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Metabolomics of the aqueous humor in the rat glaucoma model induced by a series of intracamerular sodium hyaluronate injection

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ABSTRACT

Glaucoma models are helpful to study disease characteristics and to design new therapeutic options. Metabolomic profiling approach have been used to elucidating the molecular characteristics of the aqueous humor. Juvenile male Wistar rats experimental (n = 15) and controls (n = 6) were used for these studies. Experimental rats received weekly intracamerular injection of 25 μl of sodium hyaluronate in the left eye and sterile saline solution in the right eye, consecutively for ten weeks. Rats were subjected to anterior/posterior eye segment examinations, intraocular pressure (IOP), and flash electroretinograms (ERG). The aqueous humor was collected at endpoints and analyzed by Nuclear Magnetic Resonance. Elevated IOP and significant reduction of a, b waves and amplitude of oscillatory potential was observed in the left eyes compared to control eyes. The aqueous humor metabolomic profile from control and the experimental eyes were compared. Concentrations of metabolites (amino acids, lipids and carbohydrates) significantly changed after the sodium hyaluronate injections series, compared to the sham-operated eyes. Metabolic changes in the hypertensive eyes correlated with the impaired retinal function. Observed metabolomic changes in aqueous humor in hypertensive state may play a significant role in glaucoma pathogenesis.

1. Introduction

Glaucoma is a chronic and irreversible disease characterized by increased intraocular pressure (IOP), degeneration of retinal ganglion cells (RGC) and optic nerve fibers (ONF), that manifest itself by progressive changes in retinal sensitivity and visual field performance, leading to vision loss (Adatia and Damji, 2005; Morrison et al., 2008).

Pathophysiologic mechanisms involving metabolic changes are implicated in both, the aqueous humor (AH) homeostasis and to overall AH dynamics and outflow facility, as well as the development and progression of glaucoma disease. Lower hyaluronic acid or increased fibronectin and thrombospondin AH availability (Gabelt and Kaufman, 2005) among other metabolite changes (Welge-Lussen et al., 2001; Zanón-Moreno et al., 2009; 2008) have been reported. Knowledge on the metabolomic signature of the AH can be useful for advancing in glaucoma research.

Emerging metabolomic procedures has specific advantages versus the classical diagnostic probes, such as the enzyme-linked immunosorbent assay (ELISA) (Engvall et al., 1971). This latter can only be utilized to analyze one sample or a little number of samples at a time. Furthermore, these methods cannot be used for small

Abbreviations: AA, ascorbic acid; AH, aqueous humor; ATP, adenosine triphosphates; ANOVA, analysis of variance; CG, control group; D_2O, deuterium oxide; ERG, flash electroretinogram; ELISA, enzyme-linked immunosorbent assay; FID, free induction decay; GABA, gamma-aminobutyric acid; HYA, sodium hyaluronate; IOP, intraocular pressure; ^1H, proton; NMR, nuclear magnetic resonance; ONF, optic nerve fiber; PCA, principal component analysis; PLS-DA, partial least squares discriminant analysis; RGC, retinal ganglion cell; SD, standard deviation; SSS, sterile saline solution; TSP, trimethylsilylpropionate; VLDL, very low density lipoprotein.

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sample volumes. However, metabolomics permits to monitor high amounts of metabolites from biofluids and tissues by unique assay. Most common tools comprises those based on gas or liquid chromatography coupled by mass spectrometry (Wei et al., 2012) and nuclear magnetic resonance (NMR) spectroscopy, and their corresponding bioinformatic processing patterns (Reo, 2002). Metabolomics and their complementary genomics and proteomics, contributes to better managing disease diagnosis as well as to monitor response to therapy (Bodi et al., 2012; Young and Wallace, 2009), also applicable to a wide range of ocular disorders, for which there is still much to investigate (Agudo-Barriuso et al., 2013; Galbis-Estrada et al., 2014; Nordstrom and Lewensohn, 2010; Welge-Lussen et al., 2001).

We deal with elucidating the molecular characteristics of the AH, both in healthy and pathologic eyes. Because the volume of AH that can be collected at the time of human surgery, without eye damaging, is usually not more than 0.2–0.3 ml, it is difficult to measure many substances with conventional tools (Brown et al., 1986). Efficacy of collecting low amounts of AH in rats and by measuring changes in AH composition has extensively proven before (Benozzi et al., 2002; Mayordomo, 2013; Moreno et al., 2005a; Morrison et al., 2008; Urcola et al., 2006).

Analytical platforms such as proton nuclear magnetic resonance spectroscopy (^{1}H NMR) combined with advanced multivariate analysis methodologies provide excellent options for studying complex sets of molecules without extensive sample preparation (Tkadlecova et al., 1999).

We have characterized the AH metabolic profile by high-resolution ^{1}H NMR in healthy rats as well as in the glaucoma-induced eyes by using a reproducible and effective model based on the sodium hyaluronate (HYA) intracamerular injection series (Moreno et al., 2005a; Urcola et al., 2006) to provoke a sustained IOP elevation and glaucomatous retinal damage.

2. Materials and methods

All procedures were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the corresponding Institutional Committee of the Research Center at the Faculty of Medicine of the University of Valencia (Spain) (code: A1315390015414).

2.1. Glaucoma model and sampling procedures

Male Wistar rats weighing 200 g (±40 g) and aged 7 weeks were used. Rats were housed in a standard animal room under controlled conditions of humidity and temperature (22 °C ± 2 °C) with a 12-h light/dark cycle and were allowed food and water “ad libitum”.

Fifteen rats were randomly chosen and prepared for ocular surgery. Anterior eye segment biomicroscopy was performed and morphological characteristics were registered. IOP determinations were always done at 08:00 h, to avoid circadian variations, before the eye interventions at baseline and during 10 consecutive weeks. Topic anesthetic instillation was used to reduce discomfort or pain. Additional stress was avoided to the rats during IOP determinations. Measurements were done by the same researcher by gently locating the flat-tiped cone of the veterinarian applanation tonometer (Tono-Pen® Vet, Medtronic, Madrid, Spain) over the rat corneal surface. The IOP values were mean ± standard deviation (SD) of eight to ten independent suitable readings.

To demonstrate possible changes in retinal electric activity, flash electroretinograms (ERG) were performed at baseline and at 5, 7, 9 and 10 weeks during the time-course experiments, as previously reported by Moreno et al. (2005) with minor modifications. Briefly, after 12 h of dark adaptation rats were anesthetized with isoflurane (Isoflo®, laboratorios Esteve, Barcelona, Spain) and the pupils were dilated with 1% tropicamide eye drops (®Colircusí Tropicamida 1%, laboratorios Alcon®, Barcelona, Spain). To place the active electrode (Goldring electrode, Acrivet, Hennigsdorf, Germany) on the cornea, one drop of topical anesthetic and lubricant gel (1% HA Hycare, Acrivet, Hennigsdorf, Germany) was used. The reference electrode was placed on the skin of the forehead and the grounding electrode was attached to the tail. The ERG data were recorded with the Retiport (Acrivet, Henningsdorf, Germany) previously adapted for recording each eye in separate. The white LED light stimulator was placed at a distance of 2 cm from the eye and stimuli presentation was done by a series of brief (<5 ms) LED flashes of intensities ranging from 0.03 to 3 cd s/m^2.

All surgical procedures were performed by the same researcher using a surgical microscope (OMS-90 operator, Topcon, Barcelona, Spain). The anesthetized rat was conveniently placed under the microscope to visualize the anterior eye segment. Elevated IOP was induced in the left eyes based on the descriptions of Benozzi et al. (2002) and Moreno et al. (2005), with minor modifications (Mayordomo, 2013). Using a Hamilton syringe (Hamilton GASTIGHT® Syringes, 1700 Series, Luer tip; Sigma–Aldrich, Madrid, Spain) with a 30-gauge needle, a constant amount of 25 μl of viscoelastic substance: sodium hyaluronate (HYA) (Z-Hyalin® 10 mg/ml, Imex, Valencia, Spain), was slowly injected, every Tuesday, by paracentesis (the needle, with the bevel up, was passed through the cornea, near the limbus, to the anterior chamber) into the left eye, weekly during ten consecutive weeks. HYA, the sodium salt of hyaluronic acid, administered through a series of injections into the anterior chamber results in increased viscosity of the AH, decreased AH outflow facility and elevated IOP. Then, the rat was moved to operate the right eye with the same surgical maneuver. This eye, considered as the sham-operated eye, was slowly injected with 25 μl of sterile salt solution (SSS) (B. Braun, Barcelona, Spain) in the anterior chamber, once a week for 10 consecutive weeks.

At the end of the experimental time period, the AH was obtained from fourteen rats (one rat died during anesthetic procedures) under general anesthesia with the above described protocol. The corneal paracentesis was done to proceed with the gentle aspiration of 15–20 μl of AH from the anterior chamber, and the samples were stored in Eppendorf microtubes, conveniently registered and frozen at −80 °C until processing. Finally fourteen AH samples from SSS eyes versus twelve from HYA eyes were analyzed (two sample was deleted by blood contamination).

In addition to these animals, six more rats used as the controls (CG) were destined to measure the baseline IOP, to perform the ERG and collecting 15–20 μl of AH from both anterior eye chambers without additional maneuvers. Samples were frozen and stored until metabolomic techniques. Regarding the six control rats (twelve eyes), a final number of 11 AH samples were used (one sample was lost during manipulation).

2.2. NMR spectroscopy

For NMR analysis, 15 μl of AH was mixed with 2.5 μl of deuterium oxide (D_2O), and that entire mixture was placed in a 1-mm high-quality NMR tube. For a selected group of samples, 2.5 μl of sodium-3′-trimethylsilylpropionate-2,2,3,3-d_4 (TSP; 0.5 mM) in D_2O was added for referencing purposes.

The NMR spectra were obtained with a Bruker Avance DRX 600 (Bruker Biospin GmbH, Rheinstetten, Germany) spectrometer operating at 600.13 MHz equipped with a 1-mm 1H/13C/31P TXI probe. All ^{1}H NMR spectra were acquired using a standard one-dimensional pulse sequence with water suppression. Nominal temperature of the sample was kept at 310 K. A total of 256 free induction decays (FIDs) were collected in 64k data points with a