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A diphenyl diselenide-supplemented diet and swimming exercise promote neuroprotection, reduced cell apoptosis and glial cell activation in the hypothalamus of old rats

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ABSTRACT

Aging is a process characterized by deterioration of the homeostasis of various physiological systems; although being a process under influence of multiple factors, the mechanisms involved in aging are not well understood. Here we investigated the effect of a $(PhSe)_2$-supplemented diet (1 ppm, 4 weeks) and swimming exercise (1% of body weight, 20 min per day, 4 weeks) on proteins related to glial cells activation, apoptosis and neuroprotection in the hypothalamus of old male Wistar rats (27 month-old). Old rats had activation of astrocytes and microglia which was demonstrated by the increase in the levels of glial fibrillary acidic protein (GFAP) and ionized calcium-binding adaptor molecule 1 (Iba-1) in hypothalamus. A decrease of B-cell lymphoma 2 (Bcl-2) and procaspase-3 levels as well as an increase of the cleaved PARP/full length PARP ratio (poly (ADP-ribose) polymerase, PARP) and the pJNK/JNK ratio (c-Jun N-terminal kinase, JNK) were observed. The levels of mature brain-derived neurotrophic factor (mBDNF), the pAkt/Akt ratio (also known as protein kinase B) and NeuN (neuronal nuclei), a neuron marker, were decreased in the hypothalamus of old rats. Old rats that received a $(PhSe)_2$-supplemented diet and performed swimming exercise had the hypothalamic levels of Iba-1 and GFAP decreased. The combined treatment also increased the levels of Bcl-2 and procaspase-3 and decreased the ratios of cleaved PARP/full length PARP and pJNK/JNK in old rats. The levels of mBDNF and NeuN, but not the pAkt/Akt ratio, were increased by combined treatment. In conclusion, a $(PhSe)_2$-supplemented diet and swimming exercise promoted neuroprotection in the hypothalamus of old rats, reducing apoptosis and glial cell activation.

1. Introduction

Aging is a gradual process caused by genetic, molecular and cellular changes, leading to the end of life (Harman, 2001). Many hypotheses have been proposed to explain the cause of aging and the biological bases for the functional decline (López-Otín et al., 2013). In a recent study, it was demonstrated that hypothalamus plays a key role in the development and lifespan control in rodents. Apparently, hypothalamus has a programmatic role in aging development and this is ruled by the integration between neuroimmune response and neuroendocrine activity (Zhang et al., 2013). In an aged brain, astrocytes and microglia suffer activation becoming hypertrophic and expressing more GFAP and Iba-1, markers of astrocytes and microglia activation, respectively (Blasko et al., 2004). These two types of glial cells can undergo overactivation by influence of pro-inflammatory factors (TNFα, PGE2, and INFγ) and oxidative stress (NO, H_2O_2, $O_2^{\bullet -}$). This overactivation is highly related with the generation of more cytotoxic molecules that can lead to neuroinflammation and neurodegeneration (Herman et al., 2012).

Apoptosis is involved in neuronal cell death occurring during the normal aging process being of vital importance in developing and maintaining the normal tissue homeostasis. However, excessive apoptosis seems to highlight the age-associated decline and deterioration in structures and functions of tissues and organs (Muradian and Schachtschabel, 2001). It has been reported that markers of apoptotic cell death, such as caspases, are increased in the old rat brain (Kim et al., 2010). Besides caspases, the Bcl-2 family proteins; including Bcl-2, $Bcl\text{-}2_{xl}$, Bax and Bid; also play important roles in apoptotic process determining the mitochondrial response to apoptotic stimuli, such as pro-inflammatory cytokines and free radicals (Upadhyay et al., 2003). Jun. N-terminal kinases (JNKs) are proteins that possess three isoforms and play an essential role in modulating the functions of pro- and anti-apoptotic proteins located in mitochondria (Schroeter et al., 2003). In addition, JNKs are essential mediators of relevant pro-inflammatory functions in microglia (Waetzig et al., 2005). In contrast to apoptotic proteins, the mature form of brain-derived neurotrophic factor (mBDNF) induces the activation of signaling pathways involved with

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neuronal survival, neuroplasticity and neurogenesis (Cunha et al., 2010), among them the phosphatidylinositol-3 kinase (PI3K)/Akt pathway.

Pharmacological and non-pharmacological interventions have been proposed to prevent the aging effects. Various nutritional factors are capable of restoring neuroendocrine- immune network, the metabolic harmony and the capacity to respond to oxidative stress altered by aging (Meydani, 2001).

Selenium (Se) is a trace element that has been reported to have beneficial effects on immune and antioxidant systems (Mocchegiani et al., 2014). Our previous study demonstrated that a diphenyl diselenide $(PhSe)_2$-supplemented diet improves memory and reduces circulatory levels of pro-inflammatory markers in old rats (Cechella et al., 2014; Leite et al., 2015), making it a potential intervention to alleviate deleterious processes caused by aging. In addition, evidence has been found to suggest that physical exercise reduces the risk of disorders associated with aging (Kim et al., 2003; Erickson and Kramer, 2009). In fact, physical exercise promotes the activation of numerous cell-signaling proteins which are known to be associated with neuronal survival, proliferation, and synaptic plasticity (Chen et al., 2007).

Therefore, the aim of the present study was to investigate the effect of a $(PhSe)_2$-supplemented diet and swimming exercise on proteins related to glial cells activation, apoptosis and neuroprotection in the hypothalamus of aged rats.

2. Materials and methods

2.1. Animals

Adult (4 months, 12%–16% lifespan) and old (27 months, 81%–100% lifespan) male Wistar rats were obtained from a local breeding colony and were housed in cages, with free access to food and water. They were kept in a separate air-conditioned (22 ± 2 °C) room, on a 12-h light/12-h dark cycle, with lights turned on at 07:00 a.m. The present experimental study was approved by the Institutional Ethics Committee on Care and Use of Experimental Animal Resources from the Federal University of Santa Maria, Brazil and registered under the number of 5394050115.

2.2. Drugs

Diphenyl diselenide $(PhSe)_2$ was prepared in our laboratory according to the method described by Shaaban et al. (2015) and the chemical purity (99.9%) was determined by GC/MS. Analysis of ^{1}H and ^{13}C NMR spectra showed analytical and spectroscopic data in full agreement with its assigned structure.

2.3. Experimental procedure

The animals were divided in five groups (eight animals per group): Group I (adult control) - adult rats received standard diet chow and did not perform exercise; Group II (old control) - old rats received standard diet chow and did not perform exercise; Group III ($(PhSe)_2$) - old rats received 1 ppm of $(PhSe)_2$-supplemented diet for 30 days and did not perform exercise; Group IV (exercise) - old rats received standard diet chow and performed swimming training; Group V (exercise plus $(PhSe)_2$) - old rats received 1 ppm of $(PhSe)_2$-supplemented diet and performed swimming training. With the aim to minimize observer bias, the observer was unaware of which individuals or group was subject to the treatment or procedure variables.

2.4. Dietary supplementation

Animals were fed daily with ~150 g/animal standard diet chow or chow supplemented with 1 ppm of $(PhSe)_2$ (2–3 animals per cage). The choice of protocol regimen used in this experimental study was based on our previous study (Cechella et al., 2014) that demonstrated that a diet supplemented with 1 ppm of $(PhSe)_2$ for 30 days has neuroprotective effects in old rats. The standard diet was pulverized with ethanol, whereas the supplemented diet was pulverized with $(PhSe)_2$ dissolved in ethanol (1 mg/10 ml). The standard and supplemented diets were kept at room temperature for 3 h to evaporate the alcohol and then kept at 4 °C for no >1 week (de Bem et al., 2009).

2.5. Exercise training protocol

Animals were submitted to the pre-training period of 20 min/day, 5 days (exercise and exercise plus $(PhSe)_2$ groups). After the swimming adaptation, old rats performed the swimming training with a workload (1% of body weight, 20 min per day for 4 weeks) (Ravi Kiran et al., 2004). The swimming training was performed between 09:30 and 11:00 a.m. in water at a temperature of 32 ± 1 °C. Rats from adult and old control groups and $(PhSe)_2$ group were adapted to the water by placing them to the bottom of a separate tank with shallow water (5 cm) at 32 ± 1 °C, without the workload. At the end of the exercise training, rats were towel-dried and returned to their respective cages.

Twenty-four hours after the last swimming training animals were killed by decapitation, the brains were collected and samples of hypothalamus were prepared for the western blot assay (n = 5 rats/group). For immunohistochemistry assay, rats (n = 3 rats/group) were deeply anesthetized with chloral hydrate (40%, i.p.) and were perfused through the left cardiac ventricle with 0.9% saline solution, followed by 4% paraformaldehyde in 0.1 M phosphate buffered saline (PBS), pH 7.4. After perfusion, the brains were removed, post-fixed in the same fixative solution by 24 h.

2.6. Immunohistochemistry assay

Dehydrated brains were embedded in paraffin and hypothalamic sections (8 μm) were cut in a microtome and mounted on SuperFrost-Plus glass slides (Thermo 213 Scientific, Rockford, IL, USA). Paraffin-embedded brain sections were deparaffined, rehydrated, and boiled 3 times in 10 mM citrate buffer, pH 6. Sections were then incubated for 60 min in blocking buffer, containing 10% (v/v) normal donkey serum (DS) in sodium phosphate buffer with 0.1% (v/v) Triton X-100 (PBS-Tx) at room temperature. Subsequently, the sections were incubated overnight at 4 °C with mouse anti-GFAP (Sigma, 1:400) in 1% DS diluted in 0.5% PBS-Tx. After three washes in PBS, tissue sections were incubated with anti-mouse Alexa 488 (Invitrogen, 1:400) in 1% DS diluted in 0.5% PBS-Tx for 2 h at room temperature. Thereafter, the sections were washed three times in PBS and then were incubated with 0.5 μg/ml DAPI (Invitrogen) for 10 min. The sections were washed three times in PBS and mounted on slides with Fluor Save (Merck) and covered with coverslips. Finally, images of hypothalamic sections were obtained on an Axioskop fluorescence microscope (Carl Zeiss) and examined with ImageJ software.

2.7. Western blot assay

Samples of hypothalamus were homogenized in 400 μl sodium dodecyl sulfate (SDS) 5% with protein inhibitor cocktail (Sigma-Aldrich Co., St. Louis, Missouri, USA). Hypothalamus extracts were diluted to a final protein concentration of 2 μg/μl in sample buffer (essentially constituted by Tris·HCl 0.5 M, pH 6.8 (in a final concentration of 62.5 mM), glycine, SDS, dithiothreitol (DTT), the reducing agent, and bromophenol blue, used as a marker to monitor the process of electrophoresis. The samples (20 μg of protein) and pre-stained molecular weight standards (Sigma-Aldrich Co., St. Louis, Missouri, USA) were separated by 12% and 17% SDS-PAGE electrophoresis and transferred to nitrocellulose membrane (0.45 μm, Bio-rad) using Transfer-Blot® Turbo™ Transfer System (1.0 A; 30 min for proteins above 25 kDa or 5 min for proteins below 25 kDa) and/equal protein loading was