Experimental Eye Research 138 (2015) 159–166

Contents lists available at ScienceDirect

Experimental Eye Research

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

Methods in eye research

Plate reader-based cell viability assays for glioprotection using primary rat optic nerve head astrocytes

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ARTICLE INFO

Article history:
Received 4 March 2015
Received in revised form
29 May 2015
Accepted in revised form 30 May 2015
Available online 3 June 2015

Keywords:
Optic nerve head astrocytes
Immunocytochemistry
Glioprotection
Lactate dehydrogenase
Cell viability
Oxidative stress
Drug discovery
Glaucoma

ABSTRACT

Optic nerve head astrocytes (ONHAs) are the major glia cell type in the non-myelinated optic nerve head where they contribute critically to extracellular matrix synthesis during development and throughout life. In glaucoma, and in related disorders affecting the optic nerve and the optic nerve head, pathological changes include altered astrocyte gene and protein expression resulting in their activation and extracellular matrix remodeling. ONHAs are highly sensitive to mechanical and oxidative stress resulting in the initiation of axon damage early during pathogenesis. Furthermore, ONHAs are crucial for the maintenance of retinal ganglion cell physiology and function. Therefore, glioprotective strategies with the goal to preserve and/or restore the structural and functional viability of ONHA in order to slow glaucoma and related pathologies are of high clinical relevance. Herein, we describe the development of standardized methods that will allow for the systematic advancement of such glioprotective strategies. These include isolation, purification and culture of primary adult rat ONHAs, optimized immunocytochemical protocols for cell type validation, as well as plate reader-based assays determining cellular viability, proliferation and the intracellular redox state. We validated and standardized our protocols by performing a glioprotection study using primary ONHAs. Specifically, we measured protection against exogenously-applied oxidative stress using *tert*-butylhydroperoxide (*t*BHP) as a model of disease-mediated oxidative stress in the retina and optic nerve head by the prototypic antioxidant, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox). Levels of oxidative stress were increased in the response to exogenously applied *t*BHP and were assessed by 6-carboxy-2′, 7′ dichlorodihydrofluorescein diacetate (DCFDA) fluorescence. Normalized DCFDA fluorescence showed a maximal 5.1-fold increase; the half-maximal effect (EC_{50}) for *t*BHP was 212 ± 25 μM. This was paralleled very effectively in the assays measuring cell death and cell viability with half-maximal effects of 241 ± 20 μM and 194 ± 5 μM for *t*BHP in the lactate dehydrogenase (LDH) release and the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) conversion assays, respectively. Pre-treatment with 100 μM Trolox decreased the sensitivity of ONHAs to *t*BHP. Half-maximal effects increased to 396 ± 12 μM *t*BHP in the LDH release assay and to 383 ± 3 μM *t*BHP in the MTT assay. Vehicle treatment (0.1% v/v ethanol) did not significantly affect cellular responses to *t*BHP. Antioxidant treatment increases ONHA viability and reduces the deleterious effects of oxidative stress. Our experiments provide important feasibility data for utilizing primary rat ONHAs in plate reader-based assays assessing novel therapeutics for glioprotection

Abbreviations: ANOVA, analysis of variance; BSA, bovine serum albumin; DAPI, 4′,6-diamidino-2-phenylindole dihydrochloride; DCFDA, 6-carboxy-2′, 7′ dichlorodihydrofluorescein diacetate; DIV, days *in vitro*; DMSO, dimethylsulfoxide; EAAT1, excitatory amino acid transporter 1; EtOH, ethanol; GFAP, glial fibrillary acidic protein; GLAST, glutamate/aspartate transporter; HBSS, Hank's Balanced Salt Solution; HEPES, 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid; INT, iodonitrotetrazolium chloride; IOP, intraocular pressure; LDH, lactate dehydrogenase; MPMS, 1-methoxyphenazine methosulfate; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NAD, beta-nicotinamide adenine dinucleotide sodium salt; ON, optic nerve; ONHA, optic nerve head astrocytes; PBS, phosphate-buffered saline; RGC, retinal ganglion cell; ROS, reactive oxygen species; SDS, sodium dodecyl sulfate; *t*BHP, *tert*-butylhydroperoxide; Trolox, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid.

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http://dx.doi.org/10.1016/j.exer.2015.05.023
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of the optic nerve and the optic nerve head in glaucoma and related disorders. Furthermore, our novel, standardized protocols have the potential to be readily adapted to high-throughput and high-content testing strategies.

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1. Introduction

Glaucoma is a multifactorial progressive ocular pathology, clinically presenting with damage to the retina and optic nerve (ON), ultimately leading to blindness (Casson et al., 2012). As the second leading cause of vision loss, glaucoma is estimated to affect more than 4 million individuals in the US alone (Klein and Klein, 2013) and affect an increasing number of patients worldwide (Foster et al., 2002; Quigley and Broman, 2006). However, the exact pathophysiological mechanisms underlying glaucoma still remain unknown (Bettin and Di Matteo, 2013; Chang and Goldberg, 2012). Elevated intraocular pressure (IOP) is the most clinically relevant biomarker for glaucoma and controlling IOP is currently the sole target of pharmaceutical and surgical intervention (Chang and Goldberg, 2012). While these therapies can lower IOP to slow ON damage, death of retinal ganglion cells (RGCs) and optic nerve head astrocytes (ONHAs) continues, resulting in progressive vision loss (Heijl et al., 2002; Quigley, 2011). In addition, IOP-lowering therapies often fail over time, have issues with patient compliance or have significant side effects, all of which are problematic in a chronic disease requiring long-term care. For some forms of glaucoma IOP-lowering therapies are ineffective altogether (Lee and Higginbotham, 2005). An increasing number of clinical and experimental studies suggest an IOP-independent mechanism of vision loss and highlight the need for complementary treatment approaches including neuroprotection (Chang and Goldberg, 2012; Farkas and Grosskreutz, 2001) and glioprotection (Noh et al., 2013; Qu and Jakobs, 2013). This is supported by a significant body of clinical evidence on normotensive glaucoma (Mozaffarieh and Flammer, 2013), and on glaucoma models where disease progression occurs without ocular hypertension (Mi et al., 2012). Indeed, it has been shown that neuroprotection can acieve preservation of visual function in the presence of continually elevated IOP as a disease-causing mechanism (Burroughs et al., 2011; Prokai-Tatrai et al., 2013).

Optic nerve head astrocytes (ONHAs) are the major glia cell type in the non-myelinated optic nerve head where they contribute critically to extracellular matrix synthesis (Hernandez, 2000). ONHAs exhibit complex intracellular signaling pathways (Kaja et al., 2015a), which are putative targets for glioprotection against oxidative stress. In glaucomatous retinopathy, changes in ONHAs include activation, migration, extracellular matrix remodeling, and alteration of gene and protein expression (Morrison et al., 2011).

Therefore, ONHAs are an important cellular target for drug discovery focused on glaucoma and related disorders affecting the ON and ON head. Specifically, rat primary ONHAs bear several advantages over ONHA cultures of human origin, foremost their lower genetic variability and their capacity for cryostorage.

We herein describe a complete experimental framework of optimized and validated protocols for plate reader-based assays for glioprotection using primary adult rat ONHAs.

These protocols include the *ex vivo* recovery and primary culture conditions of adult rat ONHAs, a detailed immunocytochemical characterization using a panel of relevant markers of glia cells, and protocols for plate reader-based assays determining cellular viability, proliferation, and intracellular redox state. The feasibility for testing drug candidates for glioprotection was established by testing the prototypic antioxidant 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) against exogenously-applied *tert*-butylhydroperoxide (*t*BHP)-induced oxidative stress, which is an established model for pathologic oxidative stress.

The purpose of the present study was to provide a framework and model system facilitating the discovery of novel therapeutic interventions for glaucoma and other pathologies of the ON and optic nerve head.

2. Materials and supplies

For primary ONHA culture all buffers and media were obtained from Lonza (Walkersville, MD) except trypsin-EDTA (MediaTech Inc., Manassas, VA). Serum products were purchased from Gibco® (Life Technologies, Carlsbad, CA). Tissue culture plastics were from TPP® (Techno Plastic Products AG, Trasadingen, Switzerland; sourced from Midwest Scientific, St. Louis, MO), with the exception of tissue-culture coated black/clear bottom 96-well plates for fluorescent DCFDA assays (Nunc™; Thermo Fisher Scientific, Rockford, IL). Non-tissue culture-coated 96-well plates with either optical glass bottoms (for immunocytochemistry) or LDH assays were also from Nunc™ (Thermo Fisher Scientific, Rockford, IL).

Chemicals for coating of optical glass bottom 96-well plates were obtained from Sigma Aldrich Corp. (St. Louis, MO). Poly-L-lysine-coated 12 mm glass coverslips were purchased from BD Biosciences (San Jose, CA). All antibodies and dyes used for immunocytochemistry are commercially available and listed in Table 1.

*t*BHP (Sigma Aldrich Corp., St. Louis, MO) was applied exogenously to the cell culture medium and was used to chemically induce oxidative stress, in a defined an reproducible manner. DCFDA, calcein-AM, and MTT were purchased from Life Technologies (Carlsbad, CA).

3. Detailed methods

3.1. Primary culture of rat optic nerve head astrocytes

The protocol for the studies presented herein was approved by the Institutional Animal Care and Use Committee at the University of Missouri—Kansas City, and was executed in accordance with the ARVO Statement on the Use of Animals in Ophthalmic and Visual Research.

Three month old male Brown Norway rats were euthanized and the optic nerve was carefully dissected and washed in a 35 mm dish containing ice-cold 0.1 M phosphate buffered saline pH 7.4 (PBS; Lonza, Walkersville, MD). Tissue was transferred into a second 35 mm dish containing 1 ml growth media, comprised of Dulbecco's Modified Eagle's Medium (Lonza, Walkersville, MD) supplemented with 20% fetal bovine serum (FBS; Gibco® Qualified; Life Technologies, Carlsbad, CA), 100 U/mL penicillin, and 100 μg/mL streptomycin (both from Lonza, Walkersville, MD). Optic nerve head tissue from 6 eyes was combined and subsequently cut into small pieces using a new disposable razor blade. The tissue suspension was transferred into a 5 ml microcentrifuge tube

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