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Research paper

Phosphodiesterase-5 inhibition promotes remyelination by MCP-1/CCR-2 and MMP-9 regulation in a cuprizone-induced demyelination model

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ABSTRACT

While it has recently been shown that sildenafil (Viagra®) has a protective effect on myelination/remyelination, the mechanism of this protection is still unknown. In general, cytokines, chemokines and metalloproteinases have a pro-inflammatory action, but can also exert a role in modulating glial cell activation, contributing to the balance of cell response. Investigating these molecules can contribute to clarifying the mechanisms of sildenafil neuroprotection. In addition, it is not known whether sildenafil is able to restore an already installed neurodegenerative process or if the treatment period is critical for its action. The aim of the present study was to evaluate, in a cuprizone (CPZ)-induced demyelination model, the effects and mechanisms of time-dependent treatment with sildenafil (beginning 15 days after neurodegeneration and continuing for 15 days, or starting concomitantly with neurodegeneration and continuing for 30 days) on neuroinflammation and remyelination. Neuroinflammation and demyelination induced by CPZ in rodents has been widely used as a model of multiple sclerosis (MS). In the present study, five male C57BL/6 mice aged 7–10 weeks were used per group. For four weeks, the groups received either cuprizone (CPZ) 0.2% mixed in feed or CPZ combined with the administration of sildenafil (Viagra®, Pfizer, 25 mg/kg) orally in drinking water, starting concurrently with (sild-T0) or 15 days (sild-T15) after the start of CPZ treatment. Control animals received pure food and water. The cerebella were dissected and processed for immunohistochemistry, immunofluorescence (frozen), Western blotting, Luxol fast blue staining and transmission electron microscopy. Magnetic resonance was performed for live animals, after the same treatment, using CPZ 0.3%. CPZ induced an increase in the expression of IL-1β and a decrease in MCP-1, CCR-2, MBP and GST-pi, as well as promoting damage in the structure and ultra-structure of the myelin sheath. Interestingly, the administering of sild-T0 promoted a further increase of MMP-9, MCP-1, and CCR-2, possibly contributing to changes in the microglia phenotype, which becomes more phagocytic, cleaning myelin debris. It was also observed that, after sild-T0 treatment, the expression of GST-pi and MBP increased and the myelin structure was improved. However, sild-T15 was not efficient in all aspects, probably due to the short treatment period and to starting after the installation of the degenerative process. Therefore, the present study shows that sildenafil modulates inflammation, with the involvement of MMP-9, MCP-1, and CCR-2, and also contributes to myelin repair. These protective effects were dependent on the therapeutic strategy used. This clarification can strengthen research proposals into the mechanism of action of sildenafil and contribute to the control of neurodegenerative diseases such as MS.

1. Introduction

Multiple sclerosis (MS) is a chronic immune-inflammatory disease of the central nervous system (CNS) characterized by demyelination of the white matter and axonal injury. The cerebellum has been described as an important region of the CNS that is affected by neurodegenerative diseases, such as MS, revealing severe white matter atrophy and inflammation (Riccitelli et al., 2012; Shields et al., 2012). In demyelinating diseases, important functions such as electrical conduction, connectivity and axolemal organization are compromised. Consequently, the injured axons become unable to exercise their functions efficiently, leading to severe psychomotor deficits (Bénardais et al., 2013). Therefore, research into new drugs with a

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possible remyelinating or neuroprotective role has been the subject of studies by many research groups.

Various studies have shown, for example, that the inhibition of phosphodiesterase-5 (PDE-5) by sildenafil (Viagra®) reduces neurologic deficits and increases neurogenesis and functional recovery after a stroke in rats (Charriaut-Marlangue et al., 2014; Chen et al., 2014). In terms of myelination, sildenafil has been shown to promote efficient reconstitution of the myelin sheath and control the inflammatory processes involved in demyelination models, including EAE (Experimental Autoimmune Encephalopathy) and CPZ (Cuprizone) (Nunes et al., 2012; Pifarre et al., 2011; Rapôso et al., 2013; Zhou et al., 2009). Furthermore, it has been observed that patients suffering from erectile dysfunction and, in parallel, multiple sclerosis showed an improvement in clinical status for both pathologies (Xiao et al., 2012). Thus, the beneficial implications of this drug may be associated with several factors, including cGMP accumulation in the brain.

The demyelination process is accompanied by the activation of microglial cells and astrocytes, which may assume beneficial or deleterious functions (Allan et al., 2005). The activation of these cells is associated with phenotypic changes, compatible with inflammation. Microglia and astrocytes produce pro-inflammatory cytokines (IL-1β, TNF-α), chemokines and their receptors (MCP-1/CCR-2) (Conductier et al., 2010), as well as metalloproteinase-9 (MMP-9) (Castier et al., 2009). Generally, the excessive expression of these inflammatory mediators is associated with detrimental effects on the tissue, promoting axonal injury (Kim et al., 2012).

During the remyelination process, microglia changes from a pro-inflammatory (M1) to an anti-inflammatory or immunoregulatory (M2) phenotype. Studies in vitro have observed an increase in the differentiation of oligodendrocytes when exposed to microglia M2, leading to a remyelinating state (Miron et al., 2013). The expression of MCP-1/CCR-2 by glial cells may promote this change in the phenotypes of microglia in an attempt to repair the injured environment (Low et al., 2001). Besides the regulation of these chemokines, matrix metalloproteinase (MMP-9) has an important role in controlling demyelinating diseases during the remyelination phase. MMP-9 is involved in tissue and vascular remodeling, oligodendrocyte differentiation and synaptic plasticity (Hsieh et al., 2008; Morello et al., 2011; Oh et al., 1999). A study assessing myelination by Schwann cells in the peripheral nervous system demonstrated that MMP-9 regulates the differentiation of these cells, contributing to remyelination (Kim et al., 2012).

Borán et al. (2008) showed that the cGMP–PKG pathway stimulated the regulation of microglial cell morphology, inducing a dramatic reorganization in the actin cytoskeleton compatible with a protective phenotype, which is more effective at removing dead cells. This data supports the hypothesis that cGMP–PKG signaling can modulate the pathways involved in inflammation, playing a role in the regulation of glial cell activation. This effect can contribute to the resolution of neuroinflammation. However, this mechanism remains unclear; it is possible that MMP-9 and MCP-1/CCR-2 are involved.

Therefore, the aim of the present study was to evaluate the effects and mechanisms of time-dependent treatment with sildenafil (for 15 or 30 days) on the restoration of myelin and balancing of neuroinflammation. The expression of MBP (myelin basic protein), GST-pi (mature oligodendrocytes marker), MCP-1/CCR-2, MMP-9, and IL-1β were evaluated, and the tissue structure and ultrastructure were analyzed by Luxol fast blue staining, transmission electron microscopy and magnetic resonance.

2. Materials and methods

Four groups of C57BL/6 mice aged 7 to 10 weeks and weighing 15 to 20 g were used (n = 5 per group; 20 animals total) in each processing method (80 animals for all experiments). The mice were examined for health status, acclimated to the laboratory environment at 25 °C and a 12-h light/dark photoperiod, and housed in metal cages. All experiments were carried out in compliance with the ethical guidelines for animal experimentation (L-10/2010-CEUA/FIOCRUZ).

2.1. Experiment design

The control group received a standard laboratory diet and pure water. Over a period of 30 days, the experimental groups received 0.2% cuprizone (CPZ) (oxalic bis-cyclohexylidenehydrazide — Sigma-Aldrich Inc., St. Louis, MO, USA) mixed in food. One group received no sildenafil treatment; while a second group received 25 mg/kg of sildenafil (Pfizer Inc., New York, NY, USA) from the first day of CPZ treatment (day 0) until the 30th day (day 30). This group was named T0. A third group received 25 mg/kg of sildenafil from day 15 to day 30 (T15) of CPZ treatment. Sildenafil was administered via drinking water, as previously described by Ramamurthy and Ronnett (2006), Saraiva et al. (2009), and Zhang et al. (2002). Body weight was measured every week and the drug concentration in the water was adjusted daily to maintain the dose (Fig. 1). Following treatment, the experimental and control animals were anesthetized with a mixture of ketamine (115 mg/kg, i.m.) and xylazine (10 mg/kg, i.m.) 1:1 (Sespo Comércio e Indústria Ltda., São Paulo, SP, Brazil) and euthanized as described below.

2.1.1. Immunohistochemistry (IH)

After anesthesia, the animals were transcardially perfused with physiological saline (20 ml), followed by 40 ml of 4% paraformaldehyde (Sigma-Aldrich) in 0.1 M phosphate (sodium phosphate monobasic and dibasic heptahydrate — Sigma-Aldrich) buffered saline (PBS), pH 7.2. The cerebella were immediately removed and post-fixed overnight in the same fixative. The samples were dehydrated in an ethanol series (Isofar Chemical Co., RJ, Brazil), cleared in xylene and embedded in paraffin (Merck, #1071642504). Sections (5 μm thickness) were cut on an RM 2035 microtome (Reichert S, Leica), re-hydrated, washed in 0.05 M PBS and incubated in PBS with 1% bovine serum albumin (BSA, fraction V) (Miles, Naperville, IL, USA) for 1 h. The endogenous peroxidase was blocked with 10% hydrogen peroxide (10 min), and antigen retrieval was performed by pre-treating the sections with 20 mM citrate buffer, pH 6.0, at 100 °C, for 30 min. Cerebellum sections of all groups were incubated overnight at 4 °C with the rabbit polyclonal primary antibody anti-MBP (Abcam Cambridge, USA; ab40390). After washing, sections were overlaid for 1 h with a biotin-conjugated secondary antibody, using a horseradish peroxidase (HRP) kit (DakoCytomation, CA, USA, Biotinylated Link Universal HRP; #K0690) and visualized using 3′-3-diaminobenzidine as a chromogen. Slices were then weakly counter-stained with Harris hematoxylin and mounted in Entellan (Merck, #1079610100). Reaction was performed in three slices per animal and analysis was performed in five panels per reaction (5 × 3 = 15 panels per animal). The Image Manipulation Program Gimp 2.8 (freely distributed, http://www.gimp.org/) was used in analysis.

2.1.2. Western blotting (WB)

After anesthesia, animals were euthanized and the cerebella were quickly dissected and homogenized in an extraction cocktail (10 mM EDTA, Amresco, Solon, USA; 2 mM phenylmethane sulfonyl-fluoride, 100 mM NaF, 10 mM sodium pyrophosphate, 10 mM $NaVO_4$, 10 μg of aprotinin/ml and 100 mM Tris, pH 7.4). The cerebella of five animals were mixed and homogenized to form a pool from each group. WB was performed in accordance with Nunes et al. (2012). Briefly, proteins (40 μg total) were separated with 12% (CCR-2, MCP-1, and MMP-9) or 14% (GST-pi and MBP) acrylamide gel. After overnight incubation with 5% non-fat milk, the membranes were incubated for 4 h with primary antibodies against CCR-2 (Ab10396), MCP-1 (Ab9669), MMP-9 (Ab38898), GST-pi (Ab47709) and MBP (Ab7349) (all from Abcam, Canada/US), followed by HRP-conjugated anti-rabbit (1:80,000, #A9169), anti-mouse (1:1000, #A0168) or anti-goat (1:100,000, #A5420) secondary antibodies (all from Sigma-Aldrich). For quantification, the pixel density of each immunoblot was determined using the Image J 1.38 software program (http://rsbweb.