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Research article

Characterisation of the metabolome of ocular tissues and post-mortem changes in the rat retina

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ABSTRACT

Time-dependent post-mortem biochemical changes have been demonstrated in donor cornea and vitreous, but there have been no published studies to date that objectively measure post-mortem changes in the retinal metabolome over time. The aim of the study was firstly, to investigate post-mortem, time-dependent changes in the rat retinal metabolome and secondly, to compare the metabolite composition of healthy rat ocular tissues. To study post-mortem changes in the rat retinal metabolome, globes were enucleated and stored at 4 °C and sampled at 0, 2, 4, 8, 24 and 48 h post-mortem. To study the metabolite composition of rat ocular tissues, eyes were dissected immediately after culling to isolate the cornea, lens, vitreous and retina, prior to storing at −80 °C. Tissue extracts were subjected to Gas Chromatograph Mass Spectrometry (GC-MS) and Ultra High Performance Liquid Chromatography Mass Spectrometry (UHPLC-MS). Generally, the metabolic composition of the retina was stable for 8 h post-mortem when eyes were stored at 4 °C, but showed increasing changes thereafter. However, some more rapid changes were observed such as increases in TCA cycle metabolites after 2 h post-mortem, whereas some metabolites such as fatty acids only showed decreases in concentration from 24 h. A total of 42 metabolites were identified across the ocular tissues by GC-MS (MSI level 1) and 2782 metabolites were annotated by UHPLC-MS (MSI level 2) according to MSI reporting standards. Many of the metabolites detected were common to all of the tissues but some metabolites showed partitioning between different ocular structures with 655, 297, 93 and 13 metabolites being uniquely detected in the retina, lens, cornea and vitreous respectively. Only a small percentage (1.6%) of metabolites found in the vitreous were only detected in the retina and not other tissues. In conclusion, mass spectrometry-based techniques have been used for the first time to compare the metabolic composition of different ocular tissues. The metabolite composition of the retina stored at 4 °C post-mortem is mostly stable for at least 8 h.

1. Introduction

Metabolites have many important roles in biological systems (Dunn et al., 2011a). The total complement of metabolites in a biological system is defined as the metabolome; its composition is both sample- and time-dependent (Psychogios et al., 2011; Bouatra et al., 2013). As metabolites are the end product of biological pathways, the metabolome is a sensitive measure of disease

Abbreviations: ARVO, Association for Research in Vision and Ophthalmology; FDR, False Discovery Rate; GC-MS, Gas Chromatography-Mass Spectrometry; NetCDF, Network Common Data Form; PCA, Principal Components Analysis; QC, Quality Control; UHPLC-MS, Ultra High Performance Liquid Chromatography-Mass Spectrometry.

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phenotype and a dynamic indicator of genetic, environmental or disease-specific perturbations (Dunn et al., 2011a; Kell, 2004; Raamsdonk et al., 2001).

Metabolomics is increasingly being applied as a tool in eye research. It has been used to characterise normal ocular tissues and biofluids including the tear film, lens, vitreous and retina (Chen et al., 2011; Locci et al., 2014; Yanshole et al., 2014; Mains et al., 2012; Sun et al., 2014). It has also been used to study ageing, a variety of ocular pathologies, metabolite transport in the retina, effects of drugs and metabolic changes during corneal organ culture for transplantation (Mayordomo-Febrer et al., 2015; Du et al., 2013; Kryczka et al., 2012; Song et al., 2011; Young et al., 2009).

Aqueous and vitreous are the most accessible intraocular samples that can be obtained in clinical studies, and these have been used to interrogate the metabolic activities of surrounding ocular structures (Mayordomo-Febrer et al., 2015; Young et al., 2009). However, it is unclear whether sampling ocular biofluids provides robust and reproducible information on the metabolic activity of surrounding tissues and ideally, the metabolome of the ocular tissue of interest should be studied directly. One option is to use post-mortem eyes. However, a concern with this approach is that the concentration of metabolites can alter rapidly post-mortem caused by continued metabolic activity or metabolite instability. Time-dependent post-mortem biochemical changes have been demonstrated in donor cornea (Kryczka et al., 2013) and vitreous (Boulagnon et al., 2011), but to the best of our knowledge, there have been no published studies to date that objectively measure post-mortem changes in the retinal metabolome over time. This is important baseline information for future study design.

The objectives of the study reported here were (1) to investigate post-mortem changes in the rat retinal metabolome over 48 h when eyes are stored at 4 °C (temperature at which human post-mortem eyes are stored in real-life) and (2) for the first time, annotate and characterise the metabolic composition of four ocular tissues (cornea, lens, vitreous and retina).

2. Methods

2.1. Animals

All animal experiments were conducted in accordance with the United Kingdom (UK) Home Office regulations for the care and use of laboratory animals, the UK Animals (Scientific Procedures) Act (1986), and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Twenty-one, male, Sprague Dawley rats were included in the post-mortem study (42 eyes in total) and six rats were included in the tissue comparison study; all rats were in the age-range of 7–12 weeks. The rats were kept in the same room under standard laboratory conditions and fed with standard chow. The rats were culled by carbon dioxide inhalation followed by cervical extension; this process was staggered to ensure rapid sample collection. Enucleation was performed as soon as possible (between 1 and 20 min) after death. If eyes were not dissected immediately (T = 0 h samples), they were placed in small bottles and stored on ice before transferring to a refrigerator and then stored in the dark for up to 48 h at 4°C (T = 2–48 h samples).

2.2. Dissection

Seven eyes were studied at each time point for the retinal metabolic stability study and six eyes were studied to annotate and characterise the metabolome of different ocular tissues. Dissections of the rat eyes at T = 0 were performed immediately after culling and the stored eyes were dissected at 2, 4, 8, 12, 24 and 48 h post culling to remove the retina. Under microscope guidance the cornea was carefully removed. Then the iris was divided with forceps and the lens removed in one piece to gain access to the posterior segment. Vitreous was meticulously separated from the retina. Lastly, the neurosensory retina was removed avoiding contamination with the retinal pigment epithelium/Bruch's membrane/choroid complex. All dissections were performed on a cold plate to maintain a temperature of approximately 4 °C and dissection time was between 2 and 5 min. The dissected tissues were snap-frozen with liquid nitrogen and then stored at −80 °C before extraction.

2.3. Sample preparation

Each tissue sample was carefully weighed prior to extraction, mean weights were as follows; retina 25.4 mg; vitreous 30.1 mg; lens 32.7 mg; cornea 7.9 mg. Metabolite extraction was achieved by adding 800 μL of chloroform:methanol (50:50, pre-cooled to −20 °C) followed by 50 μL of an internal standard mixture (0.1 mg/ml d_5 Benzoic acid, d_4 Succinic acid and d_5 Glycine dissolved in methanol) to each pre-weighed sample. Homogenisation/extraction was performed in a TissueLyser (Qiagen; 25 Hz, 10 min using one 3 mm tungsten carbide bead). Next, 400 μL of water was added and the mixture was vortexed and allowed to stand on ice for 10 min. The samples were then centrifuged (5 min, 13,000*g*) to allow separation of the solvents. The top hydrophilic layer (to be analysed by GC-MS) and bottom lipophilic layer (to be analysed by UHPLC-MS analysis) were transferred into separate tubes and dried overnight (Savant SPD131DDA with an RVT4104 cold trap, Thermo Scientific). The volume of polar and non-polar extractions collected was normalised to the lowest tissue weight for each tissue type in each study. Quality control (QC) samples were prepared by pooling together an aliquot of 100 μL from each retina sample after extraction and before drying. The QC samples were used for quality assurance as described by Dunn et al (Dunn et al., 2011b).

2.4. UHPLC-MS analysis

All tissue extract samples and QC samples were reconstituted in 200 μL 50:50 water:methanol prior to analysis. The samples were analysed using an Accela Ultra High Performance Liquid Chromatography system coupled to an electrospray hybrid LTQ-Orbitrap Velos mass spectrometer (ThermoFisher Scientific, Bremen, Germany). All samples were analysed separately in positive and negative ion modes. QC samples were analysed for the first ten injections and then every fifth injection. The last two injections were also QC samples. Chromatographic separations were performed, following injection of a 10 μL sample volume, onto a Hypersil GOLD column (100 × 2.1 mm, 1.9 μm; ThermoFisher Scientific, Runcorn, UK) with the column temperature set at 50 °C. The two solvents applied were solvent A −0.1% formic acid in water (vol/vol) and solvent B −0.1% formic acid in methanol (vol/vol)) at a flow rate of 400 μL/min. Solvent A was held at 100% for 0.5 min followed by an increase to 100% solvent B over 4.5 min, which was then held at 100% solvent B for a further 5.5 min. At 10.5 min, it was changed to 100% solvent A and held at 100% solvent A to equilibrate for 1.5 min. All column eluent was transferred to the mass spectrometer. Full-scan profiling data were acquired in an Orbitrap mass analyser (mass resolution 30,000 at $m/z = 400$).

Raw data files (.RAW format) generated from the UHPLC-MS system were converted to the NetCDF format using the File converter program in the XCalibur software package (ThemoFisher Scientific, Bremen, Germany). Deconvolution was performed using XCMS software (Smith et al., 2006) as described previously (Dunn et al., 2008). Whilst relative concentrations were measured no absolute quantification of metabolites to define concentrations per