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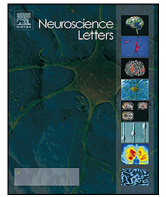

Research article

Sildenafil (Viagra®) prevents and restores LPS-induced inflammation in astrocytes

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HIGHLIGHTS

- Astrocytes are a key part of the sildenafil protective effects in the CNS.
- Sildenafil can decrease ATP-evoked intracellular Ca^{2+} release after LPS incubation.
- Sildenafil modulates the actin filaments, the cytoskeleton, in astrocytes.

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ABSTRACT

Astrocytes are effectively involved in the pathophysiological processes in the central nervous system (CNS) and may contribute to or protect against development of inflammatory and degenerative diseases. Sildenafil is a potent and selective phosphodiesterase-5 (PDE-5) inhibitor, which induces cyclic GMP accumulation. However, the mechanisms of actions on glial cells are not clear. The aim of the present work is to evaluate the role of sildenafil in lipopolysaccharide (LPS)-stimulated astrocytes. The cytoskeleton integrity and Ca^{2+} waves were assessed as indicators of inflammatory state. Two main groups were done: (A) one prevention and (B) one restoration. Each of these groups: A1: control; A2: LPS for 24 h; A3: sildenafil 1 or 10 μM for 4 h and then sildenafil 1 or 10 μM + LPS for 24 h. B1: control; B2: LPS for 24 h; B3: LPS for 24 h and then LPS + sildenafil 1 or 10 μM for 24 h. Cytoskeleton integrity was analyzed through GFAP immunolabeling and actin labeling with an Alexa 488-conjugated phalloidin probe. Calcium responses were assessed through a Ca^{2+}-sensitive fluorophore Fura-2/AM. The results show that both preventive and restorative treatments with sildenafil (in both concentrations) reduced the Ca^{2+} responses in intensity and induced a more organized actin fiber pattern, compared to LPS treated cells. This work demonstrated for the first time that astrocytes are a key part of the sildenafil protective effects in the CNS.

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1. Introduction

Sildenafil is a potent and selective phosphodiesterase-5 (PDE-5) inhibitor, which induces cyclic GMP (cGMP) accumulation. This drug was approved in 1998 for therapeutic use in erectile dysfunction and currently also being used to treat pulmonary hypertension (Revatio®) [1] and Raynaud's phenomenon [2]. Besides maintaining an excellent level of safety and tolerability in the treatment of those diseases, sildenafil also seems to provide an extended benefit to several other dysfunctions in the central nervous system (CNS). The experimental treatment with sildenafil reduced loss of memory related to age [3], reduced neurological deficits, increased neurogenesis and functional recovery after stroke in rats [4,5]. In addition, it has been reported that sildenafil improved the overall clinical

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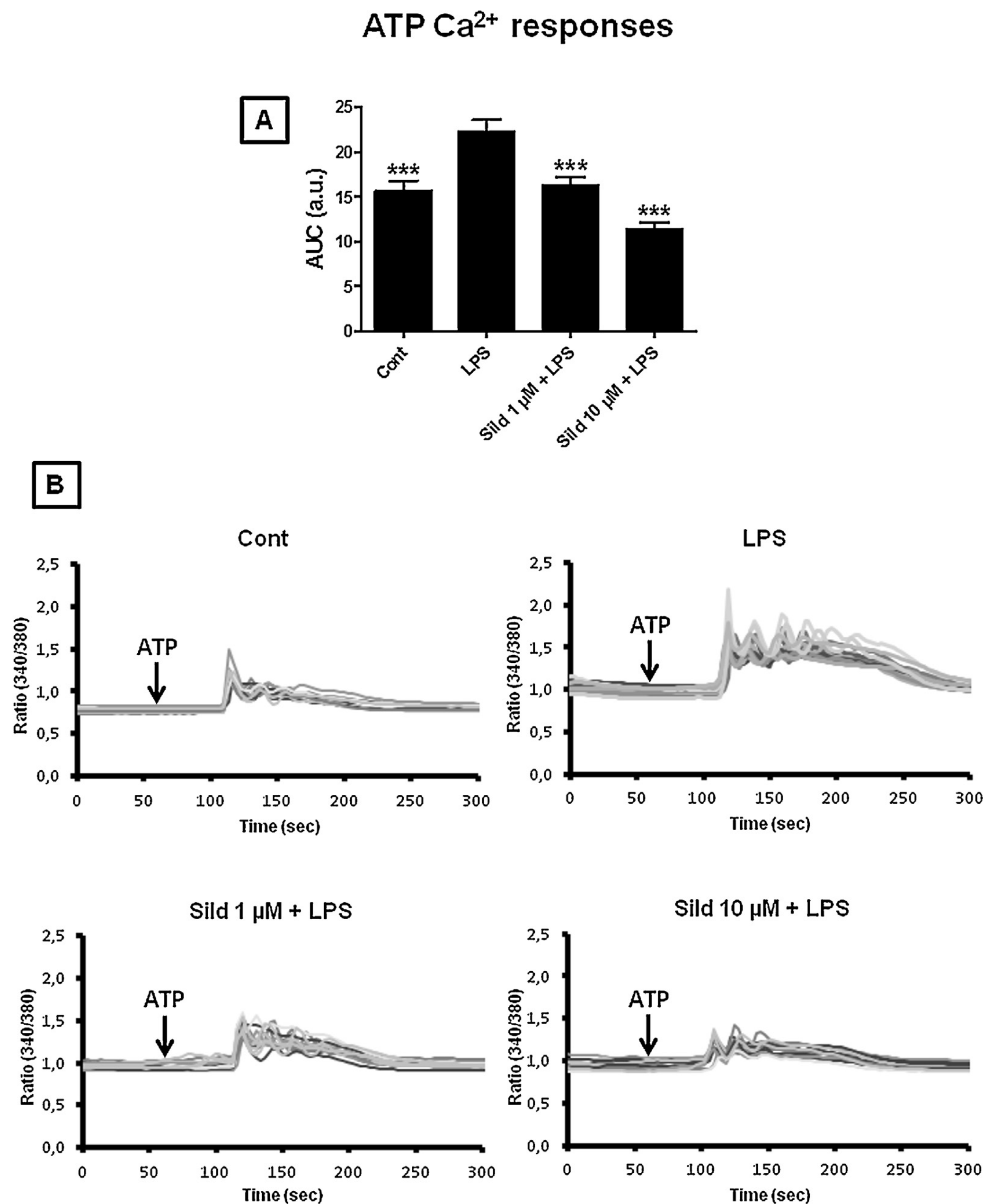

Fig. 1. Sildenafil administered before LPS (preventive treatment). The cells were loaded with the Ca^{2+} probe Fura-2/AM, stimulated with ATP and observed in a Ca^{2+} imaging system. AUC was calculated. **A.** ATP (10^{-4} M) elicited Ca^{2+} responses in astrocytes and was used as control. Astrocytes were incubated with LPS (10 ng/ml) for 24 h, or with sildenafil (1 and 10 μM, respectively) for 4 h, followed by sildenafil plus LPS for 24 h. **B.** The appearance of the Ca^{2+} transients is visualized. Results shown are from representative experiments. The cells were from four different coverslips from two different seeding times. The level of significance was analyzed using one-way ANOVA followed by Dunnett's multiple comparisons test. ***$P < 0.001$ compared to LPS-treated cells.

status of patients with multiple sclerosis (MS) [6]. It has been shown that systemic administration of sildenafil in demyelinating mice models reduced damage in the myelin sheath, decreased the release of pro-inflammatory cytokines, such as IFN-γ, TNF-α and interleukins (ILs), and reduced oxidative stress[7,8].

Modulation of Ca^{2+} responses after inflammatory stimuli can be controlled indirectly by the activity of the Na^+/K^+-ATPase [9]. Na^+/K^+-ATPase and the IP_3 receptor (IP_3R) form signaling microdomains that influence Ca^{2+} homeostasis in astrocyte networks. Preserved cytoskeleton is important for the propagation of Ca^{2+} waves in astrocytes [10,11]. After exposure to lipopolysaccharide (LPS), the Ca^{2+} signaling is overactivated in the astrocyte networks [12,13]. Furthermore, there is a disorganization of actin stress fibers [12]. Ankyrin B, a protein associated with the cytoskeleton, interacts with Na^+/K^+-ATPase and IP_3R, connecting the pump to the Ca^{2+} responses from internal cell stores and to the integrity of the cytoskeleton [14].

In spite of the great relevance of astrocytes in the development of neuroinflammatory/demyelinating diseases, the direct effects and mechanisms of sildenafil in astrocytes have not been demonstrated until now. Evidence indicates that cGMP is involved in the regulation of the Na^+/K^+-ATPase activity and cytoskeleton integrity in astrocytes [15], suggesting that stress fibers and Ca^{2+} waves can be changed by sildenafil. Ca^{2+} responses and changes in the actin cytoskeleton induced by LPS in astrocytes are parameters which can give a clue how sildenafil affects astrocytes. The present study proposes to investigate the effects of sildenafil directly in astrocytes

Fig. 2. Sildenafil administered after LPS (restorative treatment). The cells were loaded with the Ca^{2+} probe Fura-2/AM, stimulated with ATP and observed in a Ca^{2+} imaging system. AUC was calculated. **A.** ATP (10^{-4} M) elicited Ca^{2+} responses in astrocytes and was used as control. Astrocytes were incubated with LPS (10 ng/ml) for 24 h or with LPS for 24 h, followed by sildenafil (1 and 10 μM, respectively) plus LPS for 4 h. **B.** The appearance of the Ca^{2+} transients is visualized. Results shown are from representative experiments. The cells were from four different coverslips from two different seeding times. The level of significance was analyzed using one-way ANOVA followed by Dunnett's multiple comparisons test. ***$P < 0.001$ compared to LPS-treated cells.

after inflammatory stimulus induced by LPS, evaluating Ca^{2+} waves and actin cytoskeleton integrity.

2. Materials and methods

2.1. Experimental design

Rat primary cortical astrocytes were purchased from Invitrogen (Thermo Fischer Scientific, Waltham, MA, USA) and prepared according to the manufacturer's instructions with some modifications. Briefly, one vial with 1×10^6 viable cells was quickly thawed and gently rinsed with astrocyte growth medium consisting of Dulbeccoís Modified Eagle medium (DMEM) (high glucose) supplemented with 15% fetal bovine serum (both from Invitrogen). The cells were plated at a seeding density of 1×10^4 cells per cm^2 on an uncoated glass coverslip (nr 1, 20 mm in diameter) (Bergman Labora, Stockholm, Sweden) placed in a 12-well plate. The astrocytes were used after 16–17 days cultivation.

The experimental design included two main groups: (A) one prevention and (B) one restoration. Each of these groups: A1: control; A2: LPS for 24 h; A3: sildenafil 1 or 10 μM for 4 h and then sildenafil 1 or 10 μM + LPS for 24 h. B1: control; B2: LPS for 24 h; B3: LPS for 24 h and then LPS + sildenafil 1 or 10 μM for 24 h. In other words, sildenafil in both doses was administered to the astrocytes before (preventive) or after (restorative) LPS. The preventive treatment with sildenafil was done by treating astrocytes with sildenafil for

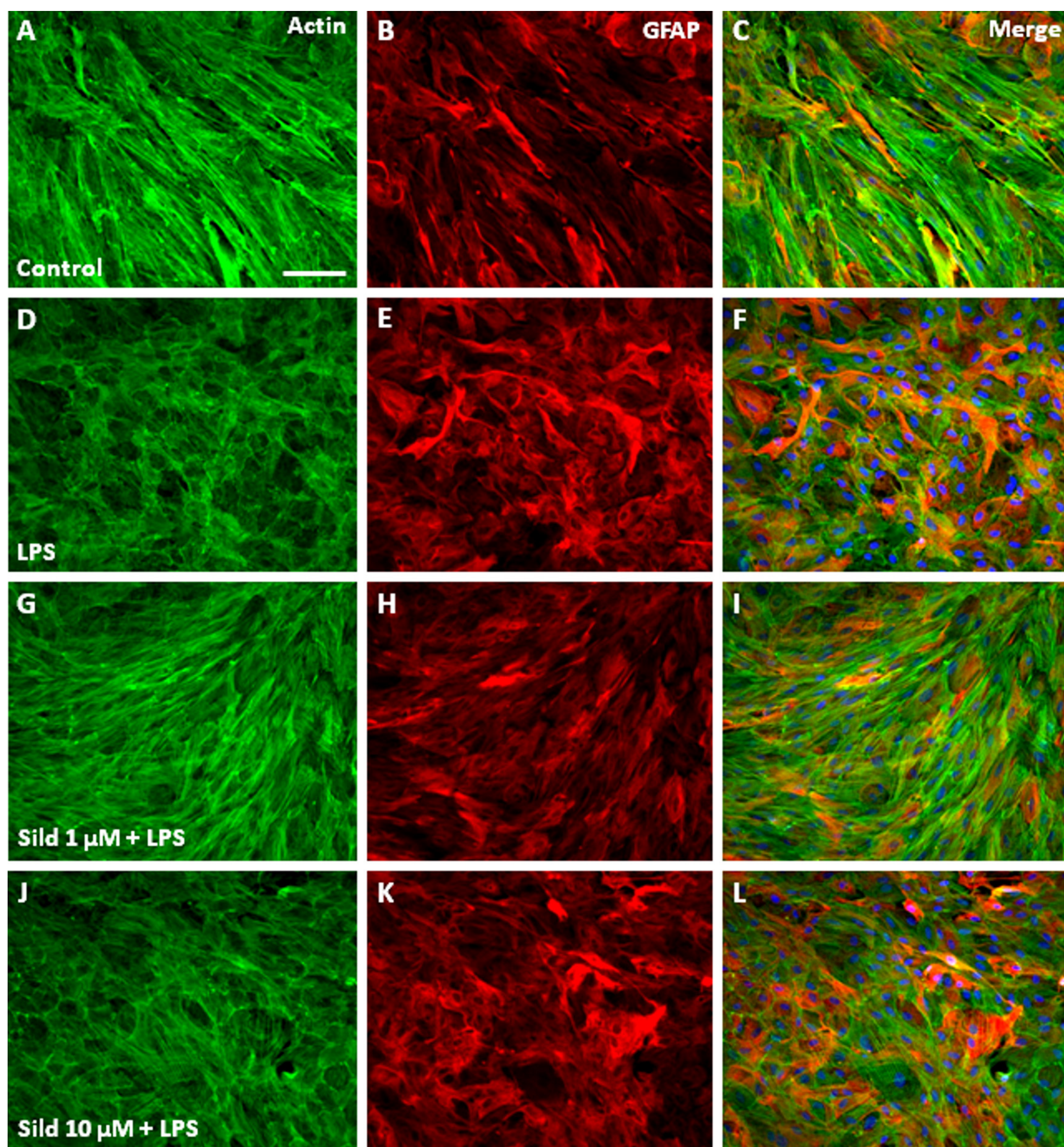

Fig. 3. Sildenafil administered before LPS (preventive treatment) induced actin filament stability. Astrocytes were stained with Alexa 488-conjugated phalloidin probe (*green*). GFAP (*red*) immunolabeling was performed to confirm the astrocytic phenotype. Merged images are also shown. The nuclei were visualized using HO33258 (*blue*). **A–C**, untreated cells showing F-actin organized in stress fibers displaying a typical morphology. **D–F**, astrocytes incubated with LPS (10 ng/ml) for 24 h. **G–L**, astrocytes incubated with sildenafil (1 μM, **G–I**; 10 μM, **J–L**) for 4 h, before LPS, followed by 24 h in sildenafil plus LPS. The cells, in both concentrations, showed a more preserved actin cytoskeleton. Scale bar = 50 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

4 h followed by LPS + sildenafil for 24 h. The restorative treatment with sildenafil was done by treating astrocytes with LPS for 24 h and then with LPS + sildenafil for 4 h.

2.2. Calcium waves and cytoskeleton labeling

After treatments, astrocytes were incubated at room temperature in the Ca^{2+}-sensitive fluorophore Fura-2/AM (Invitrogen Molecular Probes, Eugene, OR, USA) for 30 min (8 μl in 990 μl Hank's HEPES buffered saline solution (HHBSS), consisting of 137 mM NaCl, 5.4 mM KCl, 0.4 mM $MgSO_4$, 0.4 mM $MgCl_2$, 1.26 mM $CaCl_2$, 0.64 mM KH_2PO_4, 3.0 mM $NaHCO_3$, 5.5 mM glucose, and 20 mM HEPES dissolved in distilled water, pH 7.4). All substances used during the experiment were diluted in the same solution. The fluorophore was dissolved in 40 μl of dimethyl sulfoxide (DMSO) and 10 μl of pluronic acid (Molecular Probes, Leiden, The Netherlands). After incubation, the cells were rinsed three times with HHBSS. After one minute from the beginning of the experiment ATP (10^{-4} M) [16] was used as the stimulator. The experiments were performed at room temperature using a Ca^{2+} imaging system and simple software PCI (Compix Inc., Imaging Systems, Hamamatsu Photonics Management Corporation, Cranberry Twp, PA, USA) and an inverted epifluorescence microscope (Nikon ECLIPSE TE2000-E). The ATP was provided by a peristaltic pump (Instech Laboratories, Plymouth Meeting, PA, USA) at a flow rate of 600 μl/min. The substance took approximately 60 s to reach the cells through the tubes. The images were captured with a camera ORCA-12AG (C4742-80-12AG), High Res Digital cooled CCD (Hamamatsu Photonics Corporation, Hamamatsu, Japan). The total areas under curve (AUC), reflecting the amounts of Ca^{2+} released [17],

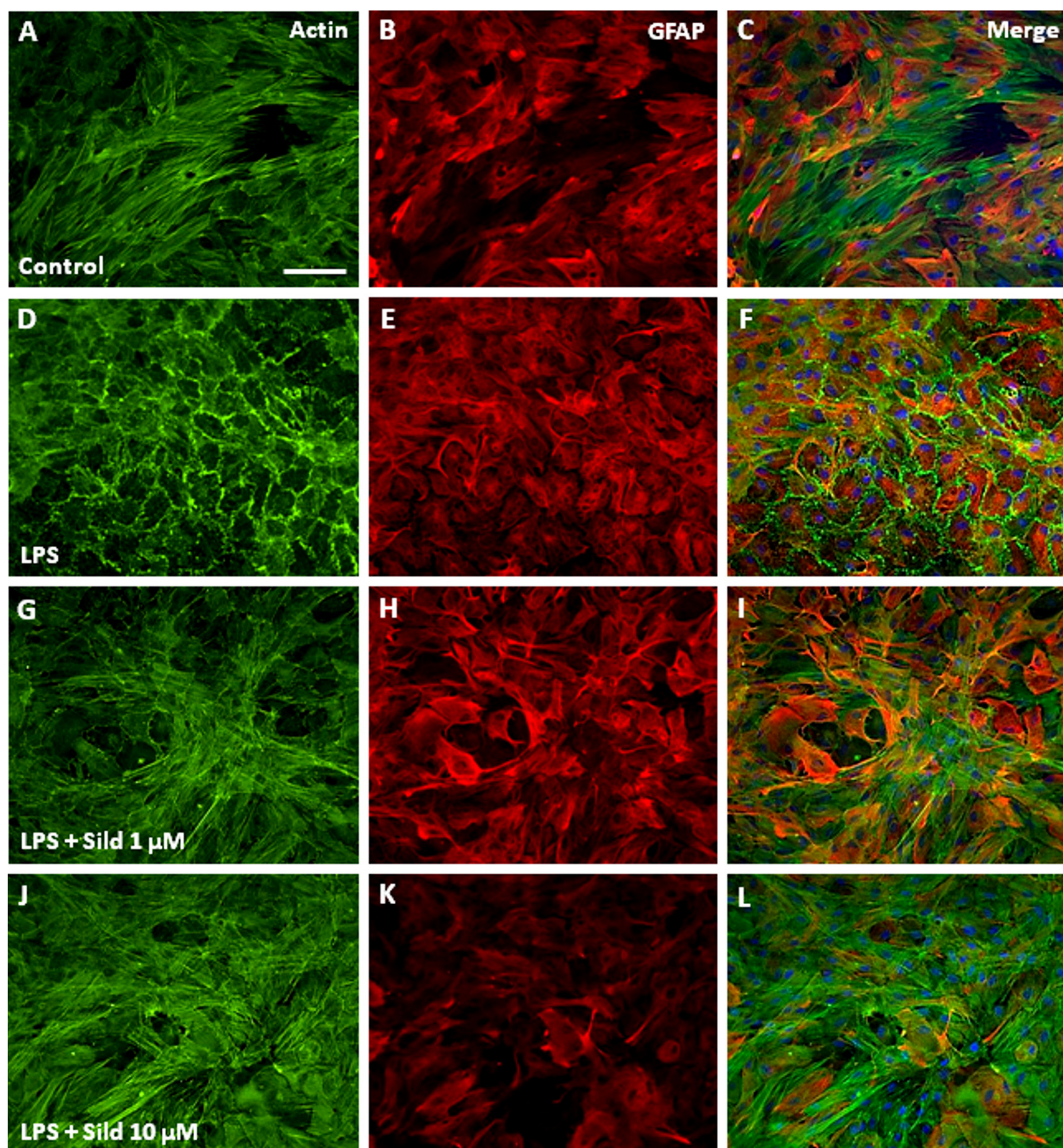

Fig. 4. Sildenafil administered after LPS (restorative treatment) induced actin filament stability. Astrocytes were stained with Alexa 488-conjugated phalloidin probe (*green*). GFAP (*red*) immunolabeling was performed to confirm the astrocytic phenotype. Merged images are also shown. The nuclei were visualized using HO33258 (*blue*). **A–C**, untreated cells showing F-actin organized in stress fibers displaying a typical morphology. **D–F**, astrocytes incubated with LPS (10 ng/ml) for 24 h. **G-L**, astrocytes incubated with LPS for 24 h, followed by sildenafil (1 μM, **G–I**; 10 μM, **J–L**) plus LPS for 4 h. The cells, in both concentrations, showed more preserved actin cytoskeleton. Scale bar = 50 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

were analyzed to provide measurements of Ca^{2+} responses (40 cells per group, per experiment; all treatments were done in triplicate). The AUC was calculated using the Origin program (Microcal Software Inc., Northampton, MA, USA). The level of significance was analyzed using one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparisons test. The error bars show the standard error of the mean (SEM).

The cultures were fixed using 4% paraformaldehyde and permeabilized using PBS–BSA–Sap, stained with an Alexa 488-conjugated phalloidin probe (Invitrogen) diluted 1:40, and a rabbit polyclonal antibody against GFAP (Dako, Glostrup, Denmark) diluted 1:100. GFAP labeling was done to confirm the astrocytes phenotype. Nuclei were stained with Hoechst 33258 diluted 1:1000 in the secondary antibody solution [18]. The coverslips were mounted and viewed under a Nikon Eclipse 80i microscope. Images were captured using a Hamamatsu C5810 color-intensified 3CCD camera.

3. Results

ATP induced Ca^{2+} responses in all analyzed astrocytes (40 of 40 cells per group) (Figs. 1 and 2), which were used as controls. The treatment with LPS induced a more oscillating and intense (increased AUC) Ca^{2+} response in astrocytes stimulated with ATP (typical response of cells in inflammatory state) (Figs. 1B and 2B), compared to the control group ($p < 0.001$). The preventive (Fig. 1) and restorative (Fig. 2) treatments with sildenafil in both concentrations reduced the Ca^{2+} responses ($p < 0.001$) evoked by ATP, compared to the LPS group. The AUCs are shown in Figs. 1A and 2A.

All cells stained for GFAP. The untreated (control) cultures predominantly contained F-actin organized in stress fibers and typically normal morphology (Figs. 3A–C and 4A–C). Treatment with LPS induced disorganization of actin filaments, with ring structures

and changed cell morphology (Figs. 3D–F and 4D–F), compared to the control group [12]. Treatment with sildenafil before (Fig. 3G–L) or after (Fig. 4G–L) LPS, in both concentrations, restored the actin filaments; astrocytes showed actin organized mainly in stress fibers, without ring structures and astrocytes morphology was similar to control group.

4. Discussion

We demonstrate for the first time that sildenafil has astrocytes as target cells. Treatment with sildenafil showed a protective role when given before and after LPS treatment. Decreased intensity of calcium responses and protection of actin fibers demonstrate the sildenafil beneficial role. The present results support previous *in vivo* studies using neurodegenerative/demyelinating models, which showed that sildenafil protects against inflammation, reducing the reactive gliosis and release of cytokines [7,19–21].

Among glial cells, astrocytes have received special attention, probably because of their close spatial relationship to neurons and synapses in the CNS, which performs bidirectional communication between astrocytes and neurons, wherein the Ca^{2+} signal by astrocytes plays an essential role [22]. Ca^{2+} responses in astrocytes act as intracellular and intercellular signals that can propagate within and between astrocytes [23,24]. Downregulation of Ca^{2+} homeostasis represents a universal mechanism of cellular pathology and inevitably causes the main mediator of necrosis or programmed (apoptosis, autophagy, anoikis, *etc.*) cell death [25–27]. Modifications of Ca^{2+} signaling may contribute to pathological progression through impacting, for example, synaptic transmission, neuronal metabolism, and ultimately in neuronal survival [28]. In addition, Ca^{2+} changes in astrocytes are involved in persistent pain [29].

Therefore, considering the role of Ca^{2+} signal in astrocytes, it is possible that sildenafil has a beneficial role by controlling Ca^{2+} responses and, consequently, balancing astrocytes' excitability and astrocyte-neuron communication, and preventing cell death and persistent pain. Corroborating with this hypothesis, it has been demonstrated that sildenafil reduces apoptosis in the brain and weakens the effects of learning and memory deficits [30,31], elevates the pain threshold level of diabetic neuropathic mice [32], reduces neurologic deficits and increases neurogenesis and functional recovery after stroke in rats [4,5]. Also, the protective effect of sildenafil in degenerative disease models such as multiple sclerosis (MS), Alzheimer's disease and Parkinson's disease [30,33–35] has been shown.

The astrocytes are potential targets during the inflammatory state [12,36,37] and the mechanisms of astrocytic inflammatory response were demonstrated. It has been reported that the actin cytoskeleton is critical for maintaining cell structure and function, cell polarity and the appropriate localization and function of critical proteins such as Na^{+}/K^{+}-ATPase [37,38]. Ankyrins are a ubiquitously expressed intracellular scaffolding protein family associated with a diverse set of membranes and cytoskeletal and cytoplasmic proteins. Both Ank-B and Ank-G have been reported to interact with Na^{+}/K^{+}-ATPase [38,39]. Also, Na^{+}/K^{+}-ATPase and the IP_3 receptor (IP_3R) form signaling microdomains that influence Ca^{2+} homeostasis. These interactions are altered in astrocytes after inflammatory stimulus, contributing to neuroinflammation [18]. It was demonstrated that cGMP-protein kinase G (PKG) regulates cytoskeleton dynamics and motility in rat astrocytes in culture [15]. If the sildenafil protection, balancing Ca^{2+} responses and maintaining the integrity of cytoskeleton in astrocytes, is mediated by its direct interaction with Na^{+}/K^{+}-ATPase as a target or if it is mediated by the cGMP accumulation and PKG activation, or both, is a speculation and need to be clarified and will be the aim of further studies by using inhibitors.

5. Conclusion

In conclusion, this work demonstrates that astrocytes are a key part of the sildenafil protective effects in the CNS. The present results suggest that sildenafil can target Na^{+}/K^{+}-ATPase in astrocytes, modulating cytoskeleton integrity and Ca^{2+} waves, with several possible beneficial effects. Sildenafil might be proposed as a potential treatment of degenerative diseases. For this reason, other studies investigating the mechanism behind its effects in astrocytes have to be made.

Conflict of interest

The authors declare no conflicts of interest.

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