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Rapid repeatable *in vivo* detection of retinal reactive oxygen species

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ABSTRACT

Oxidative injuries, such as those related to reactive oxygen species (ROS), have been implicated in various retinal and optic nerve disorders. Many ROS detection methods have been developed. Although widely utilized, many of these methods are useful only in *post mortem* tissues, or require relatively expensive equipment, or involve intraocular injection. In the present study, we demonstrated and characterized a chemiluminescent probe L-012 as a noninvasive, *in vivo* ROS detection agent in the mouse retina. Using optic nerve crush (ONC) and retinal ischemia/reperfusion (I/R) as injury models, we show that L-012 produced intensive luminescent signals specifically in the injured eyes. Histological examination showed that L-012 administration was safe to the retina. Additionally, compounds that reduce tissue superoxide levels, apocynin and TEMPOL, decreased injury-induced L-012 chemiluminescence. The decrease in L-012 signals correlated with their protective effects against retinal I/R-induced morphological and functional changes in the retina. Together, these data demonstrate the feasibility of a fast, simple, reproducible, and non-invasive detection method to monitor *in vivo* ROS in the retina. Furthermore, the results also show that reduction of ROS is a potential therapeutic approach for protection from these retinal injuries.

1. Introduction

Mitochondrial respiration is the primary source for production of reactive oxygen species (ROS), including superoxide. Ordinarily endogenous antioxidant enzymes inside or outside the mitochondria degrade these highly reactive molecules. However, excessive levels of ROS can overwhelm these intrinsic defense mechanisms leading to damage including oxidative and nitrative modifications to cellular proteins, lipid peroxidation, DNA damage, and ultimately neuronal death (Manzanero et al., 2013; Bryan et al., 2012). Mitochondrial dysfunction and increased ROS generation have been linked to numerous CNS neurodegenerative conditions, such as amyotrophic lateral sclerosis, Parkinson's disease, Alzheimer's disease, and cognitive declines observed in normal aging (Emerit et al., 2004). In addition to their pathogenic and pathophysiological roles, however, ROS is also believed to contribute to physiological processes regulating cell fate, stress responses, vascular tone, and wound healing.

Retinal neurons are particularly susceptible to oxidative stress because of the high levels of oxygen consumption, glucose oxidation, and polyunsaturated fatty acids in the retina (Arden and Sivaprasad, 2011). Unsurprisingly, ROS has been demonstrated to be involved in many retinopathies and optic neuropathies, such as age-related macular degeneration (Winkler et al., 1999), retinopathy of prematurity (Wilkinson-Berka et al., 2013), diabetic retinopathy (Wilkinson-Berka et al., 2013), retinal ischemia (Osborne et al., 2004), optic nerve trauma (Kanamori et al., 2010), and glaucoma (Aslan et al., 2008). Generation of ROS following ischemic injury, more specifically during reperfusion (Kuriyama et al., 2001), is amongst the earliest pathogenic changes leading to immune cell infiltration and neuron apoptosis (Bonne et al., 1998). Therefore, ROS represents a prime therapeutic target against injury caused by retinal ischemia.

While several strategies for removal of ROS have been developed, *in vivo* monitoring of ROS is technically challenging. Current detection methods include fluorescent probes such as

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dihydroethidium (DHE), spectrophotometric measurements, electron spin resonance spectroscopy, spin traps, and markers for oxidation and nitration of proteins, lipids, and DNA. These are all useful for assessment of oxidative stress in cultured cells, *ex vivo* biopsy, or *post mortem* tissue samples, but not for *in vivo* applications (Halliwell and Whiteman, 2004). Techniques developed for *in vivo* measurement of ROS include magnetic resonance imaging (Berkowitz et al., 2015, 2016), which requires specialized equipment not available to many laboratories, or intravitreal injection of detecting agents (Rayner et al., 2016), which limits the ability to perform repeated, frequent evaluations. Prunty and colleagues successfully used a sensitive ROS-activated fluorescent probe for *in vivo* imaging of retinal oxidative stress (Prunty et al., 2015), but this requires the animals to be kept in total darkness for 48 h to prevent photo-bleaching while the probe is accumulated in the eye, which severely limits its usefulness in detecting ROS changes shortly after injury or experimental manipulation. It is desirable to develop a technique that will overcome these limitations: requirement of relatively expensive equipment, intraocular injection, or a 48-h accumulation period.

Recent studies have demonstrated that L-012 (8-amino-5-chloro-7-phenylpyrrido [3,4-*d*]-pyridazine-1, 4 (2*H,* 3*H*) dione), a highly sensitive chemiluminescent probe (Nishinaka et al., 1993), can be used to detect subcutaneous ROS activity by *in vivo* luminescent imaging (Kielland et al., 2009; Zhou et al., 2012). Because of the ability for light to penetrate through the transparent media of the eye, we hypothesized that L-012 chemiluminescence from the retina could be detected by non-invasive *in vivo* imaging in mouse models of ocular injury. In the present study, we characterized and optimized the use of L-012 as a non-invasive *in vivo* method to assess temporal accumulation of ROS in the mouse retina following retinal ischemia/reperfusion (I/R) and optic nerve crush (ONC) injuries. Furthermore, we demonstrate the selectivity of L-012 through rapid modulation of luminescence, and the neuroprotective potential of early detection using two selective inhibitors of superoxide accumulation.

2. Materials and supplies

2.1. Animals

Female C57BL/6J mice (9- to 12-week old) were purchased from Jackson Laboratory (Bar Harbor, ME, USA) and Guangdong Animal Experimental Center (Guangzhou, Guangdong, China). All animals were maintained and handled in accordance with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research. The University of North Texas Health Center and Shenzhen Eye Hospital Institutional Animal Care and Use Committees approved the research protocol prior to initiation of the study.

2.2. Equipment

In vivo detection of L-012 signal required a small animal imaging system (IVIS Lumina *XR*™; Caliper LifeSciences, Hopkinton, MA, USA), which consists an enclosed, dark chamber with a warming pad to maintain animal body temperature. Luminescence signals were imaged by a CCD camera and were analyzed using the Living Image Software™ (PerkinElmer, Waltham, MA, USA).

Images of retina cross-sections were acquired using a Nikon Eclipse Ti inverted microscope (Nikon, Melville, NY, USA) containing the CRi Nuance FX multispectral imaging system (Caliper LifeSciences). Fluorescence was evaluated by equipping the microscope with a Semrock (Rochester, NY, USA) BrightLine TRITC-A-NTE filter cube (exciter: FF01-542/20-25, emitter: FF01-620/52-25, dichroic: FF570-Di01-25 × 36). Fluorescence was quantified using ImageJ software (NIH, Bethesda, MD, USA).

Electroretinography (ERG) was conducted using the HMsERG system (Ocuscience, Rolla, MO, USA). Amplitude and implicit times of waveforms were measured and analyzed in ERGView v4.380R software (Ocuscience).

2.3. Materials

L-012 was purchased from Wako Chemicals (catalog number: 120-04891; Richmond, VA, USA). Other materials and their respective suppliers are as follows: ketamine (Henry Schein Animal Health, Dublin, OH, USA), xylazine (Akorn, Lake Forest, IL, USA), acepromazine (Henry Schein Animal Health), isoflurane (Henry Schein Animal Health), Alcaine® ophthalmic solution (0.5% proparacaine HCl; Alcon, Fort Worth, TX, USA), Bacitracin zinc with polymixin B sulfate ophthalmic ointment (Akorn), 1% tropicamide ophthalmic solution (Bausch & Lomb Pharmaceuticals Inc., Claremont, CA, USA), Artificial Tears Solution™ (Rugby, Rockville Center, NY, USA), Gonak™ lubricant eye drop (Akorn), OxiSelect™ protein carbonyl ELISA Kit (Cell Biolabs, San Diego, CA, USA), Tissue-Tek OCT (Sakura Finetek, Torrance, CA, USA), ProlongGold anti-fade reagent with DAPI (Molecular Probes, Life Technologies, Grand Island, NY, USA), Dihydroethidium (DHE) (Thermo Fisher Scientific, Waltham, MA, USA), TEMPOL (4-hydroxy-2,2,6,6-tetramethylpiperidine-N-oxyl; Sigma-Aldrich, St. Louis, MO, USA), Apocynin (4-hydroxy-3-methoxyacetophenone; Sigma-Aldrich), Evans blue (Sigma-Aldrich).

3. Detailed methods

3.1. Mouse models of injury

Crush of the mouse ON was conducted as previously reported (Liu et al., 2014; Choudhury et al., 2015). Briefly, mice were anesthetized by intraperitoneal injection of ketamine and xylazine (100 and 10 mg/kg, respectively). Topical anesthesia of the eye was achieved by topical administration of Alcaine® ophthalmic solution. The left ON of mice in the crush group was exposed intraorbitally through a small window made between the surrounding muscles and vascular plexus, and crushed approximately 1 mm posterior to the globe with a self-closing forceps for 10 s. In the sham surgery group the left ON was exposed similarly but not crushed. Bacitracin zinc with polymixin B sulfate ophthalmic ointment was topically administered post surgery to prevent ocular infection.

Retinal I/R was induced as described previously (Kim et al., 2013; Nashine et al., 2014; Silverman et al., 2016). Briefly, mice were anesthetized with a ketamine/xylazine/acepromazine cocktail (100/10/3 mg/kg, intraperitoneally) followed by cannulation of the anterior chamber with a 30-gauge needle connected to a reservoir filled with sterile PBS. The reservoir was elevated to generate an intraocular pressure (IOP) of 120 mmHg for 1 h to induce retinal ischemia. Afterwards, the cannula was removed and retinal circulation was allowed to resume.

3.2. In vivo L-012 chemiluminescence imaging

Retinal ROS detection by the chemiluminescent probe L-012 was captured using the small animal *in vivo* imaging system. L-012 dissolved in sterilized distilled water was administered intraperitoneally using an insulin syringe with a 31-gauge needle at the indicated dose in a volume of 100 μL. (Notes: Attempts to use saline or PBS to dissolve L-012 at ≥15 mg/mL often led to precipitation. Aqueous solution of L-012 has been shown to be stable for more than 4 h at 37 °C (Zielonka et al., 2013). Frozen stocks of L-012 aqueous