Free Radical Biology and Medicine 97 (2016) 513–519

Contents lists available at ScienceDirect

Free Radical Biology and Medicine

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

Original article

Mitochondrial oxygen metabolism in primary human lens epithelial cells: Association with age, diabetes and glaucoma

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ARTICLE INFO

Article history:
Received 24 December 2015
Received in revised form
14 June 2016
Accepted 18 July 2016
Available online 19 July 2016

Keywords:
Mitochondria
Cataract
Diabetes
Glaucoma
Lens epithelial cells
Oxygen metabolism

ABSTRACT

Purpose: The hypoxic environment around the lens is important for maintaining lens transparency. Lens epithelial cells (LECs) play a key role in lens metabolism. We measured oxygen consumption to assess the role of human LECs in maintaining hypoxia around the lens, as well as the impact of systemic and ocular diagnosis on these cells.

Methods: Baseline cellular respiration was measured in rabbit LECs (NN1003A), canine kidney epithelial cells (MDCK), trabecular meshwork cells (TM-5), and bovine corneal endothelial cells (CCEE) using a XF96 Extracellular Flux Analyzer (Seahorse Bioscience, North Billerica, MA), which measures oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) in vitro. Following informed written consent, lens capsule epithelial cells were obtained from patients during cataract surgery and were divided into small explants in 96-well plates. Capsules were removed when LECs became confluent. OCR was normalized to the number of cells per well using rabbit LECs as a standard. The effect of patient age, sex, race, and presence of diabetes or glaucoma on oxygen consumption was assessed by using the Mann-Whitney *U* test and multivariate regression analysis.

Results: Primary LECs were obtained from 69 patients. The OCR from donors aged 70 and over was lower than that of those under 70 years (2.21 ± 1.037 vs. 2.86 ± 1.383 fmol/min/cell; $p < 0.05$). Diabetic patients had lower OCR than non-diabetic patients (2.02 ± 0.911 vs. 2.79 ± 1.332 fmol/min/cell; $p < 0.05$), and glaucoma patients had lower OCR than non-glaucoma patients (2.27 ± 1.19 vs. 2.83 ± 1.286 fmol/min/cell; $p < 0.05$). Multivariate regression analysis confirmed that donors aged 70 and over ($p < 0.05$), diabetic patients ($p < 0.01$), and glaucoma patients ($p < 0.05$) had significantly lower OCR, independent of other variables. Gender and race had no significant effect on OCR.

Conclusions: The lower oxygen consumption rate of human LECs in older donors and patients with diabetes or glaucoma could contribute to cataract development. Diabetes and glaucoma are particularly important factors associated with decreased OCR, independent of age. Ongoing studies are examining pO_2 at the anterior surface of the lens in vivo and oxygen consumption in the patient's LECs.

1. Introduction

Cataract is the most common cause of reversible vision loss in the world. Several risk factors for cataract formation have been reported, with age being the most impactful [1]. Age-related cataracts are responsible for nearly half of all blindness worldwide and half of all visual impairment in the United States [2]. Other intrinsic factors aside from age, such as diabetes [3], glaucoma [4], and heritability [5,6] are also known to be significant risks for cataract development. In addition, ultraviolet B radiation (UV-B) [7], body mass index (BMI) [8], smoking [5,9], diet [10], exposure to hyperbaric oxygen [11–13], and corticosteroids [14] have been identified as extrinsic or environmental risk factors for cataract.

As mentioned above, heritability is a major contributing factor towards age-related cataracts but the genes involved have not yet been identified [2,5,6,15]. Nevertheless, many inheritable mitochondrial diseases have been associated with an elevated risk of cataract. In such mitochondrial cytopathies, cataracts are of

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http://dx.doi.org/10.1016/j.freeradbiomed.2016.07.016
0891-5849/

particular interest due to the well-described MELAS (myopathy, encephalopathy, lactacidemia, and stroke-like episodes) syndrome [16], and many representative mitochondrial diseases such as mitochondrial myopathy [17], mitochondriopathy [18], chronic progressive external ophthalmoplegia [19], mitochondrial encephalomyopathy [20], and autosomal dominant optic atrophy (OPA3) [21] have all been associated with a higher risk of cataract.

Mitochondria provide energy generated by oxidative phosphorylation to the cell and at the same time play a central role in apoptosis and aging [22]. It has been reported that mitochondria decay in number and function with increasing age [23–25]. The number of mitochondria has been found to decrease in liver cells of aging mice [24,26] and humans [23,27], and the age-related decline of mitochondrial function has long been recognized to occur concomitantly with the appearance of mitochondrial morphological alterations [25] such as abnormal roundness which has been observed in aged mammals [28].

In most tissues, oxygen partial pressures below 3% (~22 mmHg) are considered hypoxic. In humans, the oxygen level near the anterior lens epithelium is approximately 0.4% (~3 mmHg) [29] indicating that the lens epithelium exists in a hypoxic environment [30,31]. The lens consumes oxygen [32] and further studies have shown that most of the small amount of oxygen derived from the iris vasculature is consumed by the lens [30,33].

Mitochondria are abundant in the lens, but only within the epithelium and differentiating fibers, as mature fibers in the core of the lens lack mitochondria. Moreover, while mitochondria account for approximately 90% of the total oxygen consumption in the lens [34], those in the lens epithelium play the most important role in lens metabolism [35,36]. Interestingly, it has been proposed that aged mitochondria consume significantly less oxygen than young mitochondria [37], and the susceptibility of the older human lens to oxygen-induced damage was directly demonstrated in a study involving patients treated with long-term hyperbaric oxygen therapy [11]. It is well known that nuclear cataracts are caused by excessive oxidation of proteins in the lens nucleus [38,39].

As described above, our previous study reported that the lens exists in a hypoxic environment which is important in maintaining lens transparency, and that oxygen levels are generally lower closer to the lens [29,30]. Exposure to increased oxygen has been identified as a risk factor for nuclear cataract, the most common type of age-related cataract [40]. Therefore, we hypothesized that decreased mitochondrial function in lens epithelial cells (LECs) plays a key role in cataractogenesis.

Recently, a noncontact method of measuring cellular respiration with an extracellular flux analyzer, the Seahorse Bioscience XF96 (North Billerica, MA, USA), has been described [43]. To test our hypothesis, we measured the oxygen consumption rate (OCR) in *ex vivo* human LECs to assess whether age, diabetes, glaucoma and other intrinsic factors contribute to mitochondrial function and, potentially, cataractogenesis.

2. Methods

2.1. Cell culture

In order to measure baseline cellular respiration, established cell lines from rabbit LECs (NN1003A), canine kidney epithelial cells (MDCK) and human trabecular meshwork cells (TM-5) were utilized. In addition, early-passage cells from bovine corneal endothelium (CCEE) were established from fresh bovine eyes. In brief, the cornea was removed and placed in a small petri dish with media and the endothelial tissue layer was dissected to small segments. In a T25 Corning flask, a media channel was made with 1.0 mL growth media (described in detail below) and one small segment of endothelium was submerged in the media. The flask was then placed in a 37 °C /5% CO_2 incubator for approximately one week until the cells grew from the tissue to a 250 mm^2 area. At that point, the tissue was slowly lifted and placed in another T25 flask for additional cell growth and eventual harvesting; this process may be repeated up to a total of three times. For cell collection each flask was trypsinized at a ratio of 1:1 with media (0.25% Trypsin-EDTA, Gibco® #25,200, Life Technologies, Grand, NY). Once the cells dissociated from the flask, three times the amount of media was added followed by slow pipetting and re-allocation of the cells and media into a 15 mL tube. The tube was spun at 700 rpm in a 4 °C centrifuge for 5–7 min, after which the supernatant was discarded, and the pellet re-suspended in media. The cells and media were aliquoted into a T75 flask and subculture was done at 65–75% confluence. The first five subcultures (passage 1 – passage 5) were used for this study.

2.2. Growth media

NN1003A were cultured in 1 g/L D-Glucose Dulbecco's Modified Eagle Medium (DMEM; Life Technologies, #11885, Grand Island, NY) with 10% fetal calf serum (FCS, Atlanta Biologicals, Flowery Branch, GA) and 1% penicillin and streptomycin (Life Technologies, Grand Island, NY). MDCK cells were cultured in Minimum Essential Medium (MEM #11090, Life Technologies, Grand Island, NY) with 10% FBS and 1% penicillin and streptomycin (Life Technologies, Grand Island, NY). TM-5 cells were cultured in DMEM (Gibco® #10566, Grand Island, NY) with 10% fetal calf serum (FCS, Atlanta Biologicals, Flowery Branch, GA) and 1% penicillin and streptomycin. CCEE cells were cultured in DMEM (Gibco® #10566, Grand Island, NY) with 10% FBS, 1% non-essential amino acid-100 × (Gibco® #11140), 2% essential amino acid-50 × (Gibco® #11130-051, Grand Island, NY),1ug/mL-Fungizone (Gibco® #15290-018, Grand Island, NY) and 2.5 μg/mL-Gentamycin (Gibco® #15750-060, Grand Island, NY).

2.3. Visualization of mitochondria by confocal laser microscopy

MitoTracker® Red CMXRos (M7512, Red CMXRos Life Technologies, Grand Island, NY) is a red-fluorescent dye (abs/em ~579/599 nm) that stains mitochondria in live cells and its accumulation is dependent upon membrane potential. MitoTracker® Red CMXRos was used to stain mitochondria in NN1003A, MDCK, TM-5 and CCEE cells. Staining of mitochondria was performed by the method described by Wolf and colleagues [41,42]. Cells growing on chambered slide-glass (Lab-Tek™ II Nunc Thurmo Scientific Inc., USA) were incubated with MitoTracker® Red CMXRos at a final concentration of 200 nM for 20 min in a 37 °C /5% CO_2 culture incubator. The chambered slide-glasses were then rinsed with culture medium followed by fixation with 4% PFA and permeabilized with PBS containing 0.2% Triton™ X-100 (Sigma-Aldrich, St. Louis, MO). Specimens were examined under a confocal laser microscope (Zeiss LSM 510 META, Jena, Germany).

2.4. Primary culture of human lens epithelial cells from donors

The human research protocol was approved by the Washington University Institutional Review Board and Human Research Protection Office, according to the tenets of the Declaration of Helsinki. Informed written consent was obtained from the subjects undergoing cataract extraction and intraocular lens implantation, as well as intraocular oxygen measurements, as described elsewhere [29]. After continuous curvilinear capsulorhexis, the capsular specimens were passed off the surgical field via forceps and