active molecules (Comparsi et al., 2014; Nogueira et al., 2004). $(PhTe)_2$ has been described as a potent neurotoxin (Caeran Bueno et al., 2013; Comparsi et al., 2014; Meinerz et al., 2014; Prauchner et al., 2013; Stangherlin and Nogueira, 2014) whose general toxicity is apparently related with the oxidation of thiol-containing proteins (Nogueira et al., 2004; Pessoa-Pureur et al., 2014). However we have recently identified that $(PhTe)_2$ modulates important signaling mechanisms initiated at the cell membrane level, which target the cytoskeleton both after *in vivo* exposure or in slices of different brain regions of young rats (Heimfarth et al., 2008, 2011, 2012a,

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http://dx.doi.org/10.1016/j.tox.2017.01.015
0300-483X/

2012b, 2013; Pessoa-Pureur et al., 2014). $(PhTe)_2$ triggers signaling pathways downstream of ionotropic and metabotropic glutamate receptors, and voltage-dependent Ca^{2+} channels (VDCC) *via* activation/inhibition of different kinase or phosphatase pathways. The final result involves the disrupted homeostasis of the cytoskeleton, particularly the phosphorylation level of intermediate filament (IF) proteins (Heimfarth et al., 2012b, 2013; Pessoa-Pureur et al., 2014).

The cytoskeleton is constituted by a complex and dynamic filamentous network of different types of proteins that together with the cytoskeletal-associated proteins form the structural scaffold, which is crucial to cell architecture and function. In addition, it is becoming widely recognized the remarkable role of the cytoskeleton in the signal transduction (Devreotes and Horwitz, 2015) *via* coordination of appropriate cell responses to intra and extracellular signals. Consequently, the neurotoxicity of $(PhTe)_2$ may be intimately associated with disruption of the normal physiology of IFs and related molecules (Pessoa-Pureur et al., 2014).

Cell specific IFs are the largest family of cytoskeletal proteins in mammalian cells. Glial fibrillary acidic protein (GFAP) is the hallmark IF protein in mature astrocytes (Hol and Pekny, 2015) implicated in important physiological roles in astrocytes (Middeldorp and Hol, 2011). In this regard, overexpression of GFAP has been associated with disruption of astrocyte homeostasis in neuropathological conditions (Zu et al., 2014) and in response to neurotoxins (Damodaran et al., 2002).

Neurofilaments (NF) are neuron-specific IFs comprised by three subunits of different molecular mass: the light (NFL; 68 kDa), middle (NFM; 145–160 kDa) and high (NFH; 160–200 kDa) molecular weight NF subunits (Sihag et al., 2007). The NFs are the most abundant elements of the mature axonal cytoskeleton where they control the axonal caliber and consequently nerve conductivity (Hoffman et al., 1987; Holmgren et al., 2012).

The IF phosphorylation is one of the most important post-translational modifications whose general role is facilitate the IF reorganization and interaction of the IF proteins with other cell elements. The phosphorylation/dephosphorylation processes take part of different signaling cascades that directly modulate the cytoskeleton assembly/disassembly (Sihag et al., 2007). In the IF subunits the phosphorylating sites are typically located in their head and tail domains. The head domain phosphorylating sites promote IF protein association/disassociation, which is a crucial factor in maintaining filament structural dynamics (Sihag et al., 2007). The GFAP and NFL phosphorylation sites are mostly located in the head domain and their phosphorylation levels (Sihag et al., 2007) provide modulation of the cytoskeleton in response to second messengers into the cell. Otherwise, NFM and NFH are highly phosphorylated in critical Ser residues located in the tail domain which are likely to have consequences for axonal caliber, stability and transport (Shea and Chan, 2008). Taking into account the complexity of the phosphorylating system directed to the cytoskeleton and the consequences of misregulated kinases and phosphatases in the equilibrium of the IF phosphorylation, the understanding of the complex interplay among the different signaling cascades targeted to the cytoskeleton is of growing interest. It is also remarkable that misregulated cytoskeleton is a hallmark of several neurodegenerative diseases (Hol and Pekny, 2015; Perrot et al., 2008) and of the action of neurotoxicant compounds (Aung et al., 2015).

Previous evidence from our group has demonstrated that the homeostasis of the cytoskeleton of primary astrocytes and neurons is disrupted by metabolites in toxic concentrations through cell specific mechanisms and that co-cultured astrocytes and neurons could interact with one another modifying the response of the cytoskeleton to the insult (Pierozan et al., 2014, 2015). In addition, we recently evidenced the cytoskeleton as an end-point of $(PhTe)_2$ toxicity in primary astrocytes and neurons (Heimfarth et al., 2016). However, until now we have not assessed the molecular mechanisms elicited by $(PhTe)_2$ in interacting astrocytes and neurons *in vitro*. Thus, the purpose of the present study was to investigate the molecular mechanisms underlying the ability of $(PhTe)_2$ to cause disruption of the cytoskeletal homeostasis in co-cultured astrocytes and neurons isolated from the cerebral cortex of rats to get light on the potential neuro-protective or neuro-detrimental interactions between neurons and astrocytes after exposure to $(PhTe)_2$. We used the phosphorylation/dephosphorylation levels of IFs as molecular endpoints of tellurium neurotoxicity. A connection between deregulation of IF phosphorylation/dephosphorylation levels with cell morphology collapse was also investigated.

2. Material and methods

2.1. Radiochemical and compounds

$[^{32}P]Na_2HPO_4$ was purchased from CNEN, São Paulo, Brazil. Benzamidine, leupeptin, antipain, chymostatin, pepstatin, acrylamide, bis-acrylamide, propidium iodode, MK-801, FK506, fostriecin, calyculin, CNQX, MPEP, Bapta, EGTA, nifedipine anti-GFAP (G3893), anti-vimentin (vim 13-2), anti-NF-L (N5264), anti-NF-M (N2787), anti-NF-H (N0142), anti-PP1, anti-PP2B (clone CN-A1), anti-PP2A (clone 7A6) anti-actin (clone AC-74), anti-βIII-tubulin, anti-mouse IgG (whole molecule), anti-mouse IgG (whole molecule)-fluorescein isothiocyante (FITC), F(ab)2fragment-Cy3 antibodies were obtained from Sigma (St. Louis, MO, USA). The chemiluminescence ECL kit peroxidase and the conjugated anti rabbit IgG (A0545) were obtained from Amersham (Oakville, Ontario, Canada). The 4,6-Diamidino-2-phenylindole (DAPI) was obtained from Calbiochem (La Jolla, CA). Fetal bovine serum (FBS), Dulbecco's modified Eagle's medium nutrient mixture F-12 (DMEM/F12), Neurobasal medium, B-27 supplement, fungizone, and penicillin/streptomycin were purchased from Gibco BRL (Carlsbad, CA). 4C3HPG and MCPG were purchased from Tocris Bioscience. The organochalcogenide $(PhTe)_2$ was synthesized using the method described by Petragnani (1994). Analysis of the ^1H NMR and ^{13}C NMR spectra showed that the compound obtained presented analytical and spectroscopic data in full agreement with their assigned structures. The purity of the compound was assayed by high resonance mass spectroscopy (HRMS) and was higher that 99.9%. $(PhTe)_2$ was dissolved in dimethylsulfoxide (DMSO) just before use. The final concentration of DMSO was adjusted to 0.1%. Appropriate controls attested that at this concentration DMSO did not interfere with the phosphorylation measurement. All other chemicals were of analytical grade and were purchased from standard commercial supplier.

2.2. Astrocyte/neuron co-culture

Astrocyte primary cultures were prepared from the cerebral cortex of newborn (0–2 day-old; P0) Wistar rats, as previously described (Loureiro et al., 2010). This work was approved by institutional Ethics Committee with the number 18275. Briefly, for primary astrocyte cultures rats were decapitated, the cerebral cortex was removed and tissue was mechanically dissociated in DMEM/F12 (pH 7.4) supplemented with glucose (33 mM), glutamine (2 mM) and sodium bicarbonate (3 mM). The dissociated cells were placed in 6-, 24- or 96-well plates in DMEM/F12/10%FBS (pH 7.4). These cells were grown in a humid incubator (37 °C; 5% CO_2), with the media replaced every 3 days.

Isolated cortical neurons were prepared from cerebral cortex of embryonic day 18 Wistar rat as previously described by Moura