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Research report

Bipolar cell reduction precedes retinal ganglion neuron loss in a complex 1 knockout mouse model

Lanying Song [a], Alfred Yu [a], Karl Murray [b,c], Gino Cortopassi [a,*]

[a] Vet Med: Molecular Biosciences, University of California, Davis, Davis, CA 95616, United States
[b] Center for Neuroscience, University of California, Davis, Davis, CA 95616, United States
[c] Department of Psychiatry & Behavioral Sciences, University of California, Davis, Davis, CA 95616, United States

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ABSTRACT

Inherited mitochondrial complex 1 deficiency causes Leber's hereditary Optic Neuropathy (LHON) and retinal ganglion cell (RGC) degeneration, and optic neuropathies are common in many inherited mitochondrial diseases. How mitochondrial defects pathomechanistically trigger optic neuropathy remains unclear. We observe that complex 1-deficient Ndufs4−/− mice present with acute vision loss around p30, and this vision loss is coincident with an 'inflammatory wave'. In order to understand what causes the inflammatory wave we explored retinal pathology that occurs from p20-p30. The results indicated that in the period p20-p30 in Ndufs4−/− retinas, there is: significant reduction in bipolar cells, RGC dendritic atrophy, reduced PSD95, increased oxidative stress as manifested by increased 4HNE, HO1 and Cuzn-SOD, increased mitochondrial biogenesis and increased apoptosis. These precede the major induction of 'inflammatory wave' at p30 shown previously, but occur earlier than frank RGC loss at p42. In general, complex 1 deficiency in retina triggers oxidative stress and mitochondrial respiratory dysfunction that causes death of the most sensitive cells, including bipolar cells and their synaptic contacts and amacrine cells in the early period, 20–24 days. The early death of these cells is the likely precursor to the sharp rise in inflammatory molecules that occurs at day 30 and coincides with vision loss, and greatly precedes the death of RGCs that occurs at p42. These data suggest that metabolic antioxidant support of the most sensitive cells in the retina, or anti-inflammatory suppression of the consequences of their death, are both rational strategies for mitochondrial blinding disease.

1. Introduction

Inherited mitochondrial deficiency causes optic neuropathy in LHON and Autosomal Dominant Optic Atrophy (ADOA) (Carelli et al., 2009). This suggests that there is some primary connection between mitochondrial dysfunction and the degeneration of retinal ganglion cells (RGCs)/optic nerve. Multiple pathomechanisms based on cell models have been suggested for how mitochondrial deficiency ultimately leads to RGC death, and include bioenergetics, excitotoxicity, and apoptosis (Danielson, Wong et al., 2002; Wong et al., 2002; Floreani et al., 2005). However, support in the context of the living vertebrate animal has awaited a construction of complex 1 deficient animal to study the pathomechanism (Kruse et al., 2008; Lin et al., 2012).

Here, we explore the role of mitochondrial complex 1 deficiency in retinal neurodegeneration using the Ndufs4 knockout mouse model. We observed retinal bipolar cell loss and post synaptic deficits that occur in days 20–30, preceding the inflammatory wave at p30 we described recently and retinal ganglion neuron loss that occurs at p42 in Ndufs4−/− retinas (Yu et al., 2015). These results support a novel pathomechanism for mitochondrial blinding disease: that mitochondrial complex 1 deficiency and oxidative stress targets a subset of sensitive cells, bipolar and amacrine cells, whose death triggers an 'inflammatory wave', which decreases RGC function, and ultimately triggers RGC death. These data suggest that metabolic antioxidant support of the most sensitive cells in the retina, or anti-inflammatory suppression of the consequences of their death, are both rational strategies for mitochondrial blinding disease.

Abbreviations: LHON, Leber's hereditary Optic Neuropathy; RGC, retinal ganglion cell; ADOA, Autosomal Dominant Optic Atrophy; OCR, oxygen consumption rates; SBACs, Starburst Amacrine Cells.

* Corresponding author at: UC Davis, Veterinary Medicine: Molecular Biosciences, 1089 Veterinary Medicine Drive VM3B 3007, Davis, CA 95616, United States.

E-mail address: gcortopassi@ucdavis.edu (G. Cortopassi).

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2. Experimental procedures

2.1. Generation of Ndufs4 knockout mice

Ndufs4 knockout mice are a gift from Richard D. Palmiter's lab (Kruse et al., 2008). To acquire Ndufs4−/− mice, we bred male Ndufs4+/− mice with female Ndufs4+/− mice. C57BL/6J mice were crossed with Ndufs4+/− mice for colony maintenance. All experiments were performed according to a protocol approved by the University of California, Davis Institutional Animal Care and Use Committee.

2.2. Immunostaining, TUNEL staining and flat-mount staining

Mice were sacrificed by cervical dislocation, eyes were fixed in 4% PFA overnight at 4 °C, then put into 30% sucrose, and embedded in OCT. For p20-p21, retinas of 8 males (4WT and 4 KO) and 5 females (3WT and 2KO) were used. For p30-32, there were 5 males (3WT and 2 KO) and 3 females (1WT and 2 KO). 20 μm thick sagittal sections were cut on a cryostat until the appearance of the optic nerve. For immunofluorescence studies, primary antibodies included Brn3a (1:200, EMD Millipore MB 5945), PSD95 (1:200, Thermo scientific MA1-046) and PKC-α (1:200, Cell signaling #59754) and β3-tubulin (1:200, Cell signaling #566). Sections were permeabilized with 0.2% Tween in PBS and incubated in primary antibodies overnight at 4 °C. Alexa488 and Alexa594 fluorescence-conjugated secondary antibodies (Invitrogen) were used. Samples were examined with Nikon Eclipse 80i fluorescent microscope. Retinas localized in the same distance from the optic nerve were selected for observing alteration of biomarkers including PKC-α, Brn3a, β3-tubulin and PSD95. The positive cell numbers of PKC-α, Brn3a, β3-tubulin and Dapi in inner nuclear layer and retinal ganglion cell layer, were counted by Nikon NIS-elements imaging software. The positive signal intensity of PSD95 was measured by Nikon NIS-elements imaging software in outer plexiform layer. For each retina, 6–9 images were collected from 3 evenly spaced sections. Non-specific background signals were subtracted from marker-specific positive signal intensity in the same section. The values of nasal and temporal portions were pooled together each section.

DeadEnd Fluorometric TUNEL System (Promega) was used to detect apoptosis in retinal section with optic nerve. The apoptotic cells were detected with an anti-Digoxigenin primary antibody and a fluorescent secondary antibody, and total nuclei was visualized with Dapi staining. Apoptotic rate was calculated as the number of apoptotic cells divided by the total number of Dapi nuclei. Equal numbers of images per age group and condition were analyzed.

For NF-H flat-mount staining, whole retinas were fixed in 4% PFA for 2 h and permeabilized with 0.5% Tween in PBS for 1 h, blocked with 5% FBS at room temperature for 1 h, then incubated with mouse anti-NF-H (1:1000, Cell Signaling #2836) at 4 °C for 48 h. Retinas were washed three times with 0.1% Tween in PBS for 3 h, and incubated with anti-mouse Alexa488 fluorescence-conjugated secondary antibodies for 48 h. Retinas were washed again three times for 3 h, and mounted in Vectashield mounting medium (Vector Laboratories). Nikon NIS-elements imaging software was used to automatically measure cell body area, dendritic field and axon diameter in the RGCs according to previous publication (Leung et al., 2011). To avoid observation bias, NF-H positive RGCs were blindly measured by two different persons, and only non- overlapping NF-H positive cells with clear dendrites were counted.

2.3. Western-blot, QRT-PCR and quantitative mitochondrial DNA

For Western-blot analysis, primary antibodies included anti-Ndufs4 (1:1000 Santa Cluz, SC-100567), Brn3a (1:1000, EMD Millipore MB5945), syntaxin-1(1:1000 EMD Millipore MB 5820), PSD95 (1:1000, Thermo scientific MA1-046), cleaved-caspase3 (1:500, Cell signaling #9664), SYT-1 (1:1000, Cell signaling #14558), MnSOD (1:1000, Abcam ab13533) and CuSOD (1:1000, Abcam ab13498), VDAC (1:1000, cell signaling), 4-HNE (1:500, Abcam ab48506), and complex II (SDHB) (1:1000 Abcam 21A11AE7). Retinas of 9 males and 7 females including 9 WT and 7 KO were dissected and homogenized in 2× cell lysis buffer (cell signaling), 10–20 μg of proteins were analyzed in 4–12% NuPAGE Bis-Tris gel (Novex). Invitrogen iBlot Gel transfer device was used to transfer proteins to nitrocellulose membranes. The membranes were incubated with primary antibodies overnight at 4 °C. Secondary antibodies were IRDye 680LT goat anti-mouse and IRDye 800CW goat anti-rabbit. Odyssey LI-COR was used to scan signals.

QRT-PCR was used to assay Caspase3, Brn3a and HO1 mRNA level in retinas. Retinas of 9 male and 7 female mice were used. Primers were Caspase3 forward, 5′-ATGGAGAACAACAAAACCTCAGT-3′, reverse, 5′-TTGCTCCCATGTATGGTCTTTAC-3′, Brn3a forward, 5′-CGGACTTTGCGAGTGTTTTGT-3′, reverse, 5′-GTGGGATGCATGG CAAAGTG-3′, and HO1 forward, 5′-GCATGCCCCAGGATTTGTC-3′, reverse, 5′-CTGACCCTTCTGAAAGTTCTTCATG-3′. Reactions were carried out in triplicate in 10 μl volume on Lightcycler 480 (Roche). Melting curve and agarose-gel electrophoresis were used to evaluate specificity of each reaction. The mRNA level of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used to normalize the mRNA levels of Caspase3, Brn3a and HO1 using ΔC_t method (Song et al., 2012).

For mouse mitochondrial copy number analysis, retinas of 10 male and 6 female mice including 9 WT and 7 KO were used to extract genomic DNA. DNA preparations (also containing mtDNA) were quantitatively analyzed through PCR on Lightcycler 480. The primers were ND1 forward, 5′-CAT GAT CTA GGA GGC TGC TGA CCT C-3′, reverse, 5′-CGT TTA CCT TCT ATA AGG CTA TGA-3′, and CFTR forward, 5′-ATG CAG CCT TTG GTG AAA CAG-3′, reverse, 5′-CTG TGA CAC GTG TGC TTT CAG-3′.

2.4. Complex1 activity assay and Seahorse analysis

Retinas of 3 male Ndufs4 KO and 3 male littermate controls were dissected and homogenized in mitochondria isolation buffer (ab110168, Abcam) using pestle A and pestle B. BCA method was used to measure homogenate protein concentration. Following complex 1 enzyme activity microplate assay kit (Abcam, ab109721) protocol, 40 μg homogenate was put in incubation solution and added microplate wells. Microplates were incubated for 3 h at room temperature. 200 μl assay solution was added to each well, POLAR star Omega fluorescence plate reader was used to measure absorbance for 30 min at 450 nm.

For Seahorse analysis, 2 male Ndufs4KO and 2 male littermate control retinas were dissected and homogenized in mitochondria solution buffer using 15 ml pestle A and pestle B, BCA method were used to measure homogenate protein concentration. Mitochondrial respiratory function in Ndufs4−/− retinas was measured by Seahorse XF24 analyzer. Briefly, the XF sensor cartridge injection ports with ADP (A), Oligomycin (B), FCCP(C), Rotenone (D) were loaded, homogenate were diluted in 1× MAS3 to yield a final concentration of 40 μg protein/ml, 50 μl of diluted homogenate was transfered into each well of a V7 XF24 tissue culture plate, 450 μl of 1.1× initial media conditions with pyruvate and malate was added. Plate measurements we made according to instructions on the instrument controller (Song et al., 2012).

2.5. Data analysis

Comparisons for differences in means were assessed by GraphPadPrism5. Statistical significance was determined using two sample independent *T*-Test. A single asterisk indicates