Experimental Eye Research 146 (2016) 118–127

Contents lists available at ScienceDirect

Experimental Eye Research

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

Research article

Negative impact of AQP-4 channel inhibition on survival of retinal ganglion cells and glutamate metabolism after crushing optic nerve

Yuko Nishikawa, Hidehiro Oku*, Seita Morishita, Taeko Horie, Teruyo Kida, Masashi Mimura, Masanori Fukumoto, Shota Kojima, Tsunehiko Ikeda

Department of Ophthalmology, Osaka Medical College, Osaka, Japan

ARTICLE INFO

Article history:
Received 28 August 2015
Received in revised form
30 December 2015
Accepted in revised form 30 December 2015
Available online 7 January 2016

Keywords:
Aquaporin-4
Optic nerve crush
TGN-020
Glutamate
Retinal ganglion cell
BAX/Bcl-2 ratio

ABSTRACT

The purpose of this study was to determine whether inhibition of aquaporin 4 (AQP4) is neuroprotective or neurodestructive after crushing the optic nerve of rats. The left optic nerves of rats were crushed, and TGN-020 (5.0 mg/kg, crush TGN-020) or its vehicle (DMSO, crush placebo) was injected intraperitoneally just after the crushing. As controls, the left optic nerves were exposed but not touched in other rats (sham controls). The retinal damages were determined by the density of retinal ganglion cells (RGCs) and the ratio of BAX/Bcl-2 on day 7. The glutamate level in the optic nerve on day 1 after the crushing was determined. The expressions of glutamine synthetase, glutamate-aspartate transporter (GLAST), and AQP4 were determined on day 3 by immunoblotting. The effects of AQP4 inhibition on the glutamate-induced changes of AQP4 expression and on the glutamate uptake were determined for optic nerve astrocytes in culture. The results showed that the density of RGCs was 2040 ± 91.3 cells/mm^2 (n = 6) in the sham control, and it was significantly decreased to 1072 ± 134.3 cells/mm^2 after crushing the optic nerve ($P < 0.0001$, crush placebo, n = 7; Fisher). An intraperitoneal injection of TGN-020 led to a further significant ($P = 0.02$, Fisher) decrease of the density of RGCs to 743 ± 371 cells/mm^2 (crush TGN-020, n = 7). The mRNA level of *BAX/Bcl-2* ratio was 0.37 ± 0.05 in the sham control (n = 6) which was significantly increased to 0.88 ± 0.10 after crushing the optic nerve (placebo crush, n = 7; $P = 0.0001$, Scheffe). TGN-020 also significantly increased the *BAX/Bcl-2* ratio to 1.29 ± 0.4 (n = 6) from the crush placebo group ($P = 0.04$, Scheffe). Immunoblotting showed similar changes in the protein levels. The glutamate level in the optic nerve was significantly increased to 53.7 ± 6.0 μM/mg/protein on day 1 (n = 4) from the sham control level of 45.9 ± 3.1 μM/mg/protein (n = 4; $P = 0.04$, *t* test). TGN-020 significantly ($P < 0.05$, Scheffe) depressed the expression of glutamate metabolism-related proteins on day 3. Exposure of cultured optic nerve astrocytes to glutamate (1.0 mM, n = 4) significantly increased the expression of AQP4 ($P < 0.001$, Scheffe) that was depressed by TGN-020 (100 nM, n = 4). In addition, glutamate uptake was inhibited by TGN-020 at 10 nM or higher. These results indicate that an inhibition of AQP4 enhances the loss of RGCs and retinal damages after crushing the optic nerve. Inhibition of AQP4 impairs glutamate metabolism which may account in part for these neurodestructive events.

1. Introduction

Aquaporin-4 (AQP4), the main water channel protein in the CNS, is chiefly expressed on the end-feet of astrocytes which envelope the capillaries in the brain (Nielsen et al., 1997). AQP4 maintains the integrity of the blood–brain barrier (Zhou et al., 2008) and also plays an important role in maintaining the homeostasis of water and ions in the CNS (Amiry-Moghaddam and Ottersen, 2003; Dibaj et al., 2007). In addition, AQP4 plays a role in the uptake of glutamate into astrocytes (Zeng et al., 2007), and thus maintain the extracellular glutamate concentration below toxic levels (Anderson and Swanson, 2000).

It has been demonstrated that AQP4 contributes to both the formation (Manley et al., 2000) and resolution (Papadopoulos et al., 2004) of brain edema. It is expected that new strategies will develop to treat brain edema by inhibiting the AQP4 channels to reduce the degree of cytotoxic edema (Manley et al., 2000;

* Corresponding author. Department of Ophthalmology, Osaka Medical College, 2-7 Daigaku-machi, Takatsuki Osaka, 569-8686, Japan.
E-mail address: hidehirooku@aol.com (H. Oku).

http://dx.doi.org/10.1016/j.exer.2015.12.012
0014-4835/

Gunnarson et al., 2008). While AQP4 inhibition is neuroprotective of retinal ischemia (Da and Verkman, 2004), it can also exacerbate diabetic retinopathy (Cui et al., 2012) and light-induced retinal damage (Li et al., 2014). Thus, inhibition of AQP4 can be both neuroprotective and neurodestructive, and which role it plays depends on the type of injury.

The optic nerve becomes edematous under various conditions including traumatic injuries, and the edema can cause additional axonal damage. We have shown that crushing the optic nerve of rats caused a 1.8-fold increase of AQP4 expression on day 1 but the level was significantly lower than that at the baseline on day 7 (Suzuki et al., 2014). Optic nerve edema, determined by its water content, was maximum on day 7 when the level of AQP4 was reduced by 28% of the baseline level (Suzuki et al., 2014). On the other hand, optic nerve injuries are known to increase the extracellular level of glutamate in the retina (Vorwerk et al., 2004). In addition, the level of the mRNA of retinal glutamate transporter is transiently increased and then decreased after optic nerve injuries (Mawrin et al., 2003). These changes indicate that the homeostasis of glutamate is likely to be impaired by crushing the optic nerve. Because of the rapid rise of AQP4 levels in the optic nerve, it is important to determine whether the inhibition of AQP4 just after an injury of the optic nerve is neuroprotective or neurodestructive for retinal ganglion cells (RGCs).

Thus, the purpose of this study was to determine whether an inhibition of AQP4 can alter the changes in the number of RGCs after the optic nerves of rats are crushed. The effects of either TGN-020, an AQP4 inhibitor, or its vehicle (DMSO) injected intraperitoneally just after the crushing the optic nerves were determined by counting the RGCs after immunohistochemical staining. We also determined the BAX/Bcl2 ratio in the retina by real-time PCR and immunoblot on day 7. In addition, we determined the changes in the expression of glutamate metabolism-related proteins in the optic nerve by immunoblot on day 3. *In vitro* experiments were performed to determine whether TGN-020 impairs the uptake of glutamate by cultured optic nerve astrocytes and TR-MUL5 cells, a rat retinal Müller cell line.

2. Methods

2.1. Animals

Nine-week-old, male Wistar rats were purchased from Japan SLC (Shizuoka, Japan) and housed in an air-conditioned room with a temperature of approximately 23 °C and humidity of 60%. The room lights were on a 12:12 light:dark cycle. All animals were handled in accordance with the ARVO Resolution for the Use of Animals in Ophthalmic and Vision Research. The experimental protocol was approved by the Committee of Animal Use and Care of the Osaka Medical College (No. 25055). A total of 81 rats was used.

2.2. Chemicals

Unless noted, all chemicals were purchased from Sigma–Aldrich (St. Louis, MO, USA). TGN-020, an AQP4 inhibitor, was dissolved in DMSO to make a 10 mM stock solution. The concentration of DMSO in the media used to culture cells was adjusted at 0.1%.

2.3. Anesthesia and euthanasia

All surgeries were performed under general anesthesia by an intraperitoneal injection of pentobarbital sodium (50 mg/kg body wt), and all efforts were made to minimize pain. Rats were euthanized by exposure to CO_2 at a rate of 6 L/min in a cage (13.8 L) with wood-shaving bedding.

2.4. Optic nerve crushing

Animals were anesthetized with an intraperitoneal injection of pentobarbital sodium, and an incision was made along the midline of the skull to expose the superior surface of the left eye. The superior rectus muscle was incised to expose the left optic nerve, and the left optic nerve 2 mm behind the eye was crushed with forceps for 10 s (Kurimoto et al., 2006). Care was taken not to occlude the blood vessels and cause retinal ischemia. We confirmed that the retinal circulation was not blocked by indirect ophthalmoscopy and also verified this by demonstrating that the HIF-1α gene was not up-regulated by real-time PCR (Tonari et al., 2012).

Immediately after crushing the optic nerve, the animals received an intraperitoneal injection of TGN-020 (5.0 mg/kg) or its vehicle (DMSO). As control, a sham operation was performed on the left eyes of other animals by exposing the optic nerve in the same way but the optic nerve was not crushed. The right eyes were untouched in both types of rats.

2.5. Labeling retinal ganglion cells

A loss of RGCs is known to occur in a delayed fashion after crushing the optic nerve; the number of RGCs remains unchanged for 5 days and then abruptly decreased to 50% on day 7 and to less than 10% on day 14 (Berkelaar et al., 1994). Thus, the loss of RGCs was determined on day 7 after crushing the optic nerve.

To study the effects of crushing the optic nerves, rats were killed on day 7, and the retinas were carefully removed from the eyes as described in detail by Winkler (Winkler, 1972). In brief, rats were euthanized by CO_2, and the globe was proptosed by placing forceps around the optic nerve just behind the eyeball. The globe was transected along the equator and the cornea and lens were removed. The retina was detached from the pigment epithelium by pressing upward with the forceps and removed by cutting its attachment to the optic nerve head. The isolated retina was placed in PBS solution immediately, and any vitreous remaining on the isolated retina was carefully removed.

The isolated retinas were then sandwiched between nylon mesh sheets and fixed in 4% PFA in PBS overnight at 4 °C. After washing in PBS and blocking in PBS containing 1.0% BS and 0.3% triton X-100, the retinas were incubated with Alexa 488-conjugated mouse monoclonal neuron-specific class III beta-tubulin (Tuj-1, 1:500) antibody (Covance, Princeton, New Jersey). Tuj-1 is a specific marker for RGCs (Cui et al., 2003; Snow and Robson, 1995), and the retinas were placed in the same medium overnight at 4 °C, washed with PBS, and cover slipped.

To determine the number of RGCs, the stained flat mounts were photographed through a fluorescent microscope (BZ X700, Keyence, Osaka, Japan). Eight areas (0.48 × 0.48 mm) from the 4 quadrants of the retina at a distance of 1.0 and 1.5 mm from the margin of the optic disc were photographed. All of the Tuj-1-positive cells in an area of 0.2 × 0.2 mm at the center of each image were counted using the NIH ImageJ program.

The mean density of the RGCs/mm^2 was calculated, and the loss of RGCs was determined by comparing the density in the retinas of animals receiving either TGN-020 or its vehicle after crushing the optic nerve to that of retinas from sham operated optic nerves ($n = 6–7$ for each group). The number of RGCs was counted by one observer (SM) who was masked to whether it was from an experimental or a sham animal.

2.6. Changes in the expression of Bax and Bcl-2 in the retina

The ratio of Bax/Bcl-2 is an indicator of neuronal death by apoptosis (Schelman et al., 2004). We determined the changes in