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Calcium signaling mechanisms disrupt the cytoskeleton of primary astrocytes and neurons exposed to diphenylditelluride

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ABSTRACT

Background: Diphenylditelluride $(PhTe)_2$ is a potent neurotoxin disrupting the homeostasis of the cytoskeleton.
Methods: Cultured astrocytes and neurons were incubated with $(PhTe)_2$, receptor antagonists and enzyme inhibitors followed by measurement of the incorporation of [32P]orthophosphate into intermediate filaments (IFs).
Results: $(PhTe)_2$ caused hyperphosphorylation of glial fibrillary acidic protein (GFAP), vimentin and neurofilament subunits (NFL, NFM and NFH) from primary astrocytes and neurons, respectively. These mechanisms were mediated by *N*-methyl-D-aspartate (NMDA) receptors, L-type voltage-dependent calcium channels (L-VDCCs) as well as metabotropic glutamate receptors upstream of phospholipase C (PLC). Upregulated Ca^{2+} influx activated protein kinase A (PKA) and protein kinase C (PKC) in astrocytes causing hyperphosphorylation of GFAP and vimentin. Hyperphosphorylated (IF) together with RhoA-activated stress fiber formation, disrupted the cytoskeleton leading to altered cell morphology. In neurons, the high intracellular Ca^{2+} levels activated the MAPKs, Erk and p38MAPK, beyond PKA and PKC, provoking hyperphosphorylation of NFM, NFH and NFL.
Conclusions: Our findings support that intracellular Ca^{2+} is one of the crucial signals that modulate the action of $(PhTe)_2$ in isolated cortical astrocytes and neurons modulating the response of the cytoskeleton against the insult.
General significance: Cytoskeletal misregulation is associated with neurodegeneration. This compound could be a valuable tool to induce molecular changes similar to those found in different pathologies of the brain.

1. Introduction

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 [36]. $(PhTe)_2$ has been described to possess some beneficial biological activities, including antioxidant properties [42].

We have previously 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 [38]. $(PhTe)_2$ triggers signaling pathways downstream of ionotropic and metabotropic glutamate receptors, and voltage-dependent Ca^{2+} channels (VDCCs) via activation/inhibition of different kinases or phosphatases. The final result involves the disrupted homeostasis of the cytoskeleton, particularly the phosphorylation level of intermediate filament (IF) proteins [15,17,18,38]. Of particular neurotoxicological importance, cytoskeletal components such as IFs, are involved in the mechanical maintenance of cell shape and are important for proper organelle localization [14]. Consequently, the disruption of cytoskeletal elements is thought to be central in cell death and neurodegeneration observed after exposure to $(PhTe)_2$ [17,18].

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. Among the important cellular processes mediated by the cytoskeleton are cell division [47], proliferation [27], migration [7], cytokinesis [28] and differentiation [26]. Moreover, it is becoming widely recognized the remarkable role of the cytoskeleton in the signal transduction [10] via coordination of appropriate cell responses to intra and extracellular signals. Consequently,

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the neurotoxicity of $(PhTe)_2$ may be intimately associated with disruption of the normal physiology of IFs and related molecules (for review see [38]).

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 [20] implicated in important physiological roles in astrocytes, for instance, in the anchoring of glutamate transporter GLAST/EAAT1 in the plasma membrane [35]. In this regard, overexpression of GFAP has been associated with disruption of astrocyte homeostasis in neuropathological conditions [55] and in response to neurotoxins [9].

Neurofilaments (NFs) 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 [44]. The NFs are the most abundant elements of the mature axonal cytoskeleton where they control the axonal caliber and consequently nerve conductivity [19,21].

The IF phosphorylation is one of the most important post-translational modifications whose general role is to facilitate the IF reorganization and interaction of the IF proteins with other cell elements. The IF phosphorylation/dephosphorylation is mediated by numerous Ser/Thr protein kinases and phosphatases, which in turn, can be independent or dependent on second messengers. The phosphorylation/dephosphorylation processes take part of different signaling cascades that directly modulate the cytoskeleton assembly/disassembly [44]. 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 [44]. The GFAP and NFL phosphorylation sites are mostly located in the head domain and the specific Ser residues at this domain are phosphorylated by second messenger-dependent kinases such as cAMP-dependent protein kinase (PKA), Ca^{2+}/calmodulin-dependent protein kinase (PKCaM) and protein kinase C (PKC) [44], providing modulation of the cytoskeleton in response to the second messenger levels into the cell. Otherwise, NFM and NFH are highly phosphorylated by Pro-directed kinases on multiple Ser residues located within the tail domain Lys-Ser-Pro (KSP) repeats, which are likely to have consequences for axonal caliber, stability and transport [43]. 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 [20,37] and of the action of neurotoxicant compounds [1].

Previous evidence from our group have demonstrated that the homeostasis of the cytoskeleton of primary astrocytes and neurons is disrupted by metabolites in toxic concentrations through cell specific mechanisms [40]. However, until now we have not assessed the molecular mechanisms elicited by $(PhTe)_2$ in the isolated cells. 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 primary astrocytes and neurons isolated from the cerebral cortex of rats using the phosphorylation/dephosphorylation levels of IFs as molecular endpoint of tellurium neurotoxicity. A connection between IF hyperphosphorylation with cell morphology collapse was also investigated.

2. Material and methods

2.1. Ethical aspects

All animal procedures were approved (approval number 18,275) by the Animal Care and Use of the Federal University of Rio Grande do Sul.

2.2. Radiochemical and compounds

The organochalcogenide $(PhTe)_2$ was synthesized using the method described by Petragnani [39]. 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 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. Radiochemical and compounds are described in Table 1. All other chemicals were of analytical grade and were purchased from standard commercial supplier.

2.3. Isolated primary culture

Astrocyte primary cultures were prepared from the cerebral cortex of newborn (0–2 day-old; P0) Wistar rats, as previously described [30]. 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. After astrocytes had reached semi-confluence (15 days in vitro [DIV]), the culture medium was removed by suction, and the cells were incubated for additional 24 or 48 h at 37 °C in an atmosphere of 5% CO_2 in DMEM/F12 without FBS or in the presence of 1% FBS, respectively, in the presence of different $(PhTe)_2$ concentrations (0–10 μM). Morphological studies were performed with cells fixed for immunocytochemistry.

Primary neuronal cultures were prepared from cerebral cortex of embryonic day 18 Wistar rat as previously described by Moura Neto et al. [34]. Briefly, single-cell suspensions were obtained by dissociating cerebral cortex cells in DMEM/F12 medium supplemented with 33 mM glucose, 2 mM glutamine, and 3 mM sodium bicarbonate. Approximately 5×10^4 cells were plated on polylisine-treated 96-well plate, 18×10^4 cells were plated on polylisine-treated coverslips placed on a 24-well plate or 1.2×10^6 cells were plated on polylisine-treated 6-well plate. The neuronal cultures were kept in Neurobasal medium supplemented with B27, 2 mM glutamine and 25 μM glutamate for 24 h. After this, the culture medium was removed by suction, and the cells were incubated in Neurobasal medium supplemented with 2 mM glutamine and B27 for additional 7 days in a humid incubator (37 °C; 5% CO_2). When neuronal cultures were 7 DIV, the culture medium was removed and cells were treated for additional 24 (8 DIV) or 48 h (9 DIV) in Neurobasal medium in the presence of different $(PhTe)_2$ concentrations (0–10 μM). Morphological studies were performed with cells fixed for immunocytochemistry.

2.4. 3-(4,5-dimethyl-2-yl)22,5-diphenyl-2H-tetrazolium bromide assay

Cell viability was determined by 3-(4,5-dimethyl-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay. MTT at 0.5 mg/ml of DMEN/F12 was added to the incubation medium after $(PhTe)_2$ treatment and incubated for 1 h at 37 °C in a humidified 5% CO_2. After incubation, the medium from each well was gently removed by aspiration and 100 μl dimethylsulfoxide (DMSO) was added to each well followed by incubation and shaking for 15 min. The formazan product generated during the incubation was solubilized in DMSO and measured at 490 and 630 nm. Only viable cells are able to reduce MTT.