Neuroscience Letters 670 (2018) 89–93

Contents lists available at ScienceDirect

Neuroscience Letters

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

Research article

# The effect of celastrol on the ocular hypertension-induced degeneration of retinal ganglion cells

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ARTICLE INFO

*Keywords:*
Celastrol
Retina
Ganglion cells
Optic nerve
Glaucoma
Neuroprotection

ABSTRACT

Celastrol, a quinine methide triterpene extracted from the perennial vine Tripterygium wilfordii, has been identified as a neuroprotective agent in various models of neurodegenerative disorders. We have reported earlier that systemic and intravitreal administration of celastrol stimulate the survival of retinal ganglion cells (RGCs) injured by optic nerve crush (ONC) and that mechanisms underlying celastrol's RGC protection may be associated with inhibition of TNF-alpha-mediated cell death. The present study evaluates the effect of celastrol on the survival of RGCs injured by ocular hypertension. Intraocular pressure (IOP) elevation resulted in approximately 23% of RGCs loss. Reduction in RGC numbers was observed in all four retinal quadrants: 30% in superior, 17% in inferior, 11% in nasal and 35% in temporal regions. Celastrol (1 mg/kg) or vehicle (DMSO) was administered three times per week by intraperitoneal injection, starting on the day of laser photocoagulation of the TM and continued for the entire duration of the experiment (5 weeks). Celastrol treatment stimulated RGC survival by an average of 24% in the entire retina compared to the vehicle-treated group. RGC numbers were increased in all four quadrants: approximately 40%, 17%, 15% and 30% more RGCs were counted in the superior, inferior, nasal and temporal regions, respectively. The average RGC numbers for the entire retinas of the celastrol/IOP group were only ~5% and 10% lower than that in vehicle- or celastrol-injected animals with normal IOP, respectively. Our data indicate a significant celastrol-mediated neuroprotection against elevated IOP-induced injury.

## 1. Introduction

Glaucomatous neuropathy affects millions of people worldwide and is often only diagnosed after patients have already suffered irreversible damage to their vision. If left untreated, glaucoma could lead to debilitating visual impairments. Vision loss in this disease is due to damage to retinal ganglion cells (RGCs) and their axons in the optic nerve. The current glaucoma treatment–lowering intraocular pressure (IOP)–has been proven to be effective in slowing the progression of the disease in most patients, however it is common for visual function to deteriorate despite IOP reduction. This dictates the necessity of developing new therapies for this disease that may be used as complementary or alternative treatment. Since the mechanisms responsible for glaucomatous damage are not well defined, multiple neuroprotective strategies that target different pathways potentially involved in this process were proposed and tested [1]. Yet, despite extensive studies in this area over the past two decades and the accumulation of a wealth of information about RGC degeneration and neuroprotection in animal models, there has been no substantial progress in developing new clinically relevant therapeutic approaches. RGCs' susceptibility to damage in glaucoma is almost certainly associated with more than one risk factor. Risk factors sufficient for or contributing to the development of the disease may vary by glaucoma type and determine not only the likelihood of developing the disease, but also its severity and rate of progression. Some of the cellular factors that are commonly associated with the pathogenesis of glaucoma include biomechanical stress associated with IOP, oxidative stress, axonal transport failure, insufficient nutrient supply, autoimmunity, protein misfolding/aggregation and glial cell dysfunction [2–4]. In our effort to simultaneously target multiple pathways that are known to be associated with RGC damage during glaucomatous neurodegeneration, we have earlier evaluated the effect of celastrol, a quinine methide triterpene extracted from the perennial vine Tripterygium wilfordii, in the optic nerve crush (ONC) model of RGC degeneration. Celastrol has been used in traditional oriental medicine for centuries as a natural remedy for inflammation and a variety of autoimmune diseases. Its neuroprotective effect has been identified in a comprehensive drug screen against various neurodegenerative diseases and furthermore its cell protective properties were

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https://doi.org/10.1016/j.neulet.2018.01.043
Received 17 October 2017; Received in revised form 8 January 2018; Accepted 22 January 2018

demonstrated in animal models of Parkinson's, Huntington's, Alzheimer's diseases, and amyotrophic lateral sclerosis [5–10]. The mechanisms underlying celastrol's neuroprotective effects are primarily associated with the induction of the heat shock response, activation of the cellular antioxidant defense system, attenuation of microglial activation, inhibition of tumor necrosis factor (TNF)-alpha, and nitric oxide synthase production [10–13]. Considering that the pathogenesis of glaucoma is thought to be associated with oxidative damage, protein misfolding/aggregation, microglial activation, and TNF alpha, celastrol could represent a therapeutic drug that targets the multifactorial nature of this disease [14–18].

Recently, we analyzed the effect of celastrol on the survival of RGCs injured by ONC [19]. Both intraperitoneal (i.p.) and intravitreal injection of celastrol stimulated the survival of injured RGCs. The average density of RGCs in animals treated with celastrol (1 mg/kg; daily i.p. injection) was increased by approximately 250%. The RGC survival was also increased by approximately 80% in ONC animals treated with a single intravitreal injection of 1 mg/kg or 5 mg/kg of celastrol. Intravitreal injection of 0.2 mg/kg of celastrol had no effect on cell survival in ONC-injured RGCs compared to DMSO-injected controls. Furthermore, to explore the mechanisms of potential celastrol-mediated RGC protection, we analyzed the expression of proteins that have been associated with celastrol-induced heat shock and antioxidant responses as well as the TNF alpha. Expression of heat shock protein 70 (Hsp70), heat shock factor 1 (Hsf1), Hsf2, and heme oxigenase 1 (HO-1) was not affected in celastrol-treated animals, whereas the level of TNF-alpha was significantly reduced, suggesting that celastrol-mediated protection of ONC-injured RGCs involves cell death pathways associated with TNF-alpha.

The current study evaluates the effect of celastrol on the survival of RGCs in an ocular hypertensive animal model of RGC degeneration. Both ONC and elevated IOP models are characterized by progressive degeneration of RGCs, however the IOP model is traditionally considered to be more relevant to human glaucoma since the elevated IOP is a well recognized risk factor for the most common forms of the disease.

## 2. Materials and methods

### 2.1. Animals

Adult Brown Norway rats (3 month-old; 250–300 g) were used to generate experimental glaucoma models. The use of animals and the procedures involving animals were approved by the Animal Research Committee of the University of California at Los Angeles and were in compliance with the National Institutes of Health Guide for the Care and Use of Animals and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Animals were housed with standard food and water provided ad libitum in a room with the temperature set at 21 °C and illuminated with fluorescent lights (330 lx) automatically turned on at 03:00 am and off at 03:00 pm The animals were kept at least 1 week in this environment before IOP measurement or trabecular meshwork (TM) laser photocoagulation. Photocoagulation was performed on one eye of each rat while the contralateral eye served as an untreated control. Celastrol (1 mg/kg) or vehicle (DMSO) was administered three times per week by i.p. injection. Four groups of animals were used in this study to evaluate celastrol's cell protective effect: Vehicle (untreated eyes of animals injected with vehicle; n = 10), Vehicle/IOP (laser-treated eyes of animals injected with vehicle; n = 10), Celastrol (untreated eyes of animals injected with celastrol; n = 14), and Celastrol/IOP (laser-treated eyes of animals injected with celastrol; n = 14).

### 2.2. Ocular hypertension model

Trabecular laser photocoagulation was used to increase IOP. After 1 week of accommodation, light and dark phase IOPs were measured in awake Brown Norway rats with Tonolab (TonoLab; Colonial Medical Supply, Franconia, NH) twice a week. Trabecular laser photocoagulation was performed as described previously (Kwong et al., 2011). Briefly, approximately 200 laser burns were delivered ab externo to the 360° TM at laser settings of 200 μm diameter, 100 mW power, and 50 m sec durations. Trabecular laser photocoagulation was repeated 2 weeks after the first treatment on eyes with no significant IOP elevation compared with contralateral control eyes. IOP elevation was sustained for 5 weeks.

### 2.3. Quantification of RGCs

Animals were deeply anesthetized with intramuscular injections of 80 mg/kg sodium pentobarbital. The eyes were enucleated, immersed in fixative for 0.5 h, and the lenses were removed. The eyecups were postfixed for another 0.5 h. Entire retinas were used for immunohistochemistry with a custom-made antibody against Rbpms according to a published protocol [20]. Briefly, the retinas were washed with PBS, incubated with 10% fetal bovine serum for 1 h to block nonspecific staining, followed by incubation with the primary antibody against Rbpms in PBS containing 1% triton, 0.5% BSA, and 0.9% sodium chloride (PBS-T-BSA) overnight at 4 °C. The retinas were washed in PBS-T-BSA and incubated with secondary Alexa Fluor 488 goat anti-rabbit IgG antibody (1/1000) overnight at 4 °C. Retinas were then washed with PBS, flat mounted with several radial cuts on a glass slide, air dried and covered with a cover-slip in an aqueous mount. Topographical analysis of RGC density was performed under a fluorescence microscope (LSM410; Carl Zeiss, Oberkochen, Germany). Three areas (0.32 × 0.24 mm each) per retinal quadrant (superior, temporal, inferior and nasal) at 1, 2, 3 and 4 mm from the optic disc were analyzed. RGCs were counted in a masked manner.

### 2.4. Statistical analysis

Data are presented as the mean ± standard deviation (SD). A repeated measures ANOVA was conducted to compare RGC density in Vehicle, Celastrol, Vehicle/IOP and Celastrol/IOP groups. All measurements at four distances and four quadrants from each animal were included in the overall ANOVA model comparing RGC density in Vehicle, Celastrol, Vehicle/IOP and Celastrol/IOP groups, and the effects of quadrants and distances were controlled by multi-factors ANOVA model. When comparisons of RGC density among groups were performed within each quadrant, all measurements at four distances from each animal were included in the ANOVA model and the distance effect was controlled by multi-factors ANOVA model. $P < 0.05$ was considered statistically significant.

## 3. Results and discussion

The rat experimental glaucoma model to investigate celastrol's cell protective effect against ocular hypertension induced RGC damage was generated by laser photocoagulation of the TM. Mean IOPs for the light and dark phases of the circadian cycle for experimental and control eyes were calculated and representative IOP profiles of vehicle-treated rat and celastrol-treated rat are shown in Fig. 1A and B respectively. TM photocoagulation to elevate IOP was performed unilaterally, while the contralateral eye received no treatment. The average light phase IOP in the Vehicle/IOP and Celastrol/IOP groups were 23.81 ± 2.68 mmHg and 21.74 ± 3.13 mmHg, respectively compared to 18.91 ± 1.07 in Vehicle group and 17.81 ± 1.06 mmHg in Celastrol group. The dark phase average IOP values were 32.67 ± 0.99, 32.15 ± 1.56, 41.17 ± 4.63 and 39.52 ± 4.99 mmHg in Vehicle, Celastrol, Vehicle/IOP and Celastrol/IOP groups, respectively. As expected, both the light and dark phase IOP in experimental eyes of vehicle-treated animals were significantly elevated after TM photocoagulation, when compared