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Memantine blocks mitochondrial OPA1 and cytochrome c release, and subsequent apoptotic cell death in glaucomatous retina

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Abstract

Purpose—To determine whether intraocular pressure (IOP) elevation alters OPA1 expression and triggers OPA1 release, as well as whether the uncompetitive N-methyl-D-aspartate (NMDA) glutamate receptor antagonist memantine blocks OPA1 release and subsequent apoptotic cell death in glaucomatous DBA/2J mouse retina.

Methods—Preglaucomatous DBA/2J mice received memantine (5 mg/kg, i.p. injection, twice a day for 3 months) and IOP in the eyes was measured monthly. RGC loss was counted following Fluoro-Gold labeling. OPA1, Dnm1, Bcl-2 and Bax mRNA were measured by Taqman qPCR. OPA1 protein was assessed by immunohistochemistry and Western blot. Apoptotic cell death was assessed by TUNEL staining.

Results—Memantine treatment significantly increased RGC survival in glaucomatous DBA/2J mice. Memantine treatment increased the 75 kDa OPA1 isoform but did not alter the 80 and 90 kDa isoforms. The isoforms of OPA1 were significantly increased in the cytosol of the vehicle-treated glaucomatous retinas but were significantly decreased in memantine-treated glaucomatous retinas. OPA1 immunoreactivity was decreased in the photoreceptors of both vehicle- and memantine-treated glaucomatous retinas but was increased in the outer plexiform layer of only the memantine-treated glaucomatous retinas. Memantine blocked apoptotic cell death in the GCL, increased Bcl-2 gene expression, and decreased Bax gene expression.

Conclusions—OPA1 release from mitochondria in glaucomatous mouse retina is inhibited by blockade of glutamate receptor activation. Because this OPA1 effect was accompanied by increased Bcl-2 expression, decreased Bax expression and apoptosis blockade, glutamate receptor activation in the glaucomatous retina may involve a distinct mitochondria-mediated cell death pathway.

Introduction

Elevated intraocular pressure (IOP) is an important risk factor for optic nerve head damage and retinal ganglion cell (RGC) death in glaucoma.¹ However, the precise pathophysiological relationship between elevated IOP and RGC death remains poorly understood. Glutamate receptor activation-induced excitotoxicity has been hypothesized to contribute to RGC death in glaucoma.² In addition, glutamate receptor activation-induced excitotoxicity has been linked to mitochondrial dysfunction in both acute and chronic neurodegenerative disorders.^{3–6}

However, the relationships among elevated intraocular pressure (IOP), glutamate receptor-mediated excitotoxicity and mitochondrial dysfunction in glaucoma remains unknown.

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. In contrast, dynamin-related protein-1 (Drp-1 in humans, Dnm1 in mice) promotes 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 humans and cause autosomal dominant optic atrophy (ADOA), a 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

Memantine, an uncompetitive N-methyl-D-aspartate (NMDA) glutamate receptor antagonist, is neuroprotective in mouse, rat, and monkey models of glaucoma.²⁵⁻²⁸ However, the downstream mechanism of this effect is poorly understood. Recent studies indicate that OPA1 mutation or deficiency in transgenic mice can cause RGC and nerve fiber degeneration, mitochondrial dysfunction, optic nerve abnormalities and visual deficits.^{29,30} Further, we reported that elevated IOP induces decreased OPA1 and increased Dnm1 gene expression as well as OPA1 and cytochrome c release in glaucomatous DBA/2J mouse optic nerve.³¹ These results suggest OPA1 involvement in degenerative RGC loss.

Hence, the present study was undertaken to determine whether memantine treatment that protects RGC death alters OPA1 and Dnm1 gene expression, OPA1 protein expression, or OPA1 protein distribution in the retina of glaucomatous DBA/2J mice, an extensively characterized strain that spontaneously develops elevated IOP.³²⁻⁴⁰ Also, we compared the progression of these changes with changes in Bcl-2 and Bax gene expression of glaucomatous DBA/2J mouse retina.

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 and 9 month-old female DBA/2J mice (The Jackson Laboratory, Bar Harbor, Maine) and 9 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. Prior studies have shown that IOP and optic nerve appearance is normal in 3 month-old DBA/2J mice.³²⁻³⁴ IOP elevation onset typically occurs between 5 and 7 months of age, and by 10 months of age, IOP-linked optic nerve axon loss is well advanced.^{32-34,39,40}

IOP Measurement

IOP measurement was performed as described previously.^{31,32,41} Each of the 7, 8 and 9 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 9 month-old non-glaucomatous C57BL/6 mice used in this study had a single IOP measurement (n=21 mice). 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 μ m 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).

Pharmacological Treatment

Two groups of mice were studied: a group treated with vehicle (0.9% Saline, i.p. injection; n=48 animals/group) and a group treated with memantine, an uncompetitive N-methyl-D-aspartate (NMDA) glutamate receptor antagonist, (Sigma, St. Louis, MO; 5 mg/kg in 0.9% saline; i.p. injection twice a day for 3 months; n=56 animals/group) given for 3 months.

Retrograde Labeling of Retinal Ganglion Cells

One week before sacrifice, Fluoro-Gold (1 μ l/injection of 4%; Fluorochrome Inc., Englewood, CO) diluted in saline was microinjected bilaterally into the superior colliculi of anesthetized mice with a mixture of ketamine (Fort Dodge Animal Health) and xylazine (Vedeco Inc.) in a stereotactic apparatus, as previously described.¹⁵ Fluoro-Gold is taken up by the axon terminals of the RGCs and transported retrogradely to the somas in the retina.⁴² The Fluoro-Gold in the RGCs persists for at least 3 weeks without significant fading or leakage.⁴³

Images were captured under Olympus spinning disk confocal microscope (Olympus America Inc., Center Valley, PA) equipped with a high-precision closed loop XY stage and closed loop Z control with commercial mosaic acquisition software from MicroBrightField (MBF Bioscience Inc., Williston, VT).^{44,45} The microscope equipped with high-resolution high sensitive CCD camera for high-speed mosaic acquisition. Images were stored in Photoshop files (Adobe Systems Inc., San Jose, 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 retinas 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 PBS, retinas were dehydrated through graded ethanols and then embedded in a polyester wax as described previously.¹⁵ For Western blot analyses, whole retinas were immediately used or frozen in liquid nitrogen and stored at 70°C until use.

Immunohistochemical Analyses

Immunohistochemical staining of 7 μ m wax sections of full thickness retina was done by the immunofluorescent method as previously described.¹⁵ Five sections per wax block from each age group (n=4 retinas/group) were used for immunohistochemical analysis. Primary antibody was a polyclonal rabbit anti-mouse OPA1 antibody (1:1000, a gift of Drs. Misaka and Kubo).⁴⁶ This antibody was produced by immunizing rabbits with a synthetic peptide corresponding

to amino acids 938–960 of mouse OPA1 protein. The serum was then affinity-purified as previously described.^{15,46} 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 OPA1 for 16 hours at 4°C. After several wash steps, the tissue was incubated with the secondary antibody, peroxidase-conjugated goat anti-rabbit IgG (1:100, Molecular Probes, Eugene, OR) for 4 hours at 4°C and then washed with PBS. The sections were counterstained with the nucleic acid stain Hoechst 33342 (1 µg/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).

Taqman Quantitative PCR

Retinas were dissected from the sclera of three 3 month-old DBA/2J mice, three vehicle-treated 9 month-old glaucomatous DBA/2J mice, and three memantine-treated 9 month-old glaucomatous DBA/2J mice, as well as three of 9 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 retinas (n=4 retinas/group) 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 OD_{260nm}/280nm absorption ratio exceeded 1.9. The cDNA was synthesized using SuperScript II first-strand RT-PCR kit (Invitrogen). OPA1, Bcl-2, and Bax gene expression were measured by qPCR (MX3000P, Stratagene, La Jolla, CA) using 25 ng of cDNA from retinas 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 OPA1, Bcl-2, Bax 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 2). The probes for OPA1, Bcl-2, and Bax were obtained from the Roche Universal Probe Library (Roche Diagnostics, Mannheim, Germany, Table 2), 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 (OPA1, Dnm1, Bcl-2 and Bax) and the endogenous reference (GAPDH). The samples were run in triplicate for each target and endogenous GAPDH control.

The number at the threshold level of log-based fluorescence (C_t) was computed automatically by the MX3000P software (Stratagene) and compared across all conditions. Delta C_t (ΔC_t) was calculated by subtracting the Normalizer C_t (GAPDH) from Target C_t (OPA1). Delta-Delta C_t ($\Delta\Delta C_t$) was calculated by subtracting the ΔC_t calibrator (control animals) from the ΔC_t Sample (experimental animals). An alternative method incorporates rates of amplification into the equation:

$$Rel. Q. = \frac{(1+E_{Norm})^{C_t Unk} / (1+E_{Norm})^{C_t Cal}}{(1+E_{Goi})^{C_t Unk} / (1+E_{Goi})^{C_t Cal}} = \frac{(1+E_{Norm})^{C_t Unk - C_t Cal}}{(1+E_{Goi})^{C_t Unk - C_t Cal}}$$

This method also improves on the $2^{(-\Delta\Delta C_t)}$ method by correcting small differences in the relationship between change in concentration and changes in C_t value.⁴⁷ OPA1, Dnm1, Bax or Bcl2 gene expression level have been automatically converted into fold changes by the MX3000P software (Stratagene).

Western Blot Analysis

Retinas were dissected from the sclera of three 3 month-old DBA/2J mice, vehicle-treated 9 month-old glaucomatous DBA/2J mice and memantine-treated 9 month-old glaucomatous. 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 MgCl₂/1 mM EDTA/1 mM EGTA/1 mM DTT/0.5% CHAPS/complete protease inhibitors; Roche Biochemicals, Indianapolis, IN). Ten microgram of pooled retinas (n=4 retinas/group) 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 BioSciences).

To assess the subcellular distribution of OPA1, the cytosolic and mitochondrial fractions were isolated from freshly isolated retinas (n=4 retinas/group) 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°C. For the cytosolic fraction, the supernatant was centrifuged at $12,000 \times g$ for 15 minutes at 4°C and the supernatant was 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°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). Good separation of the cytosolic and mitochondrial fractions was confirmed by the observation of negligible staining when cytosolic fraction blots were reprobed with antibodies to VDAC and when mitochondrial fraction blots were reprobed with antibodies to actin (data not shown).

TUNEL Staining

Retinal sections were incubated with proteinase K (10 µg/ml, 10 mM Tris, pH 7.4–8.0) for 10 min at 37°C. After rinsing in PBS, the sections were incubated with terminal deoxynucleotidyl transferase plus nucleotide mixture in reaction buffer for 60 min at 37°C (In Situ Cell Death Detection kit, Roche Diagnostics).

Statistical Analysis

Experiments presented were repeated at least three times. The data are presented as the mean $\pm$ SD. Comparison of three experimental conditions was evaluated using one-way analysis of variance (ANOVA) and the Bonferroni *t*-test. $P < 0.05$ was considered to be statistically significant.

Results

Memantine Treatment Increases RGC Survival

Previously, we reported that the peak of elevated IOP within 9–10 month-old DBA/2J mice was 21.5 ± 4.5 mmHg in the right eyes and 19.9 ± 3.7 mmHg in the left eyes ($n=98$ mice). In contrast, mean IOP within 9–10 month-old non-glaucomatous C57BL/6 mice was 14.7 ± 1.7 mmHg in the right eyes and 14.2 ± 1.8 mmHg in the left eyes ($n=21$ mice).³¹ To determine whether memantine treatment alters IOP in glaucomatous DBA/2J mice, we measured IOP in both vehicle- and memantine-treated mice. Memantine treatment did not change IOP in glaucomatous mice. The averages of IOP were 21.5 ± 5.7 mmHg in the vehicle-treated eyes ($n=48$ vehicle-treated mice) and 20.1 ± 5.2 mmHg in the memantine-treated eyes within 9 month-old glaucomatous DBA/2J mice ($n=56$ memantine-treated mice, Fig. 1A and B). In addition, no differences were found in the body weight between vehicle- and memantine-treated glaucomatous DBA/2J mice (Fig. 1C).

To assess the neuroprotective effect of memantine in RGC survival in glaucomatous retina, we retrogradely labeled RGCs by Fluoro-gold injection into the superior colliculi and analyzed RGC loss. Mean RGC density per retina for each group is presented in Table 1. Three month-old DBA/2J mouse retina had an average of 4779 RGCs in the central, 3683 RGCs in the middle, and 2720 RGCs in the peripheral areas, respectively ($n=4$ retinas, Table 1). As has been reported previously,^{25,28} memantine treatment significantly increased RGC survival by an approximate 45% compared to vehicle-treated 9 month-old glaucomatous mice.

Memantine Increases OPA1 Gene Expression and Decreases Dnm1 Gene Expression

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 retinal mRNA from 3 month-old DBA/2J mice, vehicle-treated 9 month-old glaucomatous DBA/2J mice, and memantine-treated 9 month-old glaucomatous DBA/2J mice, as well as 9 month-old non-glaucomatous C57BL/6 mice ($n=4$ retinas per pool, Table 2 and Fig. 3). Results were normalized to GAPDH mRNA. We observed that both OPA1 and Dnm1 mRNA were decreased by 0.71 ± 0.07 -fold and 0.71 ± 0.1 -fold in the retinas of 9 month-old glaucomatous DBA/2J mice, respectively, compared to 3 month-old DBA/2J mice (Fig. 3A and B). Interestingly, memantine treatment did not show a difference in OPA1 mRNA by 0.72 ± 0.06 -fold but significantly decreased Dnm1 mRNA by 0.34 ± 0.04 -fold ($P<0.05$) compared to vehicle-treated 9 month-old glaucomatous DBA/2J mice. As a positive age-matched control, we examined expression of these mRNA in the retinas of 9 month-old non-glaucomatous C57BL/6 mice. We found that expression of OPA1 mRNA was minimally different but expression of Dnm1 mRNA was significantly increased: i.e. OPA1 and Dnm1 mRNA expression in C57BL/6 mice was 1.06 ± 0.04 -fold and 1.23 ± 0.02 -fold compared to 3 month-old DBA/2J mice (Fig. 3A and B). There were no differences in the ratios of GAPDH mRNA to total RNA in the retinas between the 3 month-old DBA/2J mice, 9 month-old glaucomatous DBA/2J mice and 9 month-old non-glaucomatous C57BL/6 mice (data not shown).

Memantine Does Not Alter Total OPA1 Protein

To test whether memantine blocks alteration of total OPA1 protein expression in the retina of 9 month-old glaucomatous DBA/2J mice, we performed OPA1 Western blot and immunohistochemistry. As shown in Figure 4A, the OPA1 antibody recognized five major OPA1 isoforms; >100 kDa isoforms (called the large or L form: L2 and L3), a 90 kDa isoform (L1), an 80 kDa isoform (S1) and 75 kDa isoform (S2) in the total protein extracts of the retinas in 3 month-old DBA/2J mice. The S2 isoform of OPA1 protein was significantly increased at both vehicle- and memantine-treated 9 month-old glaucomatous DBA/2J mice compared to 3

month-old DBA/2J mice ($P<0.05$). In addition, the L3 isoform of OPA1 protein was significantly increased at memantine-treated 9 month-old glaucomatous DBA/2J mice compared to 3 month-old DBA/2J mice and vehicle-treated 9 month-old glaucomatous DBA/2J mice ($P<0.05$). In contrast, the L isoforms (L2 and L3) and S1 isoforms of OPA1 protein were not changed in both vehicle- and memantine-treated 9 month-old glaucomatous DBA/2J mice (Fig. 4A and B). When the primary antibody was omitted, as a control for OPA1 immunohistochemistry, there was no labeling by the secondary antibody in the retina of 3 month-old DBA/2J mice (Fig. 4C). In contrast, OPA1 immunoreactivity was present in the ganglion cell layer (GCL), inner plexiform layer, inner nuclear layer, outer plexiform layer, and photoreceptor layer in the retina of 3 month-old DBA/2J mice, vehicle-treated 9 month-old glaucomatous DBA/2J mice and memantine-treated 9 month-old glaucomatous DBA/2J mice (Fig. 4D-FD). Interestingly, both vehicle and memantine treatment decreased OPA1 immunoreactivity in the photoreceptors of 9 month-old glaucomatous DBA/2J mice (Fig. 4E and F). To verify that RGCs indeed express OPA1, RGCs were retrogradely labeled by Fluoro-Gold. Remarkably, all neurons containing OPA1 immunoreactivity were co-labeled by Fluoro-Gold (Fig. 4G), indicating that RGCs express OPA1 protein in DBA/2J mice.

Memantine Blocks OPA1 Release from Mitochondria

To investigate whether IOP elevation triggers OPA1 release and whether memantine treatment blocks OPA1 release in the retinas of 9 month-old glaucomatous DBA/2J mice, relative changes of OPA1 protein concentration in cytosolic and mitochondrial fractions were measured using Western blotting. Results were normalized to actin as cytosolic fraction calibrator and VDAC as mitochondrial fraction calibrator.

As shown in Figure 5A, the OPA1 antibody recognized four major OPA1 isoforms; >100 kDa isoforms (L2 and L3), a 90 kDa isoform (L1), and an 80 kDa isoform (S1) in the cytosolic fraction of 3 month-old DBA/2J mouse retina. Two major isoforms (L1 and S1) were identified in the mitochondrial fraction of both 3 month-old DBA/2J mice and 9 month-old glaucomatous DBA/2J mice. In contrast, 9 month-old glaucomatous DBA/2J mouse retina contained at least five isoforms of OPA1 in the cytosolic fractions including a small isoform (75 kDa: S2). Moreover, relative OPA1 content of five isoforms was significantly increased by 1.61 ± 0.15 -fold (L3), 2.85 ± 0.29 -fold (L2), 19.3 ± 1.53 -fold (L1), 1.58 ± 0.21 -fold (S1) and 27.2 ± 2.69 -fold (S2) in the retinas of vehicle-treated 9 month-old glaucomatous DBA/2J mice, respectively ($P<0.05$, Fig. 5A and B). Concomitantly, relative OPA1 content of both mitochondrial isoforms (L1 and S1) were significantly decreased by 0.31 ± 0.04 -fold (L1) and 0.71 ± 0.09 -fold (S1) in the retinas of vehicle-treated 9 month-old glaucomatous DBA/2J mice, respectively ($P<0.05$, Fig. 5A and B). Memantine treatment significantly decreased five cytosolic isoforms by 0.71 ± 0.08 -fold (L3), 1.1 ± 0.12 -fold (L2), 7.1 ± 0.82 -fold (L1), 0.98 ± 0.1 -fold (S1) and 13.5 ± 1.54 -fold (S2), respectively, compared to the retinas of vehicle-treated 9 month-old glaucomatous DBA/2J mice ($P<0.05$, Fig. 5A and B). In contrast, memantine treatment significantly increased the mitochondrial L isoform of OPA1 by 0.86 ± 0.09 -fold compared to the retinas of vehicle-treated 9 month-old glaucomatous DBA/2J mice ($P<0.05$, Fig. 5A and B).

Further, cytochrome c protein concentration was significantly increased by 1.33 ± 0.15 -fold in the cytosolic fraction but decreased by 0.52 ± 0.07 -fold in the mitochondrial fraction of the vehicle-treated 9 month-old glaucomatous DBA/2J mice compared to the retinas of 3 month-old DBA/2J mice ($P<0.05$, Fig. 5C). In contrast, memantine treatment significantly decreased cytochrome c protein by 0.99 ± 0.11 -fold in the cytosolic fraction but increased by 0.69 ± 0.08 -fold in the mitochondrial fraction compared to the retinas of vehicle-treated 9 month-old glaucomatous DBA/2J mice ($P<0.05$, Fig. 5C).

Memantine Increases Bcl-2 Gene Expression but Decreases Bax Gene Expression

Compared to the retinas of 3 month-old DBA/2J mice (Fig. 6A), vehicle-treated 9 month-old glaucomatous DBA/2J mice showed TUNEL-positive apoptotic cell death in the GCL (Fig. 6B). In contrast, memantine treatment blocked this apoptosis in the GCL (Fig. 6C). While vehicle-treated retinas of glaucomatous DBA/2J mice did not change Bcl-2 mRNA expression but significantly increased Bax mRNA expression compared to 3 month-old DBA/2J mice (n=4 retinas per pool, $P<0.05$, Fig. 6D and F), memantine treatment significantly increased Bcl-2 mRNA expression but decreased Bax mRNA expression (n=4 retinas per pool, $P<0.05$, Fig. 6D and F).

Discussion

These results provide the first evidence that in the RGC protective effect of glutamate receptor blockade by memantine is associated with changes in OPA1 and Dnm1 that control the mitochondrial fission/fusion balance. Specifically, memantine treatment that protects against RGC loss increased total OPA1 protein expression and significantly decreased Dnm1 gene expression. Memantine treatment also blocked OPA1 and cytochrome c release from mitochondria, increased Bcl-2 gene expression but decreased Bax gene expression in the retinas of 9 month-old glaucomatous DBA/2J mice. These findings suggest that alterations of OPA1 expression and distribution may be an important component of a biochemical cascade leading to glutamate excitotoxicity-mediated mitochondrial dysfunction in RGC death in glaucoma.

Glutamate excitotoxicity has been linked to mitochondrial dysfunction in both acute and chronic neurodegenerative disorders including glaucoma.^{3–6} Memantine inhibits glutamate-mediated excitotoxicity in neurons expressing NMDA receptor and is effective in the treatment of moderate to severe Alzheimer's disease and vascular dementia.^{48–53} Although memantine treatment provides substantial protection against RGC death in experimental models of glaucoma,^{25,28} clinical benefits for glaucoma patients taking memantine have been controversial.⁵⁴ Hence, a better understanding of the mechanism of the beneficial effects of memantine in the experimental models may provide insights that could facilitate the clinical usefulness of memantine or other glutamate receptor antagonist for treating glaucoma.

Recently, we reported that acute IOP elevation triggers mitochondrial OPA1 release in the early neurodegenerative events and that MK801, another NMDA glutamate receptor antagonist, blocks OPA1 release and apoptotic cell death, as well as increases OPA1 gene and protein expression in the ischemic rat retina.⁵⁵ In another study, we found that elevated IOP induces mitochondrial fission and triggers OPA1 and cytochrome c release in glaucomatous DBA/2J mouse optic nerve.³¹ Together with these findings, the present results suggest glutamate excitotoxicity induced by IOP elevation may directly contribute to mitochondrial dysfunction-mediated cell death in glaucomatous optic neuropathy. Moreover, they show that memantine treatment brings about a shift in proteins regulating mitochondrial fusion and fission consistent towards increased RGC survival. Further, we believe that a time-dependent progressive changes with memantine treatment may be useful for future experiments.

Closer examination of the present data provides further insights. Recent studies demonstrated that downregulation of OPA1 causes aggregation of the mitochondrial network in purified RGCs,²⁰ and that OPA1 deficiency in mouse models of ADOA impairs mitochondrial morphology, optic nerve structure, and visual function.^{29,30} In contrast, increased OPA1 expression protected cells from apoptosis by preventing cytochrome c release and by stabilizing the shape of mitochondrial cristae.^{22,56} Further, recent evidence indicated that inhibiting Drp-1-mediated mitochondrial fission selectively prevents cytochrome c release during apoptosis.⁵⁷ The present results indicate that OPA1 protein expression in the retina of eyes

with elevated IOP was increased following treatment with memantine. Conversely, memantine treatment significantly decreased Dnm1 (the mouse analogue of Drp-1) gene expression in the retina of glaucomatous DBA/2J mice. These changes suggest a shift towards favoring mitochondrial fusion. Together, these findings demonstrate a link exists between the neuron protective effect of memantine and increased OPA1 coupled with decreased Dnm1 gene expression. These results are consistent with our previous study showing that MK801 significantly increases OPA1 protein expression in ischemic retina induced by acute IOP elevation.⁵⁵ It is not clear from the present data whether these changes reflect a direct effect of memantine on the expression of the genes and protein or are indirect consequences of enhanced RGC survival. Nevertheless, they raise the possibility that direct enhancement of OPA1 retention in mitochondria or direct inhibition of Drp-1 expression may provide new strategies to protect against RGC death in glaucoma.

Recent studies from our group as well as other investigators reported that the OPA1 mRNA and protein are present in RGCs, amacrine cell and horizontal cells in the normal mouse and rat retina.^{13–16} The present study confirmed that OPA1 immunoreactivity is present in RGCs, amacrine cells, horizontal cells and photoreceptors. The effects of memantine in the retina of eyes with elevated IOP may extend beyond RGCs. Like OPA1, glutamate receptor subunits are present in horizontal cells, bipolar cells, amacrine cells, displaced amacrine cell or RGCs in the mouse retina.^{58–64} A recent study has reported that many amacrine cells in the INL were rapidly and dramatically injured or killed by glutamate or NMDA and that displaced amacrine cells in the GCL also became swollen.⁶⁴ Further, because glutamate excitotoxicity is partly responsible for glaucomatous damage in retina,^{66–69} it is possible that OPA1-positive cells in the retina of DBA/2J mice may also have glutamate receptor subunits. Hence, the possible benefits of increased OPA1 following memantine treatment in non-RGC neurons of the glaucomatous retina need to be further explored.

Although the significance of the various OPA1 subtypes is not fully clear, the present results are consistent with prior investigations of this point. Specifically, we observed that while both large (L1–L3) and small (S1 and S2) isoforms of OPA1 were significantly increased in the cytosolic fraction, only the L1 and S1 isoforms were significantly decreased in the mitochondrial fraction in the retina of glaucomatous DBA/2J retina. More importantly, however, memantine treatment blocked the reduction of L1 isoform of OPA1 and of cytochrome c release in mitochondrial fraction. This suggests a block of OPA1 or cytochrome c release from the mitochondria. The appearance of a small OPA1 immunoreactive band (75 kDa) in the cytosolic fraction in glaucomatous retina may reflect activation of rhomboid intramembrane protease (PARL), an enzyme that can cleave of OPA1 into truncated forms.⁷⁰ Thus, 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 potential proteolytic processing of OPA1 as well as the functional role of each OPA1 isoforms in the cytosolic and mitochondrial fractions of glaucomatous retina should be further explored.

A recent study reported that mitochondrial lysates prepared from C57/BL6 mouse retina recognized a set of six bands between 80 to 100 kDa using Western blots.⁷¹ In the present study, we found that DBA/2J mice showed at least four or five of OPA1 isoforms in the cytosolic fraction or whole lysates from retina tissues, and only two of OPA1 isoforms in the mitochondrial fraction, respectively. Together with these findings, our observations suggest that the appearance of OPA1 immunoreactive bands in the cytosolic fraction or whole lysates in all three experimental conditions may reflect possible posttranslational modification of OPA1 isoforms in glaucomatous retina.

Bax is a proapoptotic member of the Bcl-2 family that is essential for many pathways of apoptosis.⁷² Because Bax directly interacts with the components forming the mitochondrial permeability transition pore (MPTP) that allow proteins to escape from the mitochondria into the cytosol to initiate apoptosis,^{73–75} it could contribute to mitochondrial release of OPA1 and cytochrome c in the glaucomatous retina. Recently, we found that acute IOP elevation significantly increased Bax gene expression without altering Bcl-2 gene expression.⁵⁵ Moreover, Libby et al. reported that Bax is required for RGC apoptosis in glaucomatous DBA/2J mice.³¹ Consistent with these prior studies, we found that elevated IOP significantly increased Bax gene expression but did not alter Bcl-2 gene expression in glaucomatous DBA/2J mice. These results raise the possibility that increased Bax may facilitate OPA1 release during glaucomatous RGC death.

Although the expression of the pro-survival protein Bcl-2 was not altered by elevated IOP, we found that increased RGC survival following memantine treatment was accompanied by increased Bcl-2 gene expression. This agrees with our previous report that MK801 treatment significantly increased Bcl-2 gene expression and prevented apoptotic cell death in ischemic retina following acute IOP elevation.⁵⁵ Interestingly, however, while memantine decreased Bax gene expression in glaucomatous retina, MK801 did not change Bax gene expression in ischemic retina.⁵⁵ These differences may reflect the distinct mechanism of NMDA glutamate receptor blockade.^{51–53} Thus, unlike MK801, memantine treatment may block the Bax-mediated MPTP formation that allows OPA1 release from mitochondria by both decreasing Bax and increasing Bcl-2.

In summary, our findings demonstrate that the increased RGC survival following memantine inhibition of glutamate receptor activation reverses changes in OPA1, Dnm1, and cytochrome c release, that would otherwise occur in the glaucomatous DBA/2J mice. Although further work will be needed to determine whether these effect are primary or secondary to increased RGC survival, they raise the possibility that alterations of OPA1 and Dnm1 may be useful targets for treatments to protect RGC from pressure-related glaucoma.

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Figure 1. IOP elevation in glaucomatous DBA/2J mice following memantine treatment
The average IOP (A), the representative actual IOP values (B), and the body weight (C) in vehicle and memantine-treated eyes of mice aged 7, 8, and 9 months.

Figure 2. RGC survival in glaucomatous DBA/2J mice following memantine treatment
 The retinal flatmounts of 3 month-old DBA/2J mice (A and B), vehicle-treated 9 month-old glaucomatous DBA/2J mice (C and D), and memantine-treated 9 month-old glaucomatous DBA/2J mice (E and F). Note that high magnification showed a significant increase of RGC survival following memantine treatment compared to vehicle treatment. The quantitative analysis of RGC survival (G). Error bar represents the standard deviation (*Significant at $P < 0.05$ compared with 3 month-old DBA/2J mice or vehicle-treated 9 month-old glaucomatous DBA/2J mice, $n=4$ retinal flatmounts/mice/group). Scale bars = 50 μm (A, C and E); 100 μm (B, D and F).

Figure 3. Alterations of OPA1 and Dnm1 gene expression in the retinas of glaucomatous DBA/2J mice following memantine treatment

(A) OPA1 mRNA was significantly decreased in the retinas of both vehicle- and memantine-treated 9 month-old glaucomatous DBA/2J mice compared to 3 month-old DBA/2J mice. However, memantine treatment did not show a difference of OPA1 mRNA expression in the retinas compared to vehicle-treated 9 month-old glaucomatous DBA/2J mice. In addition, there was no significant difference of OPA1 mRNA expression in retinas between 3 month-old DBA/2J mice and 9 month-old C57BL mice. (B) Dnm1 mRNA was significantly decreased in the retinas of both vehicle- and memantine-treated 9 month-old glaucomatous DBA/2J mice compared to 3 month-old DBA/2J mice. However, memantine treatment significantly decreased Dnm1 mRNA expression in the retinas compared to vehicle-treated 9 month-old glaucomatous DBA/2J mice. In addition, Dnm1 mRNA expression was significantly increased in 9 month-old non-glaucomatous C57BL/6 mice compared to 3 month-old DBA/2J mice. Error bars represent the standard deviation (*Significant at $P<0.05$ compared with 3 month-old DBA/2J mice; **significant at $P<0.05$ compared with vehicle-treated 9 month-old glaucomatous DBA/2J mice; ***significant at $P<0.05$ compared with 3 month-old DBA/2J mice, $n=4$ retinas per pool).

Figure 4. Alteration of total OPA1 expression and distribution in the retinas of glaucomatous DBA/2J mice following memantine treatment

(A) The OPA1 protein bands show the positions of 5 major isoforms of OPA1 (>100 kDa; L2 and L3; 90 kDa; L1; 80 kDa; S1) in the retina of 3 month-old DBA/2J mouse. However, the isoform of OPA1 protein bands (75 kDa; S2) was increased in both vehicle- and memantine-treated 9 month-old glaucomatous DBA/2J mouse retina compared to 3 month-old DBA/2J mouse retina. (B) Relative intensity of chemiluminescence for each protein band was normalized using actin as cytosolic fraction calibrator. Error bars represent the standard deviation (*Significant at $P < 0.05$ compared with 3 month-old DBA/2J mice, $n = 4$ retinas per pool). (C–F) When the primary antibody for OPA1 was omitted, there was no binding of secondary antibody (C). OPA1 immunoreactivity in 3 month-old DBA/2J mouse retina (D), in vehicle-treated 9 month-old glaucomatous DBA/2J mouse retina (E), and in memantine-treated 9 month-old glaucomatous DBA/2J mouse retina (F). (G–I) OPA1 (G, green) and Fluoro-Gold (H, red) double labeling. Neurons containing OPA1 immunoreactivity were co-labeled by Fluoro-Gold (arrow heads), indicating that RGCs in the GCL contained OPA1 protein. V, vehicle; M, memantine; PR, photoreceptor; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer. Scale bar = 20 μ m (C–I).

Figure 5. Memantine prevents OPA1 and cytochrome c release in the retinas of glaucomatous DBA/2J mice

(A) OPA1 Western blot analysis of cytosolic and mitochondrial fractions from whole retinas. The OPA1 protein bands show the positions of 4 major isoforms of OPA1 (>100 kDa; L2 and L3; 90 kDa:L1; 80 kDa:S1). There was a fifth isoform of OPA1 protein band (75 kDa:S2) in the cytosolic fraction of the retinas in 9 month-old glaucomatous DBA/2J mice with vehicle and memantine treatment. 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. In vehicle-treated 9 month-old glaucomatous DBA/2J mice compared to 3 month-old DBA/2J mice, the L1–L3 and S2 isoforms of OPA1 were significantly increased in the cytosolic fraction but the L and S1 isoforms of OPA1 were significantly decreased in the mitochondrial fraction. In addition, the cytochrome c protein bands were significantly increased in the cytosolic fraction but decreased in the mitochondrial fraction. In memantine-treated 9 month-old glaucomatous DBA/2J mice compared to vehicle-treated 9 month-old

glaucomatous DBA/2J mice, the L1–L3 and S2 isoforms of OPA1 were significantly decreased in the cytosolic fraction but the L and S1 isoforms of OPA1 were significantly increased in the mitochondrial fraction. In addition, the cytochrome c protein bands were significantly decreased in the cytosolic fraction but increased in the mitochondrial fraction. Error bar represents the standard deviation (*Significant at $P<0.05$ compared with 3 month-old DBA/2J mice or vehicle-treated 9 month-old glaucomatous DBA/2J mice, $n=4$ retinas per pool). V, vehicle; M, memantine.

Figure 6. Memantine blocks apoptotic cell death, and induces increase of Bcl-2 gene expression and decrease of Bax gene expression in the retinas of glaucomatous DBA/2J mice

(A) There were no TUNEL-positive cells in the retina of 3 month-old DBA/2J mouse. (B) Apoptotic cell death was prominent present in the GCL of the retina in vehicle-treated 9 month-old glaucomatous DBA/2J mouse. (C) Memantine treatment prevents apoptotic cell death in the GCL of the retina in vehicle-treated 9 month-old glaucomatous DBA/2J mouse. (D) Vehicle treatment did not alter Bcl-2 mRNA expression in the retinas of 9 month-old glaucomatous DBA/2J mice compared to 3 month-old DBA/2J mice. However, memantine treatment significantly increased Bcl-2 mRNA expression in the retinas of 9 month-old glaucomatous DBA/2J mice compared to vehicle treatment. (E) Vehicle treatment significantly increased Bax mRNA expression in the retinas of 9 month-old glaucomatous DBA/2J mice compared to 3 month-old DBA/2J mice. However, memantine treatment significantly decreased Bax mRNA expression in the retinas of 9 month-old glaucomatous DBA/2J mice compared to vehicle treatment. Error bars represent the standard deviation (*Significant at $P < 0.05$ compared with 3 month-old DBA/2J mice or vehicle-treated 9 month-old glaucomatous DBA/2J mice, $n = 4$ retinas per pool). PR, photoreceptor; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer. Scale bar = $20\mu\text{m}$ (A–C).

Effects of the uncompetitive NMDA glutamate receptor antagonist, memantine, on the central, middle and peripheral RGC loss from 9 month-old glaucomatous DBA/2J mice.

Strain	Age (Months)	Treatment	RGC density per retina (RGCs/mm ²)		
			Central	Middle	Peripheral
DBA/2J	3	N/A	4779 ± 449	3683 ± 755	2720 ± 560
	9	Vehicle	2576 ± 533	2304 ± 636	1202 ± 230 [*]
	9	Memantine	4671 ± 873	3650 ± 761	2468 ± 709 ^{**}

Values are given as means ± SD. Comparison of three experimental conditions was evaluated using one-way analysis of variance (ANOVA) and the Bonferroni *t*-test.

^{*} Significant at *P*<0.05 compared with 3 month-old DBA/2J mice or

^{**} significant at *P*<0.05 compared with vehicle-treated 9 month-old glaucomatous DBA/2J mice, n=4 retinal flatmounts/mice/group.

Table 2

Real Time PCR primer and probe sequences for gene expression in mouse retina

Gene (Accession #)	Type	Sequences (5'-3')
OPA1 (NM_133752)	Forward	TGACAAACTTAAGGAGGCTGTG
	Reverse	CATTGTGCTGAATAACCCTCAA
Roche Applied Science	Probe	Universal Probe Library probe #91
		Cat.#04692080001
Dnm1 (NM_010065)	Forward	CGGTTAGACAGTGCACCAAG
	Reverse	GGATGTGGGTGGTCACAAT
Roche Applied Science	Probe	Universal Probe Library probe #52
		Cat.#04688490001
Bcl-2 (NM_177410)	Forward	GATCATGCCGTCCTTAGAAAA
	Reverse	CTGCTTTTATTTCATGAGGTACATT
Roche Applied Science	Probe	Universal Probe Library probe #69
		Cat.#04688686001
Bax (NM_007527)	Forward	GTGAGCGGCTGCTTGTCT
	Reverse	GGTCCCGAAGTAGGAGAGGA
Roche Applied Science	Probe	Universal Probe Library probe #83
		Cat.#04689062001
GAPDH (NM_008084)	Forward	CAACGGGAAGCCCATCAC
	Reverse	CGGCCTCACCCATTG
	Probe	CTTCCAGGAGCGAGACCCCACTAACA

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