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Research article

Topical administration of a Rock/Net inhibitor promotes retinal ganglion cell survival and axon regeneration after optic nerve injury

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ABSTRACT

Intraocular pressure (IOP)-lowering ophthalmic solutions that inhibit Rho-associated protein kinases (Rock) and norepinephrine transporters (Net) are currently under clinical evaluation. Here we evaluate topical application of one such drug for its effects on retinal ganglion cell (RGC) survival and axon regeneration after optic nerve crush injury. We performed unilateral optic nerve crush on young rats (P18) and topically applied Rock/Net inhibitor AR-13324 or placebo 3 times a day for 14 days. IOP was measured starting 3 days before and up to 9 days after injury. On day 12, cholera toxin B (CTB) was injected intravitreally to trace optic nerve regeneration. On day 14, retinas and optic nerves were collected. The retinas were flat-mounted and stained with RBPMS to quantify RGC survival and the optic nerves were sectioned for optic nerve axon quantification using fluorescent and confocal microscopy. Rock phosphorylation targets implicated in axon growth including cofilin and LIMK were examined by fluorescence microscopy and quantitative western blotting. AR-13324 lowered IOP as expected. RGC survival and optic nerve axon regeneration were significantly higher with Rock/Net inhibitor treatment compared with placebo. Furthermore, topical therapy decreased Rock target protein phosphorylation in the retinas and proximal optic nerves. These data suggest that topical administration of a Rock/Net inhibitor promotes RGC survival and regeneration after optic nerve injury, with associated molecular changes indicative of posterior drug activity. Coordinated IOP lowering and neuroprotective or regenerative effects may be advantageous in the treatment of patients with glaucoma.

1. Introduction

Failure of retinal ganglion cells (RGCs) to survive and regenerate their axons through the optic nerve underlies the permanent loss of vision in optic neuropathies such as glaucoma. Intraocular pressure lowering can slow the disease, but the search for effective therapies to protect and promote the growth of RGCs remains an important approach. Rho-associated protein kinases (Rocks) are AGC-family (PKA/PKG/PKC) serine-threonine kinases that regulate the shape and movement of cells by acting on the cytoskeleton(Maekawa et al., 1999; Riento and Ridley, 2003; Sanka et al., 2007; Wang et al., 2009). Rho GTPases and Rocks contribute to regenerative failure, and Rock inhibition has been shown to promote axon regeneration in the central nervous system (CNS).(Kubo et al., 2007; Tan et al., 2011).

In recent years, Rock inhibitors have emerged as a novel topical therapy to treat glaucoma by lowering intraocular pressures (IOP) through modulation of trabecular meshwork (TM) cells' actin cytoskeleton, resulting in increasing conventional outflow, the main route of aqueous humor outflow in human eyes(Challa and Arnold, 2014; Nakajima et al., 2005; Rao et al., 2001, 2005; Wang and Chang, 2014). In addition, Rock inhibitors have been reported to increase ocular blood flow to the optic nerve head, enhancing

Abbreviations: Rock, rho-associated protein kinases; Net, norepinephrine transporters; IOP, intraocular pressure; RGC, retinal ganglion cell; TM, trabecular meshwork; CNS, central nerve system; CTB, cholera toxin B.

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RGC survival and axon regeneration after ischemic injury(Sugiyama et al., 2011; Tokushige et al., 2011). Rock inhibitors also reduce scar formation and inflammation following glaucoma filtering surgery.(Honjo et al., 2007) These data suggest that Rock inhibitors may have additional beneficial effects for the management of glaucoma other than reduction of IOP(Challa and Arnold, 2014; Wang and Chang, 2014; Yamamoto et al., 2014).

Like Rock inhibitors, the norepinephrine transporter (Net) inhibitor has been shown to reduce IOP in animals including rabbits and monkeys(Bacharach et al., 2015; Wang et al., 2015). By combining the IOP reducing effects of both Rock and Net inhibitors, Aerie pharmaceuticals developed a novel IOP-lowering ophthalmic solution AR-13324. Previous studies have demonstrated that AR-13324 reduces IOP in normotensive monkey eyes when applied topically(Wang et al., 2015). However, the effect of topical AR-13324 on promoting RGC survival and axon regeneration has not been tested. In this report, we investigated whether topical Rock/Net inhibitor AR-13324 protects RGCs or promotes their axon regeneration following optic nerve injury, and probed potential mechanisms by examining known molecular targets of Rock's kinase activity in the retina and optic nerve.

2. Materials and methods

2.1. Animals

All procedures involving animals were in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by Institutional Biosafety Committee and the Institutional Animal Care and Use Committee at the University of California, San Diego. Wild type male and female postnatal day 18 (P18) Sprague-Dawley rats (Harlan Laboratories, Placentia, CA, USA) were used for optic nerve crush. For counting and statistical analysis of retinas and optic nerve axons, a minimum of three and up to six rats of either sex were in each group, which prior analysis indicated would give a power of 0.8 to detect a 20% difference in survival at the $p = 0.05$ level. All surgical procedures on rats were performed under general anesthesia using an intraperitoneal injection of ketamine (60 mg/kg) and xylazine (7.5 mg/kg). Rats also received subcutaneous injection of buprenorphine (0.05 mg/kg; Bedford Laboratories, Bedford, OH, USA) as postoperative analgesic. Eye ointment containing erythromycin was applied to protect the cornea.

2.2. Topical administration of AR-13324

Rock/Net inhibitor (0.06% AR-13324) ophthalmic solution and the placebo vehicle solution with same formulation were topically applied by eye dropping. Fifteen microliters of each solution were applied three times a day for 14 consecutive days immediately after injury.

2.3. Intraocular pressure (IOP) measurement

Intraocular pressure (IOP) was measured using a TonoLab tonometer (iCare, Finland) under isoflurane anesthesia. Each eye was measured 10 times per session; reported results reflect the average of these 10 measurements. The baseline IOP was determined by measuring IOP once a day for three consecutive days before any surgical or topical therapeutic application. After optic nerve injury and topical application of the Rock/Net inhibitor or placebo, we measured IOP once a day 3, 6 and 9 days post-surgery.

2.4. Optic nerve crush and quantification of regenerating axons

Unilateral optic nerve crush was performed on young adult (P18) Sprague-Dawley rats. The animal was anesthetized, and an incision was made just behind the left eye. The optic nerve was exposed and the sheath opened longitudinally. The nerve was crushed 1 mm behind the eye with number 5 forceps for 5 s, avoiding injury to the ophthalmic artery. Nerve injury was verified visually at the crush site, and the vascular integrity to the retina was evaluated by funduscopic examination. After suturing the incision, the ophthalmic formulated Rock/Net inhibitor (0.06% AR-13324, Aerie Pharmaceuticals, Bedminster, NJ, USA) was topically administered on both injured and the fellow, non-injured eye three times a day for 14 consecutive days. Twelve days after the initial injury, Alexa Fluor 488-labeled cholera toxin B (CTB-488; 10 μg/ml; Invitrogen, Carlsbad, CA, USA) was injected intravitreally with care to avoid damaging the lens. Two days later, 14 days after injury, animals were deeply anesthetized and perfused with 4% PFA in 0.1 M phosphate buffer. Optic nerves were dissected and postfixed in 4% PFA for 1 h and subsequently washed in PBS. Optic nerves were incubated in 30% sucrose at 4 °C overnight before mounting in optimal cutting temperature (OCT) compound (Sakura Finetek, Torrance, CA, USA) for longitudinal 10-μm sectioning using a Leica cryostat (Buffalo Grove, IL, USA). The nerve sections were imaged with a 10× objective using a Zeiss LSM510 confocal microscope. To quantify regenerating axons, we identified the crush site and determined the number of axons that crossed 250 μm increment distances from the crush as well as the length of the longest regenerating axon. All data were collected and analyzed by masked examiners.

2.5. Immunofluorescence staining and survival quantification of RGCs

Retinas were removed from the post-fixed eye and flat-mounted. After wash with PBS, each retina was placed into a single well on a 24-well culture plate. Retinas were blocked and permeabilized in 10% normal goat serum and 0.2% Triton X-100 for 1 h, then incubated in the same medium with the antibody against the RNA Binding Protein with Multiple Splicing (RBPMS) (Thermo Fisher Scientific Inc., Rockford, IL USA), the RGC-specific marker, (1:500) overnight at 4 °C. After washing 4 times with PBS, retinas were incubated with Alex 488-conjugated goat anti–mouse secondary antibody (1:400; Invitrogen) overnight at 4 °C. Retinas were then washed 4 times with PBS and flat-mounted with the RGC layer uppermost. Each field sampled from central, middle and peripheral zones of 4 retinal leaflets (1.6 × 1.6 mm per field, 12 fields per retina) was imaged and numbers of RGCs were counted in a masked fashion.

2.6. Western blot analysis

Retinas were lysed with lysis buffer (Cell Signaling Technology, Boston, MA, USA) containing 0.5 mM of phenylmethanesulfonyl fluoride (PMSF) (Sigma-Aldrich, St. Louis, MO, USA). Protein concentration was determined by BCA protein assay (Thermo Fisher Scientific). Samples (25 μg) were separated by SDS-PAGE in 4–20% gradient Tris-glycine precast gels (Invitrogen) and transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA). The membrane was incubated for 1 h in blocking solution containing 5% non-fat milk powder and 0.1% Tween-20, pH 7.6. This was followed by overnight incubation at 4 °C in the blocking buffer containing rabbit primary antibodies against cofilin, phospho-cofilin, and phospho-LIMK (all from Cell Signing Technology). Subsequently, the

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