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Visualization of conventional outflow tissue responses to netarsudil in living mouse eyes

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ABSTRACT

Visual impairment due to glaucoma currently impacts 70 million people worldwide. While disease progression can be slowed or stopped with effective lowering of intraocular pressure, current medical treatments are often inadequate. Fortunately, three new classes of therapeutics that target the diseased conventional outflow tissue responsible for ocular hypertension are in the final stages of human testing. The rho kinase inhibitors have proven particularly efficacious and additive to current therapies. Unfortunately, non-contact technology that monitors the health of outflow tissue and its response to conventional outflow therapy is not available clinically. Using optical coherence tomographic (OCT) imaging and novel segmentation software, we present the first demonstration of drug effects on conventional outflow tissues in living eyes. Topical netarsudil (formerly AR-13324), a rho kinase/ norepinephrine transporter inhibitor, affected both proximal (trabecular meshwork and Schlemm's Canal) and distal portions (intrascleral vessels) of the mouse conventional outflow tract. Hence, increased perfusion of outflow tissues was reliably resolved by OCT as widening of the trabecular meshwork and significant increases in cross-sectional area of Schlemm's canal following netarsudil treatment. These changes occurred in conjunction with increased outflow facility, increased speckle variance intensity of outflow vessels, increased tracer deposition in conventional outflow tissues and decreased intraocular pressure. This is the first report using live imaging to show real-time drug effects on conventional outflow tissues and specifically the mechanism of action of netarsudil in mouse eyes. Advancements here pave the way for development of a clinic-friendly OCT platform for monitoring glaucoma therapy.

1. Introduction

Optical coherence tomography (OCT) is a non-contact imaging technology that provides cross-sectional information, quantitative analysis of ocular tissues and is widely used for imaging both anterior and posterior segments. Twenty years ago the first OCT images of the cornea and anterior segment were visualized (Izatt et al., 1994). Today, anterior segment OCT imaging is a crucial tool in the clinical practice of ophthalmology, including glaucoma, the leading cause of irreversible blindness worldwide. In glaucoma practices, OCT is primarily used to monitor retinal ganglion cell axon loss, however it is also used anteriorly for angle assessment (Narayanaswamy et al., 2010) and evaluating the efficacy of laser procedures (Lee et al., 2011; See et al., 2007). Moreover, OCT is routinely used to appraise tissue responses following minimally invasive glaucoma surgeries (Jung et al., 2015; Qian et al., 2015), as well as to evaluate bleb architecture following trabeculectomy (Mastropasqua et al., 2014).

Major advancements in OCT technology for the anterior eye have resulted in increased reproducibility and accuracy in the assessment of progressive tissue damage (Kagemann et al., 2015; Li et al., 2007) and enhanced penetration of the OCT signal into ocular tissues, including conventional outflow structures (Francis et al., 2012; Kagemann et al., 2014a, 2010). Interest in imaging the

Abbreviations: OCT, optical coherence tomography; SC, Schlemm's canal; TM, trabecular meshwork; C57, C57BL/6; IOP, intraocular pressure; DBG, Dulbecco's phosphate-buffered saline containing 5.5 mM D-glucose; PBS, phosphate buffered saline; NET, norepinephrine transporter

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trabecular meshwork/Schlemm's canal (TM/SC) has increased in recent years since reduced aqueous humor drainage through these tissues is the root cause of ocular hypertension in glaucoma. For example, OCT was used to monitor mechanical responses of the TM to acute intraocular pressure (IOP) elevation in living human eyes. Elevated IOP reduced SC cross-sectional area (Kagemann et al., 2015, 2014b), which was enhanced in the presence tropicamide, a drug that disables the ciliary muscle (Kagemann et al., 2015).

Resolution limitations due to optical penetration constraints of OCT into human and non-human primate eyes, has prompted study of mouse eyes as a surrogate to learn more about conventional outflow behavior. Conventional outflow in mouse eyes is very similar to human eyes in terms of anatomy, physiology and response to a number of conventional outflow drug treatments (Aihara et al., 2003; Boussommier-Calleja et al., 2012; Li et al., 2014a; Millar et al., 2011; Overby et al., 2014a, 2014b; Smith et al., 2001). Importantly, unlike many other models, mouse eyes have a true SC, through which ~70% of aqueous humor exits (Boussommier-Calleja et al., 2012). In terms of imaging, there are great advantages in using OCT to monitor mouse outflow tissues compared to primate eyes: mouse eyes are small, have a large SC lumen, thin sclera, and can be immobilized under anesthesia during imaging sessions. For example, behavior of TM/SC can be monitored at different IOP levels (Li et al., 2014a, 2014b) and at different disease stages (Li et al., 2014b). Similar to healthy human volunteers (Kagemann et al., 2015), we observed that mouse SC dimensions are reduced in a pressure-dependent manner that is quickly reversible (Li et al., 2014a, 2014b). The mouse eyes also respond to topical cholinergic treatment similar to that of human eyes (Flocks and Zweng, 1957; Li et al., 2014a; Lutjen-Drecoll, 1973), with the ciliary muscle contracting to maintain a patent conventional outflow tract (Li et al., 2014a).

Using our custom-built OCT imaging system for mouse eyes, we set out to monitor the effects of a new class of glaucoma drug, netarsudil (Sturdivant et al., 2016), currently in late-stage human clinical trials. Netarsudil primarily targets cells in the conventional outflow tract, efficiently decreasing IOP in both human (Bacharach et al., 2015; Levy et al., 2015; Lewis et al., 2015) and non-human primate eyes (Wang et al., 2015). In addition, netarsudil has been shown to increase outflow facility in non-human primate eyes (Wang et al., 2015) and to decrease episcleral venous pressure in rabbit eyes (Kiel and Kopczynski, 2015). In the present study, we found that netarsudil lowered IOP, increased outflow facility and increased tracer deposition in the trabecular meshwork of mouse eyes. More importantly, using our newly developed OCT imaging technologies, we discovered a novel action of the drug on the living mouse outflow tissues. We observed that netarsudil induced widening of the opening to the trabecular meshwork and increases the cross-sectional area of SC without pupillary constriction.

2. Materials and methods

2.1. Animals

Mice were handled in accordance with animal care and use guidelines of Duke University and in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. C57BL/6 (C57) and CD1 mice were purchased from the Jackson Laboratory (Bar Harbor, Maine, USA), bred/housed in clear cages and kept in housing rooms at 21 °C with a 12 h: 12 h light-dark cycle. Mice were examined at ages between 2 and 5 months old.

2.2. Intraocular pressure measurement

The right eyes from age and gender-matched C57 and CD1 sedated mice (5 mice per group) were given either 10 μl 0.04% topical netarsudil mesylate (from here on referred to as netarsudil) or 10 μl placebo (CF324–01) eye drop once/day for five consecutive days. IOP of both eyes was measured prior to eye drop administration using rebound tonometry (TonoLab) between 1 and 4 pm daily (Li et al., 2014a). Briefly, mice were anesthetized with ketamine (60 mg/kg) and xylazine (6 mg/kg). IOP was immediately measured just as the mice stopped moving (light sleep). Each IOP recorded was the average of six measurements, giving a total of 36 rebounds from the same eye.

2.3. Recovery from provocative IOP elevation

The effects of netarsudil on recovery from IOP spikes in living mice were conducted by analyzing pressure decay curves in paired eyes. Mice were anesthetized by intraperitoneal injection of ketamine (100 mg/kg) and xylazine (10 mg/kg) and maintained with ketamine (50 mg/kg at 20–30 min intervals). One microglass needle was back filled with the active metabolite of netarsudil (netarsudil-M1) (100 nM) and the other with vehicle (0.001% DMSO). The two needles were connected to two fluid reservoirs and two pressure transducers (model 142PC01G; Honeywell, Fort Washington, PA). Before the two needles were placed into mouse eyes, the two needle tips were guided and immersed into tear film using micromanipulators. After pressure transducers were zeroed, anterior chambers of both eyes were cannulated simultaneously and the spontaneous IOPs were recorded. Both eyes were perfused with drug or vehicle at 15 mmHg for 30 min, allowing drug or vehicle to reach outflow tissues. The pressure in both eyes was then elevated to 40 mmHg and held for 5 min. Once the IOPs in both eyes were stable, the stopcocks were turned to close off the reservoir and maintain the opening between pressure transducers and eye interiors. Pressure was continuously recorded until returning back to spontaneous IOP. To characterize the decline in pressure over time, the data were fitted with Eq. (1).

$$p = p_{ss} + (p_0 - p_{ss})\exp(-\alpha t) \quad (1)$$

where p_0 is the IOP at the start of the experiment, which was 40 mmHg in our case, and p_{ss} is the IOP when the pressure decay reached a steady state. α is a constant that characterized the rate of pressure decay. The regression analysis yields the values of α and p_{ss} in Eq. (1).

2.4. Outflow facility measurement using iPerfusion system

A system especially designed to simultaneously measure low outflow facilities in paired mouse eyes, the iPerfusion system (Sherwood et al., 2016), was used in the present study. Each freshly enucleated mouse eye was mounted on a stabilization platform in a perfusion chamber using a small amount of cyanoacrylate glue (Loctite, Westlake Ohio, USA). The perfusion chamber was filled with pre-warmed D-glucose in phosphate-buffered saline (DBG, 5.5 mM) and temperature regulated at 35 °C. A glass microneedle, back filled with either 100 nM netarsudil-M1 or vehicle (0.001% DMSO), was connected to the system and the microneedle was inserted into anterior chamber using a micromanipulator.

Both eyes were perfused at 9 mmHg for 45–60 min to allow acclimatization and deliver the drug to the outflow pathway, followed by 9 sequential pressure steps of 4.5, 6, 7.5, 9, 10.5, 12, 15, 18 and 21 mmHg. Data analysis was carried out as described previously (Sherwood et al., 2016). Briefly, a non-linear flow-pressure model was used to account for the pressure dependence of

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