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Intraocular Pressure Elevation Induces Mitochondrial Fission and Triggers OPA1 Release in Glaucomatous Optic Nerve

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Abstract

Purpose—To determine whether intraocular pressure (IOP) elevation triggers mitochondrial fission and ultrastructural changes and alters optic atrophy type 1 (OPA1) expression and distribution in the optic nerve (ON) of glaucomatous DBA/2J mice.

Methods—IOP in the eyes of DBA/2J mice was measured and mitochondrial structural changes were assessed by conventional EM and EM tomography. Cytochrome c oxidase IV subunit 1 (COX), OPA1 and Dnm1, a rat homologue of dynamin-related protein-1, mRNA were measured by Taqman qPCR. COX and OPA1 protein distribution was assessed by immunocytochemistry and Western blot.

Results—Excavation of the optic nerve head (ONH), axon loss, and COX reduction were evident in 10 month-old glaucomatous ONH of eyes with >20 mmHg IOP elevation. EM analysis showed mitochondrial fission, matrix swelling, substantially reduced cristae volume, and abnormal cristae depletion in 10 month-old glaucomatous ONH axons. The mean length of mitochondrial cross section in these axons decreased from 916.6 ± 768.4 nm in 3 month-old mice to 582.87 ± 303.3 nm in 10 month-old glaucomatous mice ($P < 0.001$). Moderate reductions of COX mRNA were observed in the 10 month-old DBA/2J mice optic nerve heads. Larger reductions of OPA1 immunoreactivity and gene expression were coupled with larger increases of Dnm1 gene expression in 10 month-old glaucomatous ONH. Subcellular fractionation analysis indicates increased release of both OPA1 and cytochrome c from mitochondria in 10 month-old glaucomatous ONs.

Conclusions—IOP elevation may directly damage mitochondria in the ONH axons by promoting reduction of COX, mitochondrial fission and cristae depletion, alterations of OPA1 and Dnm1 expression, and induction of OPA1 release. Thus, interventions to preserve mitochondria may be useful for protecting ON degeneration in glaucoma.

Introduction

Elevated intraocular pressure (IOP) is an important risk factor for optic nerve damage in glaucoma.¹ However, the precise pathophysiological relationships among elevated IOP, glaucomatous optic nerve (ON) damage, and retinal ganglion cell (RGC) death are poorly understood. Mitochondrial changes have been identified in association with neuronal death in other models of central nervous system disease.^{2,3} In addition, there is evidence of abnormal

mitochondrial respiration in patients with glaucoma.⁴ Recently, we found that moderately elevated hydrostatic pressure can induce abnormal cristae depletion, cytochrome c release, cellular ATP reduction and translocation of dynamin-related protein 1 (Drp-1) in differentiated RGC-5 cells.⁵ Further, we also found that elevated hydrostatic pressure triggers release of optic atrophy type 1 protein (OPA1) and cytochrome c, and induces subsequent apoptotic cell death in differentiated RGC-5 cells.⁶ These observations raise the possibility that pressure-induced mitochondrial dysfunction contributes to RGC death and ON degeneration in glaucoma.

In healthy cells, mitochondria are autonomous and morphologically dynamic organelles that structurally reflect a precise balance of ongoing fission and fusion within a cell.^{7–9} This balance is regulated by a family of dynamin-related GTPases that exert opposing effects. OPA1, the human ortholog of Mgm1p/Msp1p, and the mitofusins are required for mitochondria fusion. Dynamin-related protein-1 (Drp-1) regulates mitochondrial fission.^{8,10} Mutations in OPA1, a dynamin-related GTPase which is involved in various processes related to mitochondrial inner membrane structural dynamics, are linked with neurodegenerative disease in human and cause autosomal dominant optic atrophy (ADOA), the most common form of hereditary optic neuropathy.^{11,12}

OPA1 is expressed in the soma and axons of the RGCs as well as horizontal cells.^{13–16} However, the specific functional roles of OPA1 in these cells remain unknown. Emerging evidence suggests that downregulation of OPA1 causes mitochondrial fission, leading to cytochrome c release and apoptosis in HeLa cells, as well as induces aggregation of the mitochondrial network in purified RGCs.^{17–20} Proteolytic processing of OPA1 has been observed during mitochondrial fission, although its significance is unclear.^{21–24} Also, OPA1 release during mitochondrial fission contributes to apoptotic cell death.^{19,23} Nevertheless, it is unknown whether IOP elevation can alter OPA1 expression and distribution in the ON degeneration of glaucoma.

To address these issues in an *in vivo* model of glaucoma, we evaluated whether IOP elevation triggers mitochondrial fission and ultrastructural changes and alters OPA1 expression and distribution in the ON of DBA/2J mice, an extensively characterized strain that spontaneously develops elevated IOP.^{25–33}

Materials and Methods

Chemicals

All chemicals were from Sigma (St. Louis, MO) under otherwise noted.

Animals

All procedures concerning animals were in accordance with the statement of the Association for Research in Vision and Ophthalmology for the use of animals in research. Adult 3, 6, 7–8, 9–10, and 12 month-old female DBA/2J mice (The Jackson Laboratory, Bar Harbor, Maine) and 3, 6, and 10 month-old female C57BL/6 mice (Harlan Sprague Dawley, Inc., Indianapolis, IN) were housed in covered cages, fed with a standard rodent diet *ad libitum*, and kept on a 12-h light/12-h dark cycle.

IOP Measurement

IOP measurement was performed as described previously.^{25,34} Each of the 10 and 12 month-old DBA/2J mice used in this study had a single IOP measurement per month starting at 6 months of age (to confirm development of spontaneous IOP elevation exceeding 20 mmHg). The glaucomatous DBA/2J mice that have confirmed IOP elevation were obtained in 65.3% (64/98) at 10 months of age. Also, each of the 3, 6 and 10 month-old non-glaucomatous C57BL/

6 mice used in this study had a single IOP measurement. After anesthesia with a mixture of ketamine (100 mg/kg, Ketaset; Fort Dodge Animal Health, Fort Dodge, IA) and xylazine (9 mg/kg, TranquiVed, Vedeco, Inc., St. Joseph, MO), a sterilized, water-filled microneedle with an external diameter of 50 to 70 μm was used to cannulate the anterior chamber. The microneedle was then repositioned to minimize corneal deformation and to ensure that the eye remained in its normal position. The microneedle was connected to a pressure transducer (Blood Pressure Transducer; WPI), which relayed its signal to a bridge amplifier (Quad Bridge; AD Instruments [ADI], Castle Hill, New South Wales, Australia). The amplifier was connected to an analog-to-digital converter (Power Laboratory; ADI) and a computer (G4 Macintosh; Apple Computer Inc., Cupertino, CA).

Tissue Preparations

Light-adapted mice as described above were anesthetized with isoflurane and killed by an i.p. injection of ketamine and xylazine. The ONs were dissected from the choroid and fixed with 4% paraformaldehyde in 0.1M phosphate buffer (PB, pH 7.4) for 2 hr at 4°C. After several washes in PB, ONs were dehydrated through graded ethanols and then embedded in a polyester wax as described previously.¹⁵ For Western blot analyses, whole ONs were immediately used or frozen in liquid nitrogen and stored at -70°C until use.

Immunohistochemical Analyses

Immunohistochemical staining of 7 μm wax sections of full thickness ON was done by the immuno-fluorescent method as previously described.¹⁵ Five sections per wax block from each age group (n=3 mice/group) were used for immunohistochemical analysis. Primary antibodies were mouse monoclonal antibody against COX (1:500, Molecular Probes, Eugene, OR) and polyclonal rabbit anti-mOPA1 antibody (1:1000, a gift of Drs. Misaka and Kubo).³⁵ Polyclonal rabbit anti-mOPA1 antibody was directed against amino acids 938–960 of mouse OPA1 protein was generated and peptide affinity-purified as previously described.^{15,35} To prevent non-specific background, tissues were incubated with 1% bovine serum albumin/PBS for 1 hour at room temperature and then with the primary antibody against COX or OPA1 for 16 hours at 4°C. After several wash steps, the tissue was incubated with the secondary antibodies, peroxidase-conjugated goat anti-mouse IgG or goat anti-rabbit IgG (1:100, Molecular Probes) for 4 hours at 4°C and then washed with PBS. The sections were counterstained with the nucleic acid stain Hoechst 33342 (1 $\mu\text{g}/\text{ml}$, Molecular Probes) in PBS.

Images were captured under fluorescence microscopy using a Nikon ECLIPSE microscope (E800; Nikon Instruments Inc., Melville, NY) equipped with digital camera (SPOT; Diagnostic Instrument, Sterling Heights, MI). Image exposures were the same for all tissue sections and were acquired using Simple PCI version 6.0 software (Compix Inc., Cranberry Township, PA).

Electron Microscopy

For conventional EM, two eyes from each group (n=2 mice) were fixed via cardiac perfusion with solution at 37°C in 2% paraformaldehyde, 2.5% glutaraldehyde (Ted Pella, Redding, CA) in 0.15 M sodium cacodylate (pH 7.4) and placed in pre-cooled fixative on ice for 1 hour. The following procedure was used to optimize mitochondrial structural preservation and membrane contrast.^{5,36,37} The ONHs were dissected with 0.15 M sodium cacodylate plus 3 mM calcium chloride (pH 7.4) on ice and then post-fixed with 1% osmium tetroxide, 0.8% potassium ferrocyanide, 3 mM calcium chloride in 0.1M sodium cacodylate (pH 7.4) for 1 hour, washed with ice-cold distilled water, poststained with 2% uranyl acetate at 4°C, dehydrated through graded ethanols, and embedded in Durcupan resin (Fluka, St. Louis, MO). Ultrathin (70 nm) sections were post-stained with uranyl acetate and lead salts prior to imaging using a JEOL 1200FX transmission EM operated at 80kV. The negatives were digitized at 1800 dpi using a Nikon CoolScan system, giving an image size of 4033 $\times$ 6010 pixel array and a pixel resolution

of 1.77 nm.^{38–40} The lengths of mitochondrial cross-sections at the longest extent were measured in the unmyelinated ONH as described previously.⁵ For unbiased sampling, all of the mitochondria in an image were measured.

Electron Microscope Tomography

Sections of prelaminar unmyelinated ONH axons from each group were cut at thicknesses of 400–500 nm. Sections were then stained 30 min in 2% aqueous uranyl acetate, followed by 15 minutes in lead salts. Fiducial cues consisting of 15 nm colloidal gold particles were deposited on opposite sides of the section. For each reconstruction, a series of images at regular tilt increments was collected with a JEOL 4000EX intermediate-voltage EM operated at 400 kV. The specimens were irradiated before initiating a tilt series in order to reduce anisotropic specimen thinning during image collection. Tilt series were recorded on film at 20,000 magnification with an angular increment of 2° from –60° to +60° about an axis perpendicular to the optical axis of the microscope using a computer-controlled goniometer to increment accurately the angular steps. The illumination was held to near parallel beam conditions and optical density maintained constant by varying the exposure time. The negatives were digitized with a Nikon CoolScan at 1800 dpi producing images of size 4033 × 6010 pixels. The pixel resolution was 0.7 nm. The IMOD package was used for rough alignment. Briefly, we tracked fiducial gold particles across all images of the tilt series that had been roughly aligned using cross-correlation.⁴¹ Fine alignment and volume reconstruction were performed using the TxBR package.⁴² This package removes image distortions by generating a global non-linear model of electron trajectories and then back projecting along these trajectories to build up the volume. Volume segmentation was performed by manual tracing in the planes of highest resolution with the program Xvoxtrace. The mitochondrial reconstructions were visualized using Analyze (Mayo Foundation, Rochester, MN) or the surface-rendering graphics of Synu (National Center for Microscopy and Imaging Research, La Jolla, CA) as described by Perkins et al.³⁹ These programs allow one to step through slices of the reconstruction in any orientation and to track or model features of interest in three dimensions. Movies of the tomographic volume were constructed using Amira (Visage Imaging, Inc., Carlsbad, CA).

Taqman Quantitative PCR

Eight ONHs (extending 0.25mm posteriorly from retina surface) were dissected from the sclera of four 3 month-old DBA/2J mice and four 10 month-old glaucomatous DBA/2J mice, as well as four of 10 month-old non-glaucomatous C57BL/6 mice. The tissues were stored in RNA-later (Ambion Inc, Austin, TX) at –20°C. Total RNA of pooled ONH from each group was extracted with Trizol (Invitrogen, Carlsbad, CA), purified on RNeasy mini columns (Qiagen, Valencia, CA), and treated with RNase-free DNase I (Qiagen). The RNA purity was verified by confirming that the OD260nm/280nm absorption ratio exceeded 1.9. cDNA was synthesized using SuperScript II first-strand RT-PCR kit (Invitrogen). COX, OPA1, and Dnm1 gene expression were measured by qPCR (MX3000P, Stratagene, La Jolla, CA) using 25 ng of cDNA from ONHs and 2X Taqman universal PCR master mix (Applied Biosystems, Foster City, CA) with a one-step program (95°C for 10 minutes, 95°C for 30 seconds, and 60°C for 1 minute for 50 cycles). Primers for COX, OPA1, Dnm1 and GAPDH, as well as Taqman probe for GAPDH were designed using Primer Express 2.0 software (Applied Biosystems), obtained from Biosearch Technologies (Novato, CA) (Table 1). The probes for COX, OPA1 and Dnm1 were obtained from the Roche Universal Probe Library (Roche Diagnostics, Mannheim, Germany, Table 1), and the optimal concentrations for probe and primers were determined using heart tissue. Standard curves were constructed using nine 2-fold dilutions (50ng–0.195ng) for both the targets (COX, OPA1 and Dnm1) and the endogenous reference (GAPDH). The samples were run in triplicate for each target and endogenous GAPDH control.

Western Blot Analysis

Six whole ONs (extending from ONH to optic chiasmatic nucleus) were dissected from the sclera of three 3 month-old DBA/2J mice, 8 month-old glaucomatous DBA/2J mice and 10 month-old glaucomatous DBA/2J mice. Tissues are then immediately homogenized in a glass-teflon Potter homogenizer in lysis buffer (20 mM Hepes, pH 7.5/10 mM KCl/1.5 mM $MgCl_2$ /1 mM EDTA/1 mM EGTA/1 mM DTT/0.5% CHAPS/complete protease inhibitors; Roche Biochemicals, Indianapolis, IN). Ten microgram of pooled samples from each group were separated by PAGE and electro-transferred to PVDF membranes. The membrane was blocked with 5% nonfat dry milk/0.05% Tween-20/PBS, incubated with monoclonal mouse anti-OPA1 antibody (H-300/1:1000; BD Transduction Laboratories, San Diego, CA) or monoclonal mouse anti-actin antibody (Ab-1/1:3,000; Calbiochem, La Jolla, CA), rinsed with 0.05% Tween-20/PBS, incubated with peroxidase-conjugated goat anti-mouse IgG (1:2000; Bio-Rad, Hercules, CA) or goat anti-rabbit IgM (1:5000; Calbiochem), and developed using chemiluminescence detection (ECL Plus, GE Healthcare Bio-Sciences, Piscataway, NJ). Images were analyzed by digital fluorescence imager (Storm 860; GE Healthcare Bio-Sciences) and band densities were normalized using actin as cytosolic fraction calibrator and VDAC as mitochondrial fraction calibrator with ImageQuant TL (GE Healthcare Bio-Sciences).

To assess the subcellular distribution of OPA1, the cytosolic and mitochondrial fractions were isolated from freshly isolated ONs by differential centrifugation (Mitochondrial Isolation Kit, Pierce, Rockford, IL). Briefly, the tissues were immediately homogenized in a glass-teflon Potter homogenizer in Reagent A, mixed with an equal volume of Reagent C, and then centrifuged at $700 \times g$ for 10 minutes at $4^\circ C$. For the cytosolic fraction, the supernatant was centrifuged at $12,000 \times g$ for 15 minutes at $4^\circ C$ and the supernatant were collected as cytosolic fraction. For the mitochondrial fraction, the mitochondrial pellet was lysed with 2% CHAPS in Tris buffered saline, centrifuged at $12,000 \times g$ for 15 minutes at $4^\circ C$ and the supernatant was collected. Western blot analysis was performed as above. Equal loading was confirmed by reprobing cytosolic fraction samples with actin as above, and the mitochondrial fraction samples with polyclonal rabbit anti-VDAC antibody (Ab-5/1:1000, Calbiochem). Band densities were normalized using actin as cytosolic fraction calibrator and VDAC as mitochondrial fraction calibrator with ImageQuant TL (GE Healthcare Bio-Sciences). Error bar represents the standard deviation ($P < 0.05$ by Student's t-test, $n=3$ for both 3 and 10 month-old mice). Good separation of the cytosolic and mitochondrial fractions was confirmed by the observation of negligible staining when cytosolic fraction blots were reprobbed with antibodies to VDAC and when mitochondrial fraction blots were reprobbed with antibodies to actin (data not shown).

Statistical Analysis

Experiments presented were repeated at least three times with triplicate samples. The data are presented as the mean $\pm$ SD. Comparison of two experimental conditions was evaluated using the unpaired Student's t-test. $P < 0.05$ was considered to be statistically significant.

Results

IOP Elevation Induces Mitochondrial Fission and Cristae Depletion in the ONH

Mean IOP was 15 ± 1.8 mmHg (SD) in 3 month-old DBA/2J mice. Spontaneous IOP elevation typically began by 6–8 months. The peak of IOP elevation was 21.5 ± 4.5 mmHg in the right eyes and 19.9 ± 3.7 mmHg in the left eyes within 10 month-old DBA/2J mice (Fig. 1A and B). In contrast, mean IOP was 14.7 ± 1.7 mmHg in the right eyes and 14.2 ± 1.8 mmHg in the left eyes within 10 month-old non-glaucomatous C57BL/6 mice (see Supplementary Material 1). To evaluate ON degeneration and axon loss, we analyzed cross and longitudinal sections

stained with toluidine blue. The ON in these mice has normal appearance at 3 months of age (Fig. 2A, C and E). As has been reported previously,^{29,43} substantial ON damage, including axon loss, was observed in 10 month-old glaucomatous DBA/2J mice (Fig. 2B, D, and F), confirming the presence of acquired optic neuropathy.

Mitochondrial distribution and structure were compared in the ONH of 3 month-old DBA/2J mice with normal IOP and 10 month-old glaucomatous DBA/2J mice with confirmed IOP elevation (>20 mmHg). As shown in Figure 3, COX immunoreactivity, a marker for mitochondria activity,^{44–46} was concentrated in the unmyelinated ONH of 3 month-old DBA/2J mice (Fig. 3A). In contrast, much less COX immunoreactivity was observed in the unmyelinated ONH of 10 month-old glaucomatous DBA/2J mice (Fig. 3B; see three more examples in Supplementary Material 2). Taqman qPCR using specific primers and probes for mouse COX showed that COX mRNA was significantly decreased by 0.81 ± 0.06 -fold in the ONHs of 10 month-old DBA/2J glaucomatous mice than in the ONH of 3 month-old DBA/2J mice ($n=8$ ONHs per pool, Fig. 3C, $P<0.05$). In addition, COX mRNA was decreased by 0.9 ± 0.04 fold in the ONHs of 10 month-old C57BL/6 mice than in the ONH of 3 month-old mice ($n=8$ ONHs per pool, Fig. 3C). There were insignificant differences in COX mRNA expression relative to GAPDH mRNA or in the ratios of GAPDH mRNA to total RNA between the 3 month-old DBA/2J mice ONHs and 10 month-old C57BL/6 mice ONHs (data not shown).

Transmission EM analysis showed that the axons of the unmyelinated ONH in 3 month-old DBA/2J mice contained classical elongated tubular mitochondria of various lengths (Fig. 3D). In contrast, 10 month-old glaucomatous DBA/2J mice unmyelinated ONH axons contained small rounded mitochondria with swollen matrices (Fig. 3E; see four more examples in Supplementary Material 3). Quantitative analysis showed that the mean length of mitochondrial cross section significantly decreased from 916.6 ± 768.4 nm in the unmyelinated ONH of 3 month-old DBA/2J mice to 582.87 ± 303.3 nm in the unmyelinated ONH of 10 month-old glaucomatous DBA/2J mice ($P<0.001$ by t-test, $n=216$ for both 3 and 10 month-old DBA/2J mice) (Fig. 3F).

To assess the internal mitochondrial structural changes, unmyelinated ONHs from 3 and 10 month-old DBA/2J mice were fixed to preserve mitochondrial morphology, and EM tomography was used to obtain 3D reconstructions showing detailed mitochondrial ultrastructure. Tomographic reconstructions from a 3 month-old sample showed an intact outer mitochondrial membrane (blue) and mostly lamellar cristae (various colors), occupying the mitochondrial matrix space (Fig. 4A b–d). By comparison, 3D tomographic volumes of mitochondria found in axons of 10 month-old glaucomatous DBA/2J mice often showed smaller, more globular mitochondria that were occasionally in close proximity (Fig. 4B a–e). Mitochondria of 10 month-old glaucomatous DBA/2J mice showed matrix swelling and substantially reduced cristae volume. Figure 4B a–b shows an example of a mitochondrion that is devoid of cristae in much of its volume and also displays matrix swelling (lighter regions). Mitochondrial fission also was suggested in the tomographic reconstructions (Fig. 4B a–e; arrowheads) because of the observation of closely apposed mitochondrial fragments.

IOP Elevation Alters OPA1 Gene and Protein Expression or Dnm1 Gene Expression in the ONH

OPA1 mutation or deficiency in mouse models causes RGC and nerve fiber layer degeneration, mitochondrial dysfunction, ON abnormalities and visual deficits.^{47,48} To test whether alteration of OPA1 protein expression occur in the ONH of glaucomatous mice, OPA1 immunohistochemistry was performed. As shown in Figure 5, substantial OPA1 immunoreactivity was present in the ONH of 3 month-old DBA/2J mice (Fig. 5A). Much less OPA1 immunoreactivity was observed in the ONH of 10 month-old glaucomatous DBA/2J mice than in the 3 month-old DBA/2J mice (Fig. 5B; see four more examples in Supplementary

Material 4). To determine whether there were differences in the expression of mRNA for OPA1 and Dnm1 (a mouse homologue of Drp-1) associated with advancing glaucomatous damages, Taqman qPCR was performed on ONH mRNA from 3 month-old DBA/2J mice and 10 month-old glaucomatous DBA/2J mice, as well as 10 month-old non-glaucomatous C57BL/6 mice. Results were normalized to GAPDH mRNA. We observed that OPA1 mRNA was significantly decreased by 0.65 ± 0.11 -fold in the ONHs of 10 month-old glaucomatous DBA/2J mice but was significantly increased by 1.46 ± 0.21 -fold in the ONHs of 10 month-old non-glaucomatous C57BL/6 mice ($n=8$ ONHs per pool, Fig. 5C, $P<0.05$). This result is consistent with increased mitochondrial fission in the 10 month-old glaucomatous DBA/2J mice described above. In contrast, Dnm1 mRNA was significantly increased by 1.56 ± 0.06 -fold in the ONHs of both 10 month-old glaucomatous DBA/2J mice and by 1.33 ± 0.06 -fold in the 10 month-old non-glaucomatous C57BL/6 mice ($n=8$ ONHs per pool, Fig. 5D, $P<0.05$). There were no differences in the ratios of GAPDH mRNA to total RNA between the 3 month-old DBA/2J mice ONHs, 10 month-old glaucomatous DBA/2J mice ONHs and 10 month-old C57BL/6 mice ONHs (data not shown).

IOP Elevation Triggers Mitochondrial OPA1 Release in the ONs

To investigate possible OPA1 translocation from mitochondria to the cytosol in the whole ONs of 8 and 10 month-old glaucomatous mice, relative changes of OPA1 concentration in cytosolic and mitochondrial fractions were measured using Western blotting. The blots were reprobed with actin and VDAC antibody to assess protein loading in the lanes containing cytosolic or mitochondrial proteins, respectively. Results were normalized to actin as cytosolic fraction calibrator and VDAC as mitochondrial fraction calibrator. As shown in Figure 6A, the OPA1 antibody recognized three major OPA1 isoforms; a 90 kDa (called the large or L form), an 80 kDa isoform (S1) and a 75 kDa isoform (S2) in the cytosolic fraction of 3 month-old DBA/2J mouse ON. Two major isoforms (L and S1) were identified in the mitochondrial fraction of 3 month-old mouse ON. In contrast, 8 and 10 month-old glaucomatous mouse ON contained at least four isoforms of OPA1 in the cytosolic fractions including a small isoform (~65 kDa:S3). Moreover, relative OPA1 content of all four isoforms was significantly increased by 3.05 ± 0.35 -fold (L), 4.15 ± 0.39 -fold (S1), 1.52 ± 0.16 -fold (S2), and 11.4 ± 1.25 -fold (S3) in the ONs of 8 month-old and by 5.06 ± 0.62 -fold (L), 9.61 ± 0.85 -fold (S1), 5.19 ± 0.53 -fold (S2), and 24.2 ± 2.23 -fold (S3) in the ONs of 10 month-old glaucomatous mice, respectively ($P<0.05$, Fig. 6A and B). Concomitantly, relative OPA1 content of both mitochondrial isoforms (L and S1) were significantly decreased by 0.60 ± 0.04 -fold (L) and 0.53 ± 0.04 -fold (S1) in the ONs of 8 and by 0.79 ± 0.06 -fold (L) and 0.76 ± 0.05 -fold (S1) in the ONs of 10 month-old glaucomatous mice, respectively ($P<0.05$, Fig. 6A and B).

Cytochrome c protein concentration was significantly increased by 1.79 ± 0.18 -fold and 2.89 ± 0.3 -fold in the cytosolic fraction of the ONs of 8 and 10 month-old glaucomatous mice, respectively ($P<0.05$, Fig. 6A and C). In contrast, the cytochrome c protein concentration was significantly decreased to 0.78 ± 0.06 -fold and 0.80 ± 0.07 -fold in the mitochondrial fraction of the ONs of 8 and 10 month-old glaucomatous mice, respectively ($P<0.05$, Fig. 6A and C).

Discussion

These results demonstrate that the ON degeneration that occurs in glaucomatous DBA/2J mice with elevated IOP induces COX reduction, mitochondrial fission and abnormal cristae depletion, alterations of OPA1 and Dnm1 expression, and induction of OPA1 release. These findings suggest that mitochondrial dysfunction contributes to the biochemical cascade leading to pressure-related RGC axon loss and ON degeneration in glaucoma.

Growing evidence indicates that mitochondrial structural and functional dynamics play an important role in cell and animal physiology. Imbalance in the control of mitochondrial fusion

and fission dramatically alters overall mitochondrial morphology.¹⁰ In addition, recent evidence suggests that excessive mitochondrial fission can lead to breakdown of the mitochondrial network, loss of mitochondrial DNA, and respiratory defects in mammalian cells.^{49–51} Previously, we reported that elevated hydrostatic pressure caused breakdown of the mitochondrial network by mitochondrial fission and induced abnormal cristae depletion and cellular ATP reduction in differentiated RGC-5 cells *in vitro*.⁵ This suggests that these cells may have bioenergetic impairment.^{5,38,40,52} The present results extend these findings to an *in vivo* model. The mean length of mitochondrial cross section significantly decreased in the unmyelinated ONH of 10 month-old glaucomatous mice, suggesting mitochondrial fission. In addition, EM tomography analysis showed matrix swelling, substantially reduced cristae volume, including regions devoid of cristae, and provides even stronger evidence for mitochondrial fission by allowing much more of the volume to be visualized than afforded by conventional EM. The depletion of cristae membranes is consistent with our finding of reduced COX expression in the ONH of 10 month-old glaucomatous mice. Further, the finding that Dnm1 expression had increased is reflected in the increased mitochondrial fission observed. These alterations to the 3D structure observed in 10 month-old glaucomatous mice mitochondria argue for reduced ATP generation and general mitochondrial dysfunction. Recent studies suggest that mitochondrial distribution in the ONH reflects differing energy requirements of the unmyelinated axons in comparison to the myelinated retrolaminar axons, ie. the unmyelinated portion of the ON may have greater demands for mitochondrially derived ATP than the myelinated posterior nerve.^{53–55} Also, deficiency in mitochondrially derived ATP triggers RGC death in Leber's hereditary optic neuropathy.⁵⁶ Together with these findings, our observations suggest mitochondrial dysfunction in the ONH is important during the onset of glaucomatous optic neuropathy.

In the present study, IOP elevation significantly decreased OPA1 mRNA and protein expression in the ONH of 10 month-old glaucomatous mice. Recent studies demonstrated that OPA1 protein is present in the ONH of the rat and human as well as in the RGCs of the mouse, rat and human retina,^{13–16} that downregulation of OPA1 causes aggregation of the mitochondrial network in purified RGCs,²⁰ and that these changes are linked to mitochondrial fission, mitochondrial cristae depletion and bioenergetic impairment.^{17,18,23} Because OPA1 deficiency in mouse models of ADOA impairs mitochondrial morphology, ON structure, and visual function,^{47,48} the observed reduction of OPA1 gene and protein expression in the ONH of 10 month-old glaucomatous mice with IOP elevation appears to be reflected in the structural and functional changes of mitochondria and may facilitate ONH axon loss. Further support for this idea comes from studies showing that increased OPA1 expression protects cells from apoptosis by preventing cytochrome c release and by stabilizing the shape of mitochondrial cristae.^{22,57}

In contrast to OPA1, IOP elevation significantly increased Dnm1 mRNA expression in the ONHs of 10 month-old glaucomatous mice. Recent evidence indicates that mitochondrial fission is associated with the translocation of Drp-1 from cytoplasm to defined spots on the mitochondrial membrane.^{10,50,58–60} Consistent with these prior studies, we reported that Drp-1 protein was decreased in the cytosolic fraction in the pressure-treated cells but was increased in the mitochondrial fraction, indicating that Drp-1 translocation into mitochondria in our model contributes to the mechanism of mitochondrial fission in differentiated RGC-5 cells *in vitro* after elevated hydrostatic pressure.⁵ Further evidence suggested that inhibiting Drp-1 mediated mitochondrial fission selectively prevents the release of cytochrome c during apoptosis.⁶¹ Together with these findings, the current results suggest that increase of ONH Dnm1 expression by IOP elevation may also lead to structural and functional changes of mitochondria that facilitate ON axon loss. Thus, treatments that enhance OPA1 retention in mitochondria or that inhibit Drp-1 mediated mitochondrial fission may provide a new strategy to protect against ON degeneration and RGC loss in glaucoma.

Non-glaucomatous 10 month-old C57BL/6 mice were included in our evaluations of COX, OPA1, and Dnm1 mRNA expression as an age-matched normal control strain. The absence of IOP elevation with age was confirmed (shown in Supplementary Material 1). While several mitochondrial changes occur with old age, senescence, or apoptosis, there appear to be minimal differences in neuronal mitochondria from 4 month-old young-mature rats and 13 month-old middle-aged rats.⁶² Thus, it is unlikely that there are significant differences in neuronal mitochondria of young mature normal mice (3 month-old) and middle-aged normal mice (10 month-old). Comparison of the results from the 10 month-old C57BL/6 mice with the 3 and 10 month-old DBA/2J mice results found COX mRNA was greatest in the 3 month-old DBA/2J mouse ONHs, 0.90-fold less in the 10 month-old C57BL/6 mouse ONHs, and 0.81-fold less in the 10 month-old DBA/2J mouse ONHs. This result may merely reflect less axons in the ONHs of the latter two groups as it is consistent with previous reports of modest age-related axon loss in normal C57BL/6 mice⁶³ as well as accelerated axon loss in mice with elevated IOP.^{28,43,64–66} Relative to OPA1 mRNA in the ONHs of 3 month-old DBA/2J mice, OPA1 mRNA was 0.65-fold less in glaucomatous 10 month-old DBA/2J mice but 1.46-fold greater in the non-glaucomatous 10 month-old C57BL/6 mice. Because these differences were greater in magnitude than the corresponding differences in COX mRNA, they suggest a positive association between IOP elevation and reduced expression of the OPA1 gene. Moreover, they raise the possibility that OPA1 mRNA expression changes precede IOP-associated axon loss. In contrast to OPA1, expression of Dnm1 mRNA (which codes for a protein that promotes mitochondrial fission), was least in the 3 month-old DBA/2J mouse ONHs and greatest in the 10 month-old DBA/2J mouse ONHs. This is consistent with an important role for IOP. The intermediate expression of Dnm1 mRNA in the 10 month-old C57BL/6 mouse ONHs may reflect a balance the more robust expression of OPA1 mRNA in this strain than in the 3 month-old DBA/2J mice. Together, these results support important influences of both normal aging and elevated IOP on the transition of mitochondrial fission/fusion balance to favor fission.

OPA1 release during mitochondrial fission participates in apoptotic cell death.^{19,23} Consistent with these prior studies, we also found that elevated hydrostatic pressure triggers release of OPA1 and cytochrome c, and induces subsequent apoptotic cell death in differentiated RGC-5 cells *in vitro*.⁶ In the present study, IOP elevation gradually induced OPA1 release from mitochondria to the cytosol in the ONs of 8 and 10 month-old glaucomatous mice. The actin-normalized concentrations of each of the 4 OPA1 isoforms present in the cytosol from 8 month-old ONs were intermediate between the results at 3 months of age and the results at 10 month of age. This shows the progressive nature of the increase in cytosolic OPA1 that manifest during the progression of glaucomatous damage and is further supported by the progressive increase in cytosolic cytochrome c seen in 3, 8, and 10 month-old DBA/2J mice. The greater reduction in the VDAC-normalized concentration of the 2 OPA1 isoforms in the mitochondrial fraction may reflect that mitochondrial OPA1 changes are more dramatic and precede the changes in cytosolic OPA1 and axon survival. In contrast, mitochondrial cytochrome c is reduced similarly at 8 and 10 months of age. Hence, it is possible that OPA1 release in glaucomatous ON with IOP elevation may directly contribute to ON axon loss by mediating abnormal mitochondrial structural impairment. In addition to evidence of OPA1 release to the cytoplasm, a small immunoreactive band (~65 kDa) appeared in the cytosolic fraction in the ONs of the eyes with IOP elevation. A rhomboid intramembrane protease PARL cleaves the OPA1 protein and the cleavage of OPA1 generates a pool of truncated OPA1 that is soluble in the intermembrane space.⁶⁷ Moreover, the soluble OPA1 may be crucial for the anti-apoptotic effects of PARL because it maintains the bottleneck configuration of cristae and the compartmentalization of cytochrome c.^{22,67} Thus, it is likely that the unexpected smaller molecular weight of OPA1 fragments presently observed might include the truncated forms of OPA1 that localize to in the intermembrane space or possibly one of the degradation products. The functional contributions of each of the various soluble OPA1 isoforms that are released from ON mitochondria following IOP elevation need to be further explored.

In summary, IOP elevation induces reduction of COX activity, mitochondrial fission, mitochondrial matrix swelling and cristae depletion, alterations of OPA1 and Dnm1 expression, and induction of OPA1 release from mitochondria in glaucomatous ON. Thus, these findings support the idea that interventions to protect against mitochondrial fission-related dysfunction may be beneficial for reducing glaucomatous ON degeneration and RGC loss.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. IOP elevation in glaucomatous DBA/2J mice

(A) The average IOP and (B) Representative actual IOP values in eyes of mice aged 3, 6, 7–8, 9–10 and 12 months. The peak of IOP elevation was 21.5 ± 4.5 mmHg in the right eyes and 19.9 ± 3.7 mmHg in the left eyes within 10 month-old mice.

Figure 2. ON degeneration in glaucomatous DBA/2J mice

(A, C and E) The ON of 3 month-old mice. (B, D and F). The ON of 10 month-old glaucomatous mice. Note degenerated ONH (B) and ON axon loss (D and F) in the ON of 10 month-old glaucomatous mice with elevated IOP. The specimen preservation procedure was optimized for mitochondria preservation. Scale bars = 50 μ m (A–D); 20 μ m (E and F).

Figure 3. Mitochondrial fission, matrix swelling and cristae depletion in the unmyelinated ONH of glaucomatous DBA/2J mice

(A and B) COX immunohistochemistry in the unmyelinated ONH. COX immunoreactivity was concentrated in the unmyelinated ONH of 3 month-old DBA/2J mice (A, asterisk). In contrast, much less COX immunoreactivity was observed in the unmyelinated ONH of 10 month-old glaucomatous DBA/2J mice (B, asterisk and arrow). The sections were counterstained with the nucleic acid stain Hoechst 33342 (Blue color). (C) COX mRNA expression was significantly decreased in the ONHs of 10 month-old glaucomatous DBA/2J mice (* $P < 0.05$ by Student's t-test, $n=3$ for both 3 and 10 month-old mice) but there is no significant changes between 3 month-old DBA/2J mice and 10 month-old non-glaucomatous C57BL/6 mice. (D and E) Ultrastructural changes of mitochondria in the unmyelinated ONH. Three month-old mice had elongated mitochondria of various lengths showing normal matrix

and membrane structures (D, arrowhead). In contrast, mitochondria from 10 month-old glaucomatous mice with IOP elevation (E, arrows) had small rounded organelles and significant matrix swelling. (F) Quantitative analysis of mitochondrial lengths in the unmyelinated ONH. Using conventional thin-section EM, the length of mitochondrial cross-sections at the longest extent was measured from 2D projection images. Error bar represents the standard deviation (* $P < 0.001$ by Student's t-test, $n = 216$ for both 3 and 10 month-old mice). Scale bar = 50 μm (A and B); 1 μm (C and D).

Figure 4. Three-dimensional reconstructions of mitochondria using EM tomography

Slices of 1.4 nm thickness through each reconstruction are shown through the middle of the 3 month sample (Aa) and at two different heights through the 10 month-old sample (Aa and Bb) to emphasize the normal structural features of the former and the fission and localized swelling of the latter. In the 10 month-old sample, mitochondrial fission occurred in two places giving three mitochondrial bodies. The two slices through the tomographic volume showing the closest apposition of left and middle bodies (Ba) and middle and right bodies (Bb) emphasize that the fission was completed (arrowheads). Matrix swelling and lack of cristae were observed in a localized region of the right mitochondrial body (asterisk). Top (Ab and Gc) and side views (Ac, Ad, Bd and Be) of surface-rendered volumes of the mitochondria after segmentation of the membranes show the distribution of cristae and any subvolumes devoid of cristae. The

outer membrane is shown in blue and made transparent in Ac, Bc and Bd to better visualize the shapes and sizes of cristae. The cristae are shown in various colors. Side views of the outer membrane show no ruptures of this membrane (Ad and Be). Arrowheads point to fission loci that are seen in panel Be. The subvolume with swollen matrix and devoid of cristae is also marked in Ac and Bd (asterisks). Scale bar = 200 nm (A and B). URL to 2 movies are:
<ftp://ismail@ccdb-db1.ucsd.edu/IOC2.mpg>,
<ftp://ismail@ccdb-db1.ucsd.edu/daniel2edited.mpg>, password: arr0wh6ad.

FIGURE 5. Alterations of OPA1 protein and gene expression or Dnm1 gene expression in the ONH of glaucomatous DBA/2J mice

(A and B) OPA1 immunohistochemistry in the unmyelinated ONH. OPA1 immunoreactivity was concentrated in the ONH of 3 month-old mice (A, asterisk). In contrast, much less OPA1 immunoreactivity was observed in the ONH of 10 month-old glaucomatous mice (B, asterisk and arrow). The sections were counterstained with the nucleic acid stain Hoechst 33342 (Blue color). (C) Reduction of OPA1 mRNA expression in the ONHs of 10 month-old glaucomatous DBA/2J mice. Ten month-old glaucomatous DBA/2J mice show a significant reduction of OPA1 mRNA expression in the ONHs. In contrast, 10 month-old non-glaucomatous C57BL/6 mice show a significant increase of OPA1 mRNA expression in the ONHs. (D) Increase of Dnm1 mRNA expression in the ONHs 10 month-old glaucomatous DBA/2J mice. Both 10 month-old glaucomatous DBA/2J and 10 month-old non-glaucomatous C57BL/6 mice show a significant increase of Dnm1 mRNA expression in the ONHs. Error bar represents the standard deviation (* $P < 0.05$ by Student's t -test, $n=3$ for both 3 and 10 month-old mice). Scale bar = 50 μm (A and B).

FIGURE 6. Alteration of OPA1 distribution in the ONs of glaucomatous DBA/2J mice
 (A) OPA1 Western blot analysis of cytosolic and mitochondrial fractions from whole ONs. The OPA1 protein bands show the positions of 3 major isoforms of OPA1 (90 kDa:L; 80 kDa:S1; 75 kDa:S2). There was a fourth isoform of OPA1 protein band (~65 kDa:S3) in the cytosolic fraction of the ONs in the 8 and 10 month-old glaucomatous mice. The cytochrome c protein bands show the positions of 17 kDa form of cytochrome c in cytosolic and mitochondrial fraction. (B) Relative intensity of chemiluminescence for each protein band was normalized using actin (~42 kDa) as cytosolic fraction calibrator and voltage dependent anion channel (VDAC, ~31 kDa) as mitochondrial fraction calibrator. The L, S1 and S2 isoforms of OPA1 were significantly increased in the cytosolic fraction. In contrast, the L and S1 isoforms

of OPA1 were significantly decreased in the mitochondrial fraction in the 8 and 10 month-old glaucomatous mice. The cytochrome c protein bands were significantly increased in the cytosolic fraction. However, the cytochrome c protein band was decreased in the mitochondrial fraction in the 8 and 10 month-old glaucomatous mice. Error bar represents the standard deviation (* $P < 0.05$ by Student's t-test, $n=3$ for 3, 8 and 10 month-old mice).

Table 1Primer and Probe Sequences for mouse COX, OPA1, Dnm1 and GAPDH for *Taqman* qPCR^a

Gene (Accession #)	Type	Sequences (5'–3')
COX (NM_009941)	Forward	TCACTGCGCTCGTTCTGAT
	Reverse	CGATCGAAAGTATGAGGGATG
Roche Applied Science	Probe	Universal Probe Library probe #7
		Cat.#04685059001
OPA1 (NM_133752)	Forward	TGACAAACTTAAGGAGGCTGTG
	Reverse	CATTGTGCTGAATAACCCTCAA
Roche Applied Science	Probe	Universal Probe Library probe #91
		Cat.#04692080001
Dnm1 (NM_010065)	Forward	CGGTAGACAGTGCACCAAG
	Reverse	GGATGTGGGTGGTCACAAT
Roche Applied Science	Probe	Universal Probe Library probe #52
		Cat.#04688490001
GAPDH (NM_008084)	Forward	CAACGGGAAGCCCATCAC
	Reverse	CGGCCTCACCCATTG
	Probe	CTTCCAGGAGCGAGACCCCACTAACA

^aTaqman probe containing 5' reporter FAM and 3' quencher BHQ-1 dyes (in parentheses, GeneBank accession numbers).