Journal of Neuroimmunology 321 (2018) 125–137

Contents lists available at ScienceDirect

Journal of Neuroimmunology

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

Sildenafil ameliorates EAE by decreasing apoptosis in the spinal cord of C57BL/6 mice

Eduardo Duarte-Silva[a,c], Shyrlene Meiry da Rocha Araújo[a], Wilma Helena Oliveira[a], Deniele Bezerra de Lós[d], Maria Eduarda Rocha de França[b], Amanda Pires Bonfanti[e], Gabriela Peron[e], Livia de Lima Thomaz[e], Liana Verinaud[e], Ana Karolina de Santana Nunes[b], Christina Alves Peixoto[a,f,*]

[a] Laboratory of Ultrastructure, Aggeu Magalhães Institute (IAM), PE, Brazil
[b] Postgraduate Program in Biological Sciences/Center of Biosciences, Federal University of Pernambuco (UFPE), Recife, PE, Brazil
[c] Postgraduate Program in Biosciences and Biotechnology for Health (PPGBBS), Oswaldo Cruz Foundation (FIOCRUZ-PE)/Aggeu Magalhães Institute (IAM), Recife, PE, Brazil
[d] Postgraduate Program in Biotechnology/Northeast Network in Biotechnology (RENORBIO), Federal University of Pernambuco (UFPE), PE, Brazil
[e] Departament of Structural and Functional Biology, University of Campinas (UNICAMP), Campinas, São Paulo, Brazil
[f] Institute of Science and Technology on Neuroimmunomodulation (INCT-NIM), Brazil

ARTICLE INFO

Keywords:
Apoptosis, C57BL/6 mice
EAE
MS
Sildenafil
Remyelination

ABSTRACT

Apoptosis is one form of cell death that is intimately related to health and pathological conditions. In most neuroinflammatory and/or neurodegenerative diseases, apoptosis is associated with disease development and pathology and inhibition of this process leads to considerable amelioration. It is becoming evident that apoptosis also participates in the pathogenesis of Multiple Sclerosis (MS) and its animal model, Experimental Autoimmune Encephalomyelitis (EAE). Drugs such as Sildenafil, a Phosphodiesterase type 5 Inhibitor (PDE5I), have proven to be neuroprotective in MS models. However, it is not known whether Sildenafil is able to modulate cell death, specifically apoptosis, in EAE mice. Therefore, the aim of this study was to determine the effects of Sildenafil on extrinsic and intrinsic apoptosis pathways in the spinal cord of C57BL/6 mice with EAE. TUNEL analysis showed that EAE mice had elevated number of TUNEL$^+$ cells and that treatment with Sildenafil led to reduced number of dying cells, indicating that Sildenafil was able to inhibit cell death. We observed that both extrinsic and intrinsic pathways of apoptosis were governing the dynamics of EAE progression. We showed that in EAE mice there were increased levels of extrinsic (Caspase-8, -3, TNF-α, FADD) and intrinsic (Caspase-9, Bax and Cytochrome C) apoptosis markers. Bcl-2, an anti-apoptotic protein, was downregulated in EAE mice. We also demonstrated that EAE mice had increased levels of non-caspase mediators of cell survival/cell death (p-IκBα and p-MAPK-p38). Besides, EAE mice presented augmented demyelination. Nevertheless, this is the first research to demonstrate that Sildenafil, when administered concomitant to disease induction, modulated the expression of pro- and anti-apoptotic proteins of the extrinsic and intrinsic pathways, as well as diminished the expression of non-caspase mediators and promoted remyelination in the spinal cord, indicating neuroprotective effects. Thus, the present study demonstrated that Sildenafil inhibits apoptosis by two distinct, although interconnected, mechanisms: directly by modulating caspase expression (through extrinsic and intrinsic pathways) and indirectly by modulating the expression of molecules involved in cell death and/or cell survival.

1. Introduction

Multiple Sclerosis (MS) is a complex chronic disease of the central nervous system (CNS) that has an autoimmune and inflammatory nature (Broux et al., 2013). In spite of all efforts and research done so far, there is not a single animal model that can capture the whole complexity of this pathology in human beings (Denic et al., 2011). Nevertheless, there are some widely used animal models of MS, such as Experimental Autoimmune Encephalomyelitis (EAE), which share a lot of similarities with MS, like inflammation and axonal degeneration (Steinman and Zamvil, 2005). Besides, Copaxone, Mitoxantrone and Natalizumab, drugs approved for MS treatment, were first tested in this

* Corresponding author at: Laboratory of Ultrastructure, Aggeu Magalhães Institute (IAM), PE, Brazil.
E-mail addresses: peixoto.christina@gmail.com, cpeixoto@cpqam.fiocruz.br (C.A. Peixoto).

https://doi.org/10.1016/j.jneuroim.2018.06.002
Received 22 February 2018; Received in revised form 16 May 2018; Accepted 4 June 2018

model. Thus, EAE is a relevant model for studying the effects of drugs in animals with MS-like symptoms (Steinman and Zamvil, 2006).

Apoptosis is one form of programmed cell death that plays a major role in normal and pathological conditions. For instance, during normal development, specifically in the brain development, there is elimination of unwanted extra cells, such as neurons, in order to have their number precisely regulated (Bibel and Barde, 2000; Singh, 2007). On the other hand, deregulated apoptosis is related to initiation and progression of infectious, autoimmune and neurodegenerative diseases, and also cancer (Singh, 2007). Apoptosis is triggered either by intrinsic or extrinsic stimuli. The extrinsic pathway is activated when a specific ligand, such as TNF or Fas ligand, binds to the death receptor in the cell surface, such as TNFR and FasR. This binding induces the activation of the receptors, which in turn rearrange in order to form a homotrimeric complex, which then participates in the recruitment of TNFR1-associated death domain protein (TRADD) and Fas-associated death domain protein (FADD) (Kiechle and Zhang, 2002). The intrinsic pathway is related to the mitochondria and happens when this organelle responds to stress signals releasing pro-apoptotic factors, such as Apoptosis-inducing factor (AIF), Second mitochondria-derived activator of caspase/direct inhibitor of apoptosis-binding protein with low pI (SMAC/DIABLO), Endonuclease G and Cytochrome C (Kiechle and Zhang, 2002).

Many studies demonstrate that apoptosis is one of the pathological mechanism in MS and EAE (Das et al., 2008; Dasgupta et al., 2012, 2013; Meyer et al., 2001). For that reason, studies addressing the apoptotic pathway are essential, once new molecular targets can be discovered and new drugs can be used to treat neuroinflammatory diseases, such as MS.

Phosphodiesterase-5 (PDE-5) is an enzyme responsible for the regulation of intracellular levels of the second messenger 3,5-cyclic guanosine monophosphate (cGMP) (Corbin, 2004; Corbin and Francis, 1999), which is involved in a plethora of cellular processes, such as cell proliferation and differentiation (Hertz and Beavo, 2011), neuroplasticity (Reierson et al., 2011) and memory (Domek-Łopacińska and Strosznajder, 2005). Interestingly, a study suggests that increased levels of cGMP is associated with anti-inflammatory effects (Rapôso et al., 2014). Sildenafil (Viagra ®) is a PDE-5 selective inhibitor and it has classically been used as a treatment for erectile dysfunction (ED) (Goldstein et al., 1998) and pulmonary hypertension (Shekerdemian et al., 2002). Besides, some studies showed that Sildenafil's use is correlated with angiogenesis and axonal remodeling (Ding et al., 2008, 2011), oligodendrogenesis (Zhang et al., 2012), improvement in memory, cognition and learning (Cuadrado-Tejedor et al., 2011; Shahidi et al., 2014), decreased microglial activation and reduction in pro-inflammatory cytokines secretion and oxidative stress (Nunes et al., 2012; Raposo et al., 2013). Some studies show that the PDE5 inhibitors (PDE5Is) can interfere with apoptosis, either by inducing or preventing it from happening. One study about idiopathic pulmonary arterial hypertension (IPAH) showed that administration of Tadalafil led pulmonary arterial smooth muscle cells (PASMCs) to apoptosis with subsequent amelioration of IPAH (Yamamura et al., 2017). Mei et al. (2015) showed that Sildenafil induced apoptosis of human colorectal cancer cells (Mei et al., 2015). On the other hand, Bolnick et al. (2015) demonstrated that Sildenafil was able to prevent the apoptosis of the human first-trimester trophoblast cells when they were exposed to oxidative stress (Bolnick et al., 2015). Another study revealed that Sildenafil prevented apoptosis of cardiomyocytes in an animal model of cardiotoxicity (Fisher et al., 2005). Nevertheless, more research is yet to be performed in order to elucidate its therapeutic potential, mechanisms of action and safety regarding its use in CNS diseases. Therefore, the aim of this study was to evaluate the effects of Sildenafil on apoptosis in C57BL/6 mice spinal cord.

2. Material and methods

2.1. Animals

A total of 37 C57BL/6 female mice aged 8–12 weeks and weighing 25–30 g from Aggeu Magalhães Institute were used and distributed in the following experimental groups: a) Control (n = 9) - they received only water; b) EAE (n = 13) – EAE was induced and they received only vehicle (water); c) SILD (n = 14) - EAE was induced and they received 25 mg/kg of Sildenafil subcutaneously (s.c) during 21 days. Mice were kept under a controlled temperature (22 °C) and photoperiod environment (12 h/12 h light/dark) and received water and standard chow *ad libitum* throughout the entire experiment. The experiment was approved by and performed in accordance with the guidelines of the Aggeu Magalhães Institute Ethics Committee/Oswaldo Cruz Foundation (87/2015 CEUA/FIOCRUZ).

2.2. Experimental autoimmune encephalomyelitis (EAE) induction

The EAE was induced as described elsewhere (Verinaud et al., 2015), with a few modifications. Briefly, each animal was immunized with 200 μg of MOG-$_{35-55}$ (Genscript, USA) emulsified in Complete Freund's Adjuvant (CFA, Sigma, USA) supplemented with dried out *M. tuberculosis* (H37RA) through an injection *via* s.c. in the upper back (the region immediately after the head) and in the tail base (100 μL of emulsion in each region). Pertussis toxin (Ptx, Sigma) (240 ng) was administered *via* i.p. at 0 h and 48 h after the immunization. Clinical signs of the disease were followed and graded daily according to a 0–5 score scale, in which: 0, no sign; 1, limp tail; 2, hind limbs weakness; 3, hind limb paralysis; 4, hind limb paralysis and fore limb weakness; 5, full paralysis/death. In the last day of experiment, mice were anesthetized *via* i.p with 80 mg/kg of ketamine and 10 mg/kg of xylazine. The spinal cord was removed by ejection. After mice dissection for vertebral column removal, a 10 mL syringe with PBS (1 ×) was inserted in the spinal canal lumen of the thoracic or sacral region and pressure was applied until the spinal cord was completely removed. The spinal cord was washed in PBS (1 ×) and used for analysis of the inflammatory and apoptotic pathways.

2.3. Sildenafil treatment

Sildenafil treatment (Viagra®, Pfizer) was initiated in the first day after the immunization. The drug was diluted in a salt solution (NaCl 0.2%) at a dose of 25 mg/kg and was administered subcutaneously (s.c) twice a day (8 h interval between doses) during 21 days.

2.4. Immunohistochemistry

The immunohistochemical analysis was performed as described elsewhere (Oliveira et al., 2016). Briefly, after anesthesia, the animals were transcardially perfused with 20 mL of physiological saline followed by 40 mL of 4% paraformaldehyde (PFA, Sigma) in 0.1 M phosphate sodium phosphate monobasic and dibasic heptahydrate – Sigma-Aldrich) buffered saline (PBS), pH 7.2. After euthanasia, mice spinal cord was removed and *overnight* (O/N) post-fixed in the same fixative. The samples (n = 3 per group) were processed for paraffin, as routine, and transverse sections with a thickness of 5 μm were cut on an RM 2035 microtome (Reichert S, Leica). The endogenous peroxidase activity was blocked with hydrogen peroxide (3%) diluted in methanol for 20 min and unspecific sites with blocking solution of bovine serum albumin (1%) (Miles, Naperville, IL, USA), TWEEN 20 and PBS (1 ×) for 1 h room temperature. Next, the samples were incubated O/N at 4 °C with the following primary antibodies: Caspase-8 (Santa Cruz, sc5263, 1:100), Caspase-3 (ABCAM, ab4051, 1:100) and TNF-α (Prepotech, #500-P64, 1:50). After being washed with PBS (1 ×), the slides were incubated for 1 h at ambient temperature with a biotin-conjugated