Experimental Eye Research 148 (2016) 90–96

Contents lists available at ScienceDirect

Experimental Eye Research

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

Research article

Increased production of omega-3 fatty acids protects retinal ganglion cells after optic nerve injury in mice

Shanshan Peng [a, c, d], Zhe Shi [a], Huanxing Su [b], Kwok-Fai So [a, c, d], Qi Cui [a, c, d, *]

[a] GHM Institute of CNS Regeneration, Jinan University, Guangzhou, China
[b] State Key Laboratory of Quality Research in Chinese Medicine, Institute of Chinese Medical Sciences, University of Macau, Macau, China
[c] Guangdong Key Laboratory of Brain, Function and Diseases, Jinan University, Guangzhou, China
[d] Joint International Research Laboratory of CNS Regeneration, Ministry of Education of PRC, Jinan University, Guangzhou, China

ARTICLE INFO

Article history:
Received 26 October 2015
Received in revised form
19 May 2016
Accepted in revised form 1 June 2016
Available online 3 June 2016

Keywords:
Optic nerve injury
Retinal ganglion cell
Survival
Omega-3 fatty acids
ERK1/2 pathway
cAMP

ABSTRACT

Injury to the central nervous system causes progressive degeneration of injured axons, leading to loss of the neuronal bodies. Neuronal survival after injury is a prerequisite for successful regeneration of injured axons. In this study, we investigated the effects of increased production of omega-3 fatty acids and elevation of cAMP on retinal ganglion cell (RGC) survival and axonal regeneration after optic nerve (ON) crush injury in adult mice. We found that increased production of omega-3 fatty acids in mice enhanced RGC survival, but not axonal regeneration, over a period of 3 weeks after ON injury. cAMP elevation promoted RGC survival in wild type mice, but no significant difference in cell survival was seen in mice over-producing omega-3 fatty acids and receiving intravitreal injections of CPT-cAMP, suggesting that cAMP elevation protects RGCs after injury but does not potentiate the actions of the omega-3 fatty acids. The observed omega-3 fatty acid-mediated neuroprotection is likely achieved partially through ERK1/2 signaling as inhibition of this pathway by PD98059 hindered, but did not completely block, RGC protection. Our study thus enhances our current understanding of neural repair after CNS injury, including the visual system.

1. Introduction

The central nervous system has a limited ability to repair itself after traumatic injury, resulting in the progressive degeneration of the injured axons and parent neurons followed by the loss of certain neurological functions. In fact, it has been shown that neuronal survival after injury is the crucial precondition for successful axon regeneration and recovery of lost functions. Recently, a number of studies have shown that omega-3 fatty acids alleviate the risks associated with neuronal loss after traumatic brain and spinal cord injuries (Lim et al., 2013; Kumar et al., 2014; Michael-Titus and Priestley, 2014). Dietary supplementation with omega-3 polyunsaturated fatty acids also robustly promotes neurovascular restorative dynamics and improves neurological functions in stroke patients (Zhang et al., 2015). The neuroprotective mechanisms involved include decreased neuro-inflammation and oxidative stress, neurotrophic support, and activation of cell survival pathways (Michael-Titus and Priestley, 2014; Zendedel et al., 2015).

Adenosine 3′,5′-cyclic monophosphate (cAMP), an important factor in the second messenger system, plays a diverse role in neuronal function. It is known to be involved in neuronal survival (Rydel and Greene, 1988; Hanson Jr. et al., 1998), process expression (Song and Poo, 1999), and enhancement of neurite outgrowth (Jo et al., 1999; Kao et al., 2002). cAMP is also sensitive to neurotrauma, and the levels of cAMP in neurons drop after axotomy (Shen et al., 1999; Cui et al., 2003). This decrease in endogenous cAMP expression is thought to underlie the resulting poor survival and neurite outgrowth, and numerous attempts to enhance these processes by increasing intracellular cAMP have been made over the past few years (Kashimoto et al., 2008; Montoya et al., 2009; Metcalfe et al., 2012; Lau et al., 2013; Drummond et al., 2014; Kranz et al., 2014). The pro-survival pathway in neurons also appears to involve extracellular signal-regulated kinase (ERK) 1/2 (Klocker et al., 2000; Park et al., 2004; Marques-Fernandez et al., 2013), with various trophic factors acting through ERK1/2 to modulate neuroprotection (Hetman et al., 1999; Sanchez et al., 2012). In fact, we previously showed that cAMP potentiated

* Corresponding author. GHM Institute of CNS Regeneration, Jinan University, Guangzhou, China.
E-mail address: qicui@yahoo.com (Q. Cui).

http://dx.doi.org/10.1016/j.exer.2016.06.001
0014-4835/

ciliary neurotrophic factor (CNTF) function during retinal ganglion cell (RGC) survival and axonal regeneration, and that the ERK pathway participated in these CNTF-dependent processes (Park et al., 2004).

Notably, the visual system in rodents has been widely used as a model of the central nervous system (CNS) to study neuroprotection and axonal regeneration (Shen et al., 1999; Watanabe et al., 2003; Yin et al., 2003; Park et al., 2004; Kashimoto et al., 2008; Charalambous et al., 2013; Li et al., 2013). In fact, using an optic nerve (ON) crush model in a transgenic mouse line overproducing omega-3 fatty acids (fat-1) and wild type (wt) C57BL/6 mice, we investigated the effects of increased production of omega-3 fatty acids on RGC survival. Effect of cAMP elevation on RGC viability was also examined by intraocular injection of the cAMP analogue 8-(4-chlorophenylthio)-adenosine 3′:5′-cyclic monophosphate (cpt-cAMP) which led to persistent elevation of cAMP expression in the eye (Cui et al., 2003). In the present study, we further investigated the role of the intracellular ERK1/2 signaling pathway responsible for the observed changes in cell protection using intraocular injection of the pathway inhibitor 2-(2-diamino-3-methoxyphenyl-4H- 1-benzopyran-4-one (PD98059) in order to determine whether inhibition of this pathway influenced the increased production of omega-3 fatty acid and the resulting changes in RGC survival after ON crush.

2. Materials and methods

2.1. Animals and surgical procedures

Thirty-six wt C57BL/6 mice and 51 fat-1 mice were used to examine RGC viability in this study. We obtained fat-1 breeders (Kang et al., 2004) on a C57BL/6 background from Dr. Jing X. Kang (Harvard Medical School) and raised them in the Laboratory Animal Center, University of Macau. The production of this fat-1 line mice has been reported (Kang et al., 2004), and we have used this line in previous studies (Liu et al., 2014). Mice were housed in a temperature-controlled, 12:12 light/dark room and were allowed free access to water and food. The heterozygous fat-1 mice and wt littermates were obtained by mating male heterozygous fat-1 mice with female C57BL/6 mice. The fat-1 phenotypes of each animal were characterized using isolated genomic DNA and fatty acid composition analysis from mouse tails. Both the fat-1 and wt mice were fed a modified diet containing 10% corn oil (TROPHIC Animal Feed High-tech Co., Ltd, China), with a fatty acid profile high in ω-6 PUFAs (mainly linoleic acid) and low in ω-3 PUFAs (~0.1% of the total fat supplied). All experiments performed were approved by the University of Macau and Jinan University Animal Experimentation Ethics Committees. All possible measures were taken to minimize suffering and limit the number of mice used in this study. The wt mice were divided into six groups, one group was used as the normal control group to obtain the number of RGCs in the intact mice; three groups received intravitreal injections of saline, 0.1 mM CPT-cAMP (a nondegradable membrane-permeable analogue; Sigma, St. Louis, MO), or 5 mM PD98059 (an ERK1/2 pathway inhibitor PD98059; Sigma) and were examined two weeks after ON crush; and the last two groups received ON crush injury but were examined 1 week and 3 weeks after injury, respectively. The fat-1 mice were also divided into the same six groups. All intravitreal injections were 3 μl in volume, and each mouse received two intravitreal injections of the same molecule on post-injury days 3 and 8. The mice were sacrificed 2 weeks after ON crush. The ON crush procedures have been described previously (Yin et al., 2003). Briefly, after anesthesia with ketamine and xylazine the left ON was exposed through a posterior temporal intraorbital approach. Using a pair of fine forceps, the ON was completely crushed about 1 mm behind the optic disc.

We chose to utilize CPT-cAMP and PD98059 in this study as we previously showed that intravitreal injections of CPT-cAMP can successfully enhance cAMP expression in the eye for a prolonged period of time (Cui et al., 2003) and intravitreal injections of PD98059 can effectively inhibit ERK1/2 signaling transduction (Park et al., 2004). To verify that ERK1/2 signaling had been successfully inhibited by PD98059, another 36 fat-1 mice were used for Western blotting experiments. Owing to the small size of the mouse retina, three retinas of the same condition were pooled together as one sample, and three samples from each group were obtained for analysis. The mice were evenly divided into 4 groups: intact, ON crush + saline, ON crush + CPT-cAMP, and ON crush + PD98059.

2.2. Immunohistochemistry

Examination of the surviving RGCs was performed using immunohistochemistry. The mice were killed with an overdose of anesthetics and perfused with cold 4% paraformaldehyde in phosphate-buffered saline (PBS). The retinas were then dissected out and post-fixed in the same fixative for 1 h. After washing the retinas for 5 min × 3 times with PBS, non-specific binding was blocked in PBS containing 10% normal goat serum and 0.2% Triton for 1 h, and then the retinas were incubated with goat primary antibody against Brn3a (1:500, Santa Cruz, USA) and mouse ED1 antibody overnight at 4 °C. Brn3a is known to specifically label RGCs in the retina (Charalambous et al., 2013), whereas the ED1 antibody has been widely used to label macrophages (Yin et al., 2003; Luo et al., 2007). Retinas were rinsed with PBS and then incubated with fluorescein isothiocyanate (FITC)-conjugated secondary antibody (1:500; DebCo, USA) overnight at 4 °C. The retinas were then examined using a fluorescent microscope. The Brn3a-positive RGCs were counted. Owing to the small size of the mouse retina, only two fields at each quadrate, thus eight fields for each retina, were selected along the axis toward the ON head to determine the density of Brn3a-positive RGCs and ED1-positive macrophage under 20× magnification.

To stain the regenerating axons, 16-μm thick longitudinal ON sections were cut and immunostained with GAP-43 primary antibody overnight at 4 °C, followed by FITC-conjugated secondary antibody (1:800; DebCo, USA) for 2 h at room temperature. Axon growth was quantified using a calibrated ocular to measure distance (Yin et al., 2003). We counted the number of GAP-43-positive axons crossing a line at a distance of 0.5 mm from the end of the crush site.

2.3. Western blotting procedures

Retinas were collected three days after intravitreal injection. Each sample was homogenized using cell lysis buffer and centrifuged in order to isolate the cell supernatant for further examination. Protein content was quantitatively analyzed using the BCA protein quantitative kit (Beyotime). Protein preservation was achieved by adding SDS-PAGE blue loading buffer to the protein (5:1) and boiling for 5 min. Then, 30 μl of sample was loaded onto a 10% acrylamide-Bis solution gel for separation (Bio-Rad). Protein was transferred onto PVDF membrane and incubated with phosphorylated primary anti-ERK1/2 (1:2000; Promega) overnight at 4 °C, then incubated with HRP conjugated secondary antibody (Beyotime) for 1 h at 37 °C. The labeled proteins were detected using the ECL agent (Beyotime), and the membranes were imaged.