Behavioural Brain Research 247 (2013) 241–247

Contents lists available at SciVerse ScienceDirect

Behavioural Brain Research

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

Research report

p,*p*′-Methoxyl-diphenyl diselenide protects against amyloid-β induced cytotoxicity *in vitro* and improves memory deficits *in vivo*

Simone Pinton[a], Ana Cristina Souza[a], Marcel H.M. Sari[a], Rita M. Ramalho[b], Cecília M.P. Rodrigues[b,c,**], Cristina W. Nogueira[a,*]

[a] *Laboratório de Síntese, Reatividade e Avaliação Farmacológica e Toxicológica de Organocalcogênios, Centro de Ciências Naturais e Exatas, Universidade Federal de Santa Maria, Santa Maria, CEP 97105-900, RS, Brazil*
[b] *Research Institute for Medicines and Pharmaceutical Sciences (iMed.UL), Faculty of Pharmacy, University of Lisbon, Lisbon 1649-003, Portugal*
[c] *Department of Biochemistry and Human Biology, Faculty of Pharmacy, University of Lisbon, Lisbon 1649-003, Portugal*

HIGHLIGHTS

- *p*,*p*′-Methoxyl-diphenyl diselenide [$(MeOPhSe)_2$] protected against amyloid-β induced cytotoxicity.
- $(MeOPhSe)_2$ prevented Aβ-induced cell death by inhibiting apoptosis and downregulating JNK phosphorylation.
- $(MeOPhSe)_2$ treatment attenuated Aβ-induced memory deficits in mice.

ARTICLE INFO

Article history:
Received 17 December 2012
Received in revised form 20 March 2013
Accepted 23 March 2013
Available online 1 April 2013

Keywords:
p,*p*′-Methoxyl-diphenyl diselenide
Amyloid-β peptide
Apoptosis
c-Jun N-terminal kinase
Memory impairment

ABSTRACT

Behavioral evidence suggests that the organoselenium compound *p*,*p*′-methoxyl-diphenyl diselenide [$(MeOPhSe)_2$] ameliorates memory and learning performance in rodents. Here, we investigated the molecular mechanism of $(MeOPhSe)_2$ neuroprotection in cortical neurons exposed to amyloid-β (Aβ) peptide as well as in Aβ-infused mice. For this purpose, primary cultures of rat cortical neurons were pre-incubated with 10 μM of $(MeOPhSe)_2$ or vehicle, followed by exposure to 25 μM Aβ fragment 25–35 or vehicle. Furthermore, the therapeutic effect of $(MeOPhSe)_2$ (5 mg/kg, oral route, daily for 5 days) on memory deficits was evaluated in mice exposed to Aβ fragment 25–35 (3 nmol/3 μl/per site, intracerebroventricular infusion). The results demonstrate that $(MeOPhSe)_2$ prevented Aβ-induced cell death *in vitro*, associated with inhibition of caspase-3 and -9 activities, poly (ADP-ribose) polymerase (PARP) cleavage and c-Jun N-terminal kinase (JNK) activation. Further, $(MeOPhSe)_2$ rescued Aβ-induced memory impairment in mice. In conclusion, $(MeOPhSe)_2$ is neuroprotective *in vitro* and *in vivo*, suggesting that this organoselenium compound offers a potential treatment option for Alzheimer's disease.

1. Introduction

The deposition of amyloid-β peptide (Aβ) into dense senile plaques and the formation of neurofibrillary tangles (NFT) are main neuropathological hallmarks of Alzheimer's disease (AD), which is characterized by progressive loss of neurons and deterioration of memory and intellectual capacities [1,2]. Aβ plays a key role in the development of AD, with multiple neurotoxic effects ranging from Tau hyperphosphorylation and subsequent NFT formation, oxidative stress, excitotoxicity, neuroinflammation, neural dysfunction and cell death [1–4].

AD pathogenesis is considered complex and involves multiple pathways of neuronal damage. Recent data suggest that the c-Jun N-terminal kinase (JNK) intracellular signaling pathway may be involved in AD pathogenesis. In fact, JNK is activated in the brain of AD patients [5–8]. JNK is sensitive to oxidative stress and can be activated by Aβ, leading to phosphorylation of transcription

Abbreviations: AChE, acetylcholinesterase; AD, Alzheimer's disease; Aβ, amyloid-β; DMSO, dimethyl sulfoxide; DTT, dithiothreitol; HBSS, Hank's balanced salt solution; i.c.v., intracerebroventricular; JNKc-, Jun N-terminal kinase; MAP2, microtubule-associated protein 2; $(MeOPhSe)_2$, *p*,*p*'-methoxyl-diphenyl diselenide; MTS, tetrazolium [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt]; NFT, neurofibrillary tangles; PARP, poly (ADP-ribose) polymerase; PBS, phosphate buffered saline; STZ, streptozotocin; ^{13}C NMR, carbon-13 nuclear magnetic resonance; ^{1}H NMR, proton nuclear magnetic resonance.

* Corresponding author. Tel.: +55 55 32208140; fax: +55 55 32208978.
** Corresponding author at: Research Institute for Medicines and Pharmaceutical Sciences (iMed.UL), University of Lisbon, Av. Prof. Gama Pinto, Lisbon 1649-003, Portugal. Tel.: +351 21 794 6490; fax: +351 21 794 6491.
E-mail addresses: cmprodrigues@ff.ul.pt (C.M.P. Rodrigues), criswn@quimica.ufsm.br (C.W. Nogueira).

0166-4328/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.bbr.2013.03.034

factors that control the apoptotic process [7,9,10]. Furthermore, JNK activation triggers the phosphorylation of Tau and amyloid precursor protein (APP), leading to enhanced amyloidogenic cleavage [6,8]. The critical involvement of JNK in modulation of memory has been supported by the fact that inhibition of JNK phosphorylation reverses memory deficits in AD models [11,12]. Thus, several studies have demonstrated that the blockade of JNK activation could provide an alternative therapeutic strategy to AD [8,11–13].

Organoselenium compounds are promising pharmacological agents with neuroprotective properties [14–17]. Importantly, these compounds have been proven to ameliorate memory and cognitive function in rodents [18–22]. Moreover, we recently demonstrated that *p*,*p*′-methoxyl-diphenyl diselenide [$(MeOPhSe)_2$] improves memory in the model of sporadic dementia of Alzheimer's type induced by streptozotocin (STZ). The neuroprotective action of $(MeOPhSe)_2$ can be attributed to antioxidant/antinitrosative properties [23,24], inhibition of acetylcholinesterase (AChE) activity [23,25], and suppression of neuroinflammation [26]. However, the molecular mechanism of $(MeOPhSe)_2$ action is completely unknown as well as its possible effects on other models of AD. Therefore, in the present study we evaluated the effect of $(MeOPhSe)_2$ in Aβ-induced toxicity in primary cultures of rat cortical neurons. Furthermore, we assessed whether $(MeOPhSe)_2$ alleviates memory deficits induced by intracerebroventricular (i.c.v.) infusion of Aβ in mice.

2. Materials and methods

2.1. In vitro assays

2.1.1. Isolation and culture of rat cortical neurons

Primary cultures of rat cortical neurons were prepared from 17- to 18-day-old fetuses of Wistar rats as previously described [27], with minor modifications. In short, pregnant rats were CO_2 gas-anesthetized and decapitated. The fetuses were collected in Hank's balanced salt solution (HBSS-1; Invitrogen, Grand Island, NY, USA) and rapidly decapitated. After removal of meninges and white matter, the brain cortex was collected in Hank's balanced salt solution without Ca^{2+} and Mg^{2+} (HBSS-2). The cortex was then mechanically fragmented, transferred to a 0.025% trypsin in HBSS-2 solution, and incubated for 15 min at 37 °C. Following trypsinization, cells were washed twice in HBSS-2 containing 10% fetal calf serum and re-suspended in Neurobasal medium (Invitrogen), supplemented with 0.5 mM L-glutamine, 25 μM L-glutamic acid, 2% B-27 supplement (Invitrogen), and 12 mg/mL gentamicin. Neurons were then plated on tissue culture plates, precoated with poly-D-lysine at 1×10^6 cells/ml, and maintained at 37 °C in a humidified atmosphere of 5% CO_2. All experiments were performed using cells cultured for 14 days in fresh medium, supplemented with B-27 and L-glutamine. Cells were characterized by phase contrast microscopy and indirect immunocytochemistry for neurofilaments and glial fibrillary acidic protein. Neuronal cultures were >95% pure. After 14 days in culture, isolated rat neurons were incubated with 10 μM $(MeOPhSe)_2$ or vehicle dimethyl sulfoxide (DMSO) for 12 h. Cortical neurons were then co-incubated with 25 μM Aβ fragment 25–35 (Bachem AG, Budendorf, Switzerland), or vehicle (water) for 24 h. Adequate controls using Aβ fragment 35–25 reverse peptide (Bachem AG) were also performed. $(MeOPhSe)_2$ was synthesized as previously reported [28]. Analysis of the ^1H NMR and ^{13}C NMR spectra showed analytical and spectroscopic data in full agreement with its assigned structure. The chemical purity of 99.9% was determined by gas chromatography-mass spectrometry.

2.1.2. Measurement of cell death

Cell viability was measured by the tetrazolium [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS] viability assay (CellTiter 96®AQueous Non-Radioactive Cell Proliferation Assay; Promega) according to the manufacturer's instructions. In addition, Hoechst labeling of cells followed by morphological evaluation was used to determine levels of apoptosis. Apoptotic nuclei were detected by Hoechst labeling after cell fixation with 4% formaldehyde in phosphate buffered saline (PBS), for 10 min at room temperature. Following incubation with Hoechst dye 33258 (Sigma Chemical Co., St. Louis, MO, USA) at 5 μg/mL in PBS for 5 min, and PBS washes, slides were mounted with PBS:glycerol (3:1, v/v) and fluorescence visualized with an Axioskop fluorescence microscope (Carl Zeiss GmbH, Hamburg, Germany). Fluorescent nuclei were scored and categorized according to the condensation and staining characteristics of chromatin. Normal nuclei showed non-condensed chromatin dispersed over the entire nucleus. Apoptotic nuclei were identified by condensed and fragmented chromatin contiguous to the nuclear membrane, as well as nuclear fragmentation and presence of apoptotic bodies.

2.1.3. Caspase activity

General caspase-like activities were determined by colorimetric enzymatic cleavage of chromophore *p*-nitroanilide (pNA) from the specific substrate *N*-acetyl-Asp-Glu-Val-Asp-pNA, for caspase-3; *N*-acetyl-Val-Glu-Ile-Asp-pNA, for caspase-6; *N*-acetyl-Ile-Glu-Thr-Asp-pNA, for caspase-8; and *N*-acetyl-Leu-Glu-His-Asp-pNA, for caspase-9 (Sigma Chemical Co.). Samples were homogenized in isolation buffer, containing 10 mM Tris–HCl buffer, pH7.6, 5 mM $MgCl_2$, 1.5 mM potassium acetate, 2 mM dithiothreitol (DTT), and protease inhibitor cocktail tablets (Complete; Roche Applied Science, Mannheim, Germany). The proteolytic reaction was carried out in isolation buffer containing 50 μg cytosolic protein and 50 μM of each specific caspase substrate. The reaction mixtures were incubated at 37 °C for 1 h, and the formation of pNA was measured at 405 nm using a 96-well plate reader.

2.1.4. Immunoblotting

Steady-state levels of procapase-3 and cleaved caspase-3, full-length poly (ADP-ribose) polymerase (PARP) and cleaved PARP, JNK and p-JNK proteins were determined by Western blot analysis. Briefly, 100 μg of total protein extracts were separated on 12% SDS-polyacrylamide electrophoresis gels. Following electrophoretic transfer onto nitrocellulose membranes, immunoblots were incubated with 15% H_2O_2 for 15 min at room temperature. After blocking with 5% milk solution, the blots were incubated overnight at 4 °C with primary mouse monoclonal antibodies reactive to JNK and p-JNK, or primary rabbit polyclonal antibodies to caspase-3 and PARP (Santa Cruz Biotechnology, Santa Cruz, CA, USA); and finally with secondary antibodies conjugated with horseradish peroxidase (Bio-Rad Laboratories, Hercules, CA, USA) for 3 h at room temperature. The membranes were processed for protein detection using Super Signal™ substrate (Pierce, Rockford, IL, USA). β-Actin was used as a loading control. Protein concentrations were determined using the Bio-Rad protein assay kit according to the manufacturer's specifications.

2.1.5. Immunohistochemistry

Immunohistochemistry was performed in fixed neural cells to detect JNK expression and cellular localization. Briefly, cells were fixed with 4% formaldehyde in PBS, for 10 min at room temperature, washed, and incubated in serum blocking solution (Santa Cruz Biotechnology) with 0.3% Triton X-100 for 1 h. Specimens then were incubated with both monoclonal p-JNK and polyclonal MAP2 (Santa Cruz Biotechnology) antibodies overnight at 4 °C. After rinsing, specimens were incubated with either fluorescently labeled anti-mouse or anti-rabbit (CyTM2 andCyTM5, respectively) (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) for 2 h at room temperature. Adequate controls were prepared omitting the primary antibody or using preimmune IgG. Finally, nuclei were stained with Hoechst 33258 as described above. Samples were then mounted and visualized in an Axioskop fluorescence microscope (Carl Zeiss).

2.2. In vivo experiments

2.2.1. Experimental procedure

In vivo experiments were conducted using male Swiss mice (30–35 g), approximately 60 days old. Animals were maintained at 22–25 °C with free access to water and food, under a 12:12 h light/dark cycle with lights on at 7:00 a.m. All manipulations were carried out between 08:00 a.m. and 01:00 p.m. Animals were used according to the guidelines of the Committee on Care and Use of Experimental Animal Resources and with the approval of the Animal Use Committee (23081.007005/2010-96), Federal University of Santa Maria, Brazil. All efforts were made to minimize animal suffering and reduce the number of animals used in the experiments. Mice were divided into six groups (n = 8–10): I) Sham; II) Aβ; III) $(MeOPhSe)_2$; IV) Donepezil; V) Aβ + $(MeOPhSe)_2$; and VI) Aβ + Donepezil. Mice of groups II, V and VI received Aβ fragment 25–35 aggregated (3 nmol/3 μl/per site; i.c.v.). Aβ-peptide was dissolved in sterile filtered water and aggregated by incubation at 37 °C for 4 days before use [29]. Animals received $(MeOPhSe)_2$ (5 mg/kg) or Donepezil (5 mg/kg; positive control) or vehicle (canola oil) at 10 ml/kg body weight per oral route, *via* gavage by 5 days, after i.c.v. infusion of Aβ peptide or vehicle (water). The dose of Aβ was chosen according to Wang et al. [30] and infusion of Aβ peptide or vehicle was performed by a "free hand" protocol with the bregma fissure as a reference point [31].

2.2.2. Morris water maze test

Spatial learning and memory were accessed using the Morris water maze task [32]. The water maze consisted of a basin (180 cm × 40 cm) made of black plastic and filled with water (22 ± 2 °C) at a height of 30 cm. Black plastic beads were evenly spread over the water surface to camouflage the escape platform (diameter: 10 cm) made of black plastic and covered with a wire mesh grid to ensure a firm grip. The pool was placed in a room with several extra maze visual cues. For the acquisition phase, mice were placed next to and facing the wall successively in north, south, east and west positions. The escape platform was hidden 1 cm below water level in the middle of the northwest quadrant. Behaviors were videotaped *via* TV camera. The equipment was hidden from the view of the animals, but was able to follow their