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Research paper

Apoptosis inducing factor deficiency causes retinal photoreceptor degeneration. The protective role of the redox compound methylene blue

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ABSTRACT

Dysfunction in mitochondrial oxidative phosphorylation (OXPHOS) underlies a wide spectrum of human ailments known as mitochondrial diseases. Deficiencies in complex I of the electron transport chain (ETC) contribute to 30–40% of all cases of mitochondrial diseases, and leads to eye disease including optic nerve atrophy and retinal degeneration. The mechanisms responsible for organ damage in mitochondrial defects may include energy deficit, oxidative stress, and an increase in the NADH/NAD^+ redox ratio due to decreased NAD^+ regeneration. Currently, there is no effective treatment to alleviate human disease induced by complex I defect.

Photoreceptor cells have the highest energy demand and dependence on OXPHOS for survival, and the lowest reserve capacity indicating that they are sensitive to OXPHOS defects. We investigated the effect of mitochondrial OXPHOS deficiency on retinal photoreceptors in a model of mitochondrial complex I defect (apoptosis inducing factor, AIF-deficient mice, Harlequin mice), and tested the protective effect of a mitochondrial redox compound (methylene blue, MB) on mitochondrial and photoreceptor integrity. MB prevented the reduction in the retinal thickness and protein markers for photoreceptor outer segments, Muller and ganglion cells, and altered mitochondrial integrity and function induced by AIF deficiency. In rotenone-induced complex I deficient 661 W cells (an immortalized mouse photoreceptor cell line) MB decreased the NADH/NAD^+ ratio and oxidative stress without correcting the energy deficit, and improved cell survival. MB deactivated the mitochondrial stress response pathways, the unfolding protein response and mitophagy. In conclusion, preserving mitochondrial structure and function alleviates retinal photoreceptor degeneration in mitochondrial complex I defect.

1. Introduction

The energy necessary for retinal function originates mostly from mitochondrial oxidative phosphorylation (OXPHOS) in which the transport of electrons from respiratory substrates through the electron transport chain (ETC) complexes is coupled with the generation of the inner membrane proton motive force used to generate ATP. As it transfers electrons from NADH to ubiquinone, complex I is the major NADH consumer and NAD^+ generator. Inherited OXPHOS deficiencies cause a large spectrum of human primary mitochondrial diseases of which 30–40% are caused by a complex I defect [1]. An ocular phenotype occurs in approximately 50% of OXPHOS defects in human subjects [2,3]. While missense mutations of mtDNA complex I genes cause retinal ganglion cell death in Leber hereditary optic neuropathy, a disease of the inner retina [4–6], the damage of the outer retina caused by mitochondrial defects has been reported as a rare condition [6]. However, mitochondria are present at the highest density in all outer retinal layers including retinal pigment, photoreceptor [7,8], and Muller glial cells [8], raising the possibility that a decrease in oxidative metabolism is a major pathogenic factor for outer retinal disorders [6].

The Harlequin (Hq) mouse is a model of neuronal degeneration [9] induced by an ecotropic proviral insertion in the intron 1 of the gene encoding Apoptosis Inducing Factor (AIF) leading to decreased AIF protein expression. AIF is a mitochondrial intermembrane space protein [10] that is loosely associated with the inner membrane [11], which promotes apoptosis when translocated to the nucleus [10]. AIF also has cellular functions that are independent from its role in the execution of apoptosis [12–14]. Interestingly, AIF deficiency decreases mitochondrial oxidative phosphorylation (OXPHOS) rates due to a reduced amount of fully assembled complex I [15]. AIF maintains the integrity and mitochondrial import of CHCHD4.1 (Coiled-coil-helix-coiled-coil-helix domain containing 4.1, the human equivalent of the yeast mitochondrial intermembrane space import and assembly protein 40, Mia40/Tim40) that catalyzes oxidative folding and import of OXPHOS

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protein subunits [16]. Therefore, AIF deficiency causes a posttranslational downregulation of OXPHOS complexes including complex I [1,16–19]. Mice with either a systemic hypomorphic AIF mutation (Hq mice) [9] or tissue-specific AIF knockout [17,18] develop a neuromuscular and retinal mitochondrial cytopathy. In humans, AIF mutations also manifest as familial X-linked mitochondriopathies [20–22]. While retinal ganglion neurons are reported sensitive to the AIF-induced complex I defect [9], its impact on retinal photoreceptors has not been studied.

There is currently no proven treatment to prevent or reverse the retinal degeneration induced by mitochondrial complex I defects. Although oxidative stress is considered a key pathogenic factor for organ damage, antioxidants have shown only modest protective effects in vivo [23,24]. Parallel pathways for electron transport may be induced in mitochondria, and are reported to rescue mitochondrial function in diseases induced by OXPHOS deficiencies. For example, treatment with the coenzyme Q10 derivative idebenone, that shuttles electrons from complex I to complex III, demonstrated promising results in human subjects [25]. A natural homolog of vitamin K rescued pink1 deficient mitochondria—a model of Parkinson's disease—due to its ability to shuttle electrons from complexes I and II to III [26]. The redox compound methylene blue (MB) is reduced by flavin-dependent enzymes (i.e., complex I) to MBH2 whereas cytochrome c is reported to reoxidize MBH2 to MB [27]. Its low redox potential (11 mV) would allow MB to receive electrons from either FMN or Fe-S centers in complex I, and facilitate NADH oxidation. MB is a FDA-approved pharmacological drug that has been used to treat various ailments for more than a century. Chronic administration of low-dose MB enhances memory [28], is neuroprotective against retinal optic neuropathy induced by rotenone-induced complex I inhibition [29], and alleviates cardiac arrest-induced brain damage [30] and neuron loss [31]. It is reported that MB is neuroprotective by normalizing ATP production and decreasing ROS generation [27].

The goals of our study were to establish the value of the Hq mouse in studying complex I-induced degeneration of the outer retina, and determine the therapeutic benefit of a mitochondrial redox compound in protecting the integrity of mitochondria and outer retinal photoreceptor cells. We found that MB prevented the reduction in retinal thickness and the decrease in protein markers for photoreceptor outer segments, Muller and ganglion cells, and preserved mitochondrial integrity and function. In rotenone-induced complex I deficient 661 W cells (immortalized mouse photoreceptor cell line) MB improved cell survival, normalized the NAD^+/NADH ratio and decreased oxidative stress without correcting the energy deficit. MB deactivated the mitochondrial stress response pathways, unfolding protein response and mitophagy.

2. Material and methods

2.1. Animals

Animal experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 2011), and approved by Central Michigan University Institutional Animal Care and Use Committees. Hemizygous (Hq/Y) males were obtained by mating Hq/X female with Hq/Y males. These mice and their appropriate wild-type controls were obtained from Jackson Laboratory. Mice were housed with a 12-h light-dark cycle, and have free access to food and water. Baldness was assessed as the percentage of the body surface area without hair, and considered a hallmark of the Hq phenotype. To confirm, male mice were genotyped on tail samples as described [1], and phenotyped by determining the AIF protein in retinal homogenates by western blot analyses.

MB shows opposite effects at low versus high doses [32]. Low MB concentrations favor reduction, whereas at higher concentrations MB may “re-route” electrons away from the electron transport chain thus acting as a mitochondrial uncoupler and disrupting the redox balance [33]. For this study MB was administered in drinking water after weaning. Water intake was monitored weekly in order to adjust the MB concentration in drinking water and provide a concentration of MB of 10 mg/kg/day. Our dose selection was based on the reported dose of 1–10 mg/kg in acute intraperitoneal administration that improved memory in mice [34,35]. In our hands, the oral dose of 10 mg/kg improved heart function in a murine model of diabetes [36].

Mice were sacrificed at 11 months of age, and retina were harvested for either immediate or future use (frozen at −80 °C).

2.2. Retinal thickness

Retinas were harvested, fixed and sectioned at 12 μm on a cryostat as described [37]. Formalin-fixed paraffin retinal sections were stained with toluidine blue, and visualized with light microscopy for morphometric studies [38]. Multiple images were taken from the mid-retina and four additional locations (both sides of the optic nerve) at 4×, and the thickness (from the top of the inner nuclear layer to the external side of the pigmental layer) was assessed using a Retiga camera attached to a Nikon Biophot light microscope with Qcapture software (QImaging, Burbay, BC, Canada). Retinal thickness was measured using OpenLab software (Improvision, Lexington, MA). Representative images are shown from the same region, and the average of the five measurements was used for comparison between individual animals.

2.3. Confocal microscopy

Retina cryosections were fixed with pre-cooled acetone, air dried at room temperature, rinsed with 10 mM phosphate buffer, blocked in PBS with 10% FBS, and incubated with primary antibody (dilution 1:500 in PBS with 0.5% BSA) overnight at 4 °C. Retinal sections were probed with fluorescent secondary antibodies (Thermo Fisher) (dilution 10 μg/mL). After washing, slides were air dried, mounted with DAPI (Vector), and examined with a fluorescent microscope. The thickness of the retinal nuclear layers was assessed by measuring the corrected total cell fluorescence (CTCF) as described [39] using Image J (http://rsbweb.nih.gov/ij/download.html).

2.4. Electron microscopy

Posterior eyecups were harvested directly into triple aldehyde-DMSO, sequentially exposed to ferrocyanide-reduced osmium tetroxide and acidified uranyl acetate, dehydrated in ascending concentrations of ethanol, and passed through propylene oxide before being embedded in Poly/Bed resin (Polysciences Inc., 21844-1). Acidified uranyl acetate was used to stain thin sections before examination using a JEOL 1200EX electron microscope. All EM images were independently analyzed, and observed in a blind fashion at the Electron Microscopy Core Facility, Case Western Reserve University. Quantification of the outer segment thickness and damaged mitochondria was performed on EM photographs.

2.5. Cell culture

The photoreceptor-like cells (661 W) were generously provided by Dr. Al-Ubaidi Muayyad from University of Houston, and suspended in growth media conditions as described [40]. Depending upon the experiment, cells were grown in either 35-or 100-mm cell culture dishes, or 96-well culture plates. On attaining confluence, these cells were divided into three experimental groups; control cells, cells treated with rotenone (20 μM), and cells incubated with both rotenone and methylene blue (0-30μM) for 24 h. Cells are harvested and used in various experiments discussed below.