Suppression of Mitochondrial Oxidative Stress Provides Long-term Neuroprotection in Experimental Optic Neuritis

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PURPOSE. Axonal loss is thought to contribute to the persistence of visual loss in optic neuritis and multiple sclerosis (MS). The mechanisms of injury are poorly understood. The authors investigated the contribution of mitochondrial oxidative stress and the effects of modulating mitochondrial antioxidant gene expression in the optic nerves of mice induced with experimental allergic encephalomyelitis (EAE), with a focus on long-term neuroprotection.

METHODS. Optic nerves from mice with EAE were probed for reactive oxygen species (ROS) with the use of dichlorofluorescein diacetate (DCFDA), dihydroethidium, and cerium chloride. To modulate mitochondrial oxidative stress, recombinant AAV containing the human SOD2 gene or a ribozyme targeting murine SOD2 was injected into the vitreous. Control eyes received the recombinant virus without a therapeutic gene. Mice were sensitized for EAE and were monitored by serial contrast-enhanced MRI. The effects of SOD2 modulation on the EAE optic nerve were gauged by computerized analysis of optic nerve volume, myelin fiber area, and retinal ganglion cell loss at 1, 3, and 12 months after sensitization for EAE.

RESULTS. ROS were detected in the EAE optic nerve as early as 3 days after antigenic sensitization. Colocalization suggested mitochondria as the source of ROS activity in the absence of inflammation. The ribozyme suppressing SOD2 gene expression increased myelin fiber injury by 27%. Increasing SOD2 levels twofold in the optic nerve by virally mediated gene transfer ameliorated myelin fiber injury by 51% and RGC loss fourfold, limiting it to 7% in EAE at 1 year.

CONCLUSIONS. Amelioration of mitochondrial oxidative stress by SOD2 gene delivery may be a therapeutic strategy for suppressing neurodegeneration in optic neuritis. (Invest Ophthalmol Vis Sci. 2007;48:681–691) DOI:10.1167/iovs.06-0553

Experimental autoimmune encephalomyelitis (EAE) is an autoimmune inflammatory disorder of primary central nervous system (CNS) demyelination that is frequently used as an animal model system for studying the pathogenesis and testing the efficacy of treatments for multiple sclerosis (MS). In both disorders, axonal and neuronal loss is thought to play a key role in irreversible loss of function and disability.

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The mechanisms leading to this neurodegeneration, however, are poorly understood. A major hypothesis is that axons are transected by inflammatory cells, but this does not explain the degeneration of neurons seen before the inflammatory cell infiltration or the progressive loss of function after the inflammatory phase has subsided.

Our previous work focused on the role of inflammatory cells as mediators of optic nerve injury by the release of reactive oxygen species (ROS) into the extracellular microenvironment. Recent studies have demonstrated that retinal ganglion cell and optic nerve injury induced by axotomy are accompanied by increased ROS activity. This appears to be mediated by mitochondria. Mitochondria are the primary source of cellular ROS. Increased ROS activity is linked to many neurodegenerative diseases that have axonal and neuronal loss as a major feature. Although ROS have been recognized as key mediators of CNS injury in patients with EAE and multiple sclerosis (MS), the contributions of mitochondria to ROS activity and cell death have not.

We hypothesize that mitochondrial dysfunction plays an important role in the neurodegeneration of optic neuritis and MS and that this process begins much earlier than believed. Here we explore the role of mitochondrial oxidative stress and the potential of gene-based modulation of antioxidant gene expression in the visual system of mice induced with EAE, with a focus on lasting suppression of axonal and neuronal degeneration.

METHODS

Recombinant Adeno-Associated Virus

The adeno-associated AAV vector backbone pTR-UF was used to accept the SOD2 and RzSOD2 cDNAs. Gene expression was driven by the hybrid cytomegalovirus (CMV) and chicken β-actin promoter (SOD2 and RzSOD2). The resultant pTR-SOD2 and pTR-RzSOD2 plasmids were amplified, then purified and packaged as AAV serotype 2 vectors. The resultant rAAV-packaged SOD2 and humanized GFP control viruses were assayed. Each virus preparation contained 1011 to 1012 genome copies/mL and 109 to 1010 infectious center units/mL.

Cell Culture and Immunochromic and RNA Analyses of SOD2

Mouse fibroblasts (NIH/3T3) and retinal ganglion cells (RGC-5) were grown in Dulbecco modified Eagle medium (DMEM; Fisher Scientific, Pittsburgh, PA) supplemented with 10% heat-inactivated fetal bovine serum and 1% penicillin streptomycin (Sigma, St. Louis, MO) at 37°C with 5% CO2. Cells were grown in 15-cm dishes and were infected at a multiplicity of infection (MOI) of 5000 particles per cell. Two days after AAV infection, cells were harvested and mitochondria were isolated from AAV-SOD2 transgenic cells, and controls were infected with AAV-GFP. Briefly, this involved washing the trypsinized cells in cold PBS, followed by resuspension in a buffer consisting of 50 mM Tris-HCl, 0.21 M D-mannitol, 70 mM sucrose, 0.1 M phenylmethylsulfonyl fluoride (PMSF), 3 mM CaCl2, 20 mM EDTA, pH 7.5. Cells were then manually homogenized. Homogenates were centrifuged at 1200g
for 10 minutes at 4°C. The resultant supernatant containing the mitochondrial fraction was collected and then centrifuged at 12,000g for 20 minutes at 4°C. The pellet containing the mitochondria was washed and resuspended in buffer consisting of 50 mM Tris-HCl, 10 mM EDTA, and 20% sucrose, pH 7.5, and was stored at -80°C for later analysis.

For immunodetection, 15 μg protein from the isolated mitochondrial pellet was separated on a 10% SDS polyacrylamide gel and electrotransferred to a polyvinylidene fluoride membrane (Bio-Rad, Hercules, CA). Protein content of the samples was measured (Dc Protein Assay; Bio-Rad). We immunostained the membrane with polyclonal anti–SOD2 antibodies (Stressgen Bioreagents, Victoria BC, Canada) and then with goat anti–rabbit IgG horseradish peroxidase (HRP)-conjugated secondary antibodies (Sigma). We detected complexes using the enhanced chemiluminescence (ECL) system (Amersham Pharmacia Biotech, Piscataway, NJ). Immunostained fragments were quantified by densitometry with the use of NIH Image (available by ftp from zippy.nlm.nih.gov/or from http://rsb.info.nih.gov/nih-image). Anti–mouse β-actin antibody was used as an internal control for protein loading.

A superoxide dismutase assay kit (Calbiochem, San Diego, CA) was used to test SOD2 activity in the mitochondrial isolates according to the manufacturer’s instructions. Briefly, the isolated mitochondria were incubated with 1-methyl-2-vinylpyridinium (R2) at 37°C for 1 minute. The reagent 5,6,6a,11b-tetrahydro-3,9,10-trihydroxybenzo[c]-]fluorine (R1) was then added. The R1 reagent undergoes alkaline autoxidation, which is accelerated by superoxide dismutase and yields a chromophore. Kinetic measurement of the 525-nm absorbance change was performed with the use of a plate reader (Power Wave; Bio-TEK Instruments Inc., Winooski, VT) after the addition of R1. SOD activity was determined from the ratio of the auto-oxidation rates measured in the samples and in the assay control, deionized water. One SOD 525 activity unit is defined as the activity that doubles the auto-oxidation rate of the assay control. SOD activity was expressed in SOD 525 U/mg protein.

To quantify SOD2 mRNA levels, total RNA of SOD2-transfected murine 3T3 cells was extracted with a kit (RNeasy Mini Kit; Qiagen, Valencia, CA), according to the manufacturer’s specifications. For detection of transfected human SOD2 RNA, we used a full-length probe of human SOD2 cDNA. Mouse GAPDH DNA probe (905 bp/fragment) used as an internal control was purchased (Ambion, Austin, TX). All probes used in Northern blot analysis were labeled with α-[32P]ATP. Twenty micrograms total RNA was fractionated in a 1.2% agarose gel containing 1% formaldehyde, transferred to a nylon membrane, and fixed by ultraviolet (UV) light cross-linking. The filter was hybridized at

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**Figure 1.** Fluorescence microscopy of ROS. Superoxide anion was undetectable in a normal optic nerve cross-section (A) that showed labeling of mitochondria with green labeling (B, C). Three days after sensitization for EAE, red labeling with dihydroethidium revealed superoxide anion in the optic nerve (D). ROS activation was associated with diminished green labeling of mitochondria (E) relative to the normal optic nerve (B). Some colocalization is shown in the merged panel (F). SOD2 gene inoculation attenuated superoxide in the 3-day EAE optic nerve (G) relative to untreated EAE (D) with diminished mitochondrial labeling (H), particularly at foci at which superoxide anion was highly expressed (I). In a longitudinal section of the 6-day EAE optic nerve, red labeling of mitochondria (J) was associated with hydrogen peroxide detected by green labeling with DCFDA (K), often colocalizing with red labeling except at several perivascular foci (arrows) where hydrogen peroxide was highly expressed (L).
Induction of EAE
All mice in this study were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and with the approval of the University of Florida Institutional Care and Use Committee. Experimental allergic encephalomyelitis was induced in mice by sensitization with 0.2 mL sonicated homologous spinal cord emulsion in complete Freund adjuvant (Difco, Detroit, MI) injected subdermally into the nuchal area 2 weeks after intraocular injection of rAAV or on the same day as rAAV injections.

Intraocular Injections
Two microliters rAAV (RzSOD2-RzSOD20 mice, SOD2-SOD50 mice) was injected into the vitreous cavity of DBA/1J mice. These mice were simultaneously sensitized for EAE. To allow sufficient time for expression of the AAV-delivered transgene (SOD2) during early EAE, however, 10 mice received intravitreal injections of AAV-SOD2 into the right eyes, but they were sensitized for EAE 2 weeks after intraocular injections and then humanely killed 3 days later. As internal controls for the placebo effects of ocular injection, the left eyes of mice sensitized for EAE received AAV-GFP. Ten mice that received no intraocular injection served as controls for unadulterated EAE. Ten unadulterated mice were sensitzed for EAE 2 weeks after intraocular injection and then humanely killed 3 days later.

Detection of ROS and Mitochondrial Selective Probes
Mice received overdoses of sodium pentobarbital 3 days (10 mice) and 6 days (10 mice) after antigenic sensitization. Globes and attached optic nerves were immediately dissected and then processed for fluorescent double staining for comparison with ocular specimens obtained from normal animals (10 mice) not sensitized for EAE. To detect intracellular ROS generation, we used two probes (Molecular Probes, Eugene, OR). The probe 2′,7′-dichlorofluorescein diacetate (DCFDA) was used to detect hydrogen peroxide. DCFDA has no fluorescence until it passively diffuses into cells, where intracellular esterase cleaves the acetates and the oxidation of DCFDA by H2O2 produces a green fluorescent signal. Dihydroethidium (DHE) was used to detect mitochondrial superoxide (O2\textsuperscript{-}). Superoxide oxidizes DHE to a red fluorescent signal. Dyes (MitoTracker; Molecular Probes; red, M-7512; green, M-7514) were costained with ROS probes. After a brief rinse in PBS, tissues were incubated for 20 minutes at 37°C with a mixture of 10 μM DCFDA plus 0.3 μM red or 2.5 μM dihydroethidium plus 0.1 μM green (both MitoTracker; Molecular Probes). Tissues were washed with PBS, fixed with cold 4% paraformaldehyde for 2 hours, processed for cryomicroscopy, then observed under a fluorescence microscope (Leitz, Wetzlar, Germany) or confocal microscope (Bio-Rad).

Immunohistochemistry
Retinas and optic nerves were dissected from 10 mice 3 days after antigenic sensitization for EAE and from 10 mice 6 days after EAE sensitization, for comparison with 10 control mice 3 days after inoculation with the AAV-GFP vector. Tissue sections were stained with an anti–macrophage antibody (green) and an anti–oligodendrocyte antibody (red) and revealed TUNEL-positive cells (arrows) as oligodendrocytes (E). Oligodendrocytes (arrows) in adjuvant-inoculated nerves were TUNEL negative (F).

FIGURE 2. Inflammation. Toluidine blue staining (A) and immunofluorescence labeling with an anti–macrophage antibody (B) show no inflammatory cells in the 3-day EAE optic nerve. In the 30-day EAE optic nerve, inflammatory cells (C, arrows) were identified as macrophages (D).

FIGURE 3. Apoptosis. TUNEL-positive cells were seen in the ganglion cell layer of the retina in 3-day EAE animals (A) but not in controls inoculated with the adjuvant (B). In addition, the 3-day EAE optic nerve revealed TUNEL-positive cells (C, arrows). Optic nerves of animals inoculated with the adjuvant were TUNEL negative (D). Colocalization with an anti–oligodendrocyte antibody identified TUNEL-positive cells (arrows) as oligodendrocytes (E). Oligodendrocytes (arrows) in adjuvant-inoculated nerves were TUNEL negative (F).
FIGURE 4. Modulation of antioxidant genes in acute optic neuritis. Relative to the normal optic nerve head (A, arrows) and displacement of the peripapillary retina are seen in acute EAE (B, arrows). A ribozyme suppressing SOD2 expression markedly increases optic nerve head swelling in EAE-sensitized mice (C, arrows), whereas SOD2 overexpression suppresses it (D, arrows). Retinas of a normal animal (E) and a 1-month-old EAE animal (F) contrast with the severe loss of the RGC layer in a 1-month EAE animal inoculated with the SOD2 ribozyme (G) and with SOD2 treatment (H). Relative to the normal unmyelinated optic nerve head (I), transmission electron microscopy reveals hydropic degeneration of axons and mitochondria (arrow) in a 1-month EAE animal (J) that is exacerbated by suppressed SOD2 expression with the ribozyme (K, asterisks) and is ameliorated by SOD2 overexpression (L). Compared with the normal retrobulbar optic nerve in which myelinated axons (a) are evident (M), inflammatory cells (IC) and demyelinated axons (a) are seen in a 1-month EAE animal (N). The ribozyme against SOD2 increased axonal loss and myelin loss (O), whereas SOD2 gene transfer ameliorated them (P). Compared with axons and mitochondria of the normal nerve (Q), electron-dense cerium perhydroxide reaction product (arrows) is evident within mitochondria, some swollen with dissolution of cristae, in the optic nerve of a 1-month EAE animal (R). The ribozyme against SOD2 increased hydropic degeneration of mitochondria (arrows) in EAE-sensitized animals even in myelinated axons (S), whereas SOD2 suppressed mitochondrial (arrows) and axonal injury in acute optic neuritis (T). (U) Histogram of myelin fiber area in the normal optic nerve, uninoculated EAE, RzSOD2 treatment in EAE,
lotion with only Freund adjuvant and with 10 normal unsensitized mice. After washes in increasing concentrations of sucrose PBS buffer, isolated tissues were snap-frozen and stored at -20°C. Tissues were sectioned on a cryostat (Leitz). After blocking in 5% normal goat serum, they were reacted with primary anti-macrophage (epitope, F4/80 antigen) or anti–oligodendrocyte (epitope, full-length cyclic nucleotide phosphodiesterase [CNPase]) antibodies (Abcam, Inc., Cambridge, MA) followed by incubation with Cy2-conjugated anti-mouse secondary antibodies (Jackson Laboratory, Bar Harbor, ME), then visualized by fluorescence microscopy. Additionally, apoptotic cell death was assessed with terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) according to the specifications of the manufacturer of the reaction kit (Roche, Indianapolis, IN).

GFP Expression

Retinas and optic nerves of GFP-infected eyes were sectioned and examined for GFP expression 1 month after sensitization for EAE. To determine whether inflammatory cells or oligodendrocytes expressed GFP, we reacted the sectioned tissues against the anti–macrophage or anti–oligodendrocyte antibody counterstained red with Cy3. Tissues were examined by fluorescence microscopy for colocalization or GFP with the cell markers.

MRI Analysis

At 2 weeks and at 1, 3, 5, 6, and 12 months after EAE sensitization and viral inoculation, high-resolution 3-dimensional (3D) MRI of mouse optic nerve was performed with the use of a 4.7-Tesla magnet (Oxford Instruments Limited, UK). Animals were anesthetized with isoflu- ranne 1.5%-2% (IsoFlo; Abbott Laboratories, Queenborough, Kent, UK) while lying in a prone position with their heads firmly fixed in a purpose-built surface coil. T1-weighted 3D image acquisitions were performed immediately after intraperitoneal administration of gadolinium (Gd)-DTPA (Berlex Laboratory, Wayne, NJ) at a dose of 0.2 mmol/kg body weight. With the use of an appropriate workstation and software (O2 workstation, Paravision 2.212 [Silicon Graphics, Mountain View, CA]; MRI core software [University of Florida Brain Institute, Gainesville, FL]), the volume of the optic nerve was quantified. For statistical analysis, AAV-GFP–inoculated left eyes were compared with right eyes that underwent AAV-SOD2 rescue gene. Statistical analysis was performed by Student’s t test for unpaired data.

Light and Electron Microscopy

Mice received overdoses of sodium pentobarbital 1, 3, and 12 months after viral inoculation. They then underwent immediate intracardiac perfusion with fixative consisting of 4% paraformaldehyde and 2% glutaraldehyde in 0.1 M PBS buffer (pH 7.4). For detection of in vivo H2O2, we perfused with a mixture consisting of 2 mM cerium chloride, 10 mM 3-amino-1,2,4-triazole, 0.8 mM NADH, 0.1 M PBS buffer (pH 7.5), and 7% sucrose followed by perfusion with the fixative. Eyes with attached optic nerves were dissected and further processed by immersion in 2.5% glutaraldehyde and postfixed in 1% osmium tetroxide and 0.1 M sodium cacodylate-HCl buffer (pH 7.4). Tissue was dehydrated through an ethanol series to propylene oxide, infiltrated, and embedded in epoxy resin polymerized at 60°C overnight. Semithin longitu- dinal sections (0.5-1 μm) of the optic nerve head and retrobulbar nerve were made and stained with toluidine blue. Ultrathin sections (90 nm) were cut and placed on nickel grids for immunocytochemistry. Nonspecific binding of antibodies was blocked by floating the grids on 5% normal goat serum in 0.01 M Tris-buffered saline (pH 7.2) with Tween 20 (TBST) for 30 minutes. They were then reacted with rabbit anti-MnSOD antibodies. After washes in 0.1 M PBS, the grids were reacted with the secondary goat anti-rabbit IgG anti- bodies conjugated to 10 nm gold for 1 hour at room temperature. After washes in buffer, grids were rinsed in deionized water. For examination by low-magnification transmission electron microscopy, we used a kit to enlarge the immunogold particles by silver enhancement (Ted Pella, Redding, PA). To check for nonspecific binding of the secondary antibody, control grids were incubated in the buffer, followed by the gold-labeled antibody. Immunolabeled and control specimens were photographed by transmission electron microscopy with and without post-staining (model H-7000 or H7600; Hitachi, Tokyo, Japan) operating at 75 to 80 kV.

Morphometric Analysis

Morphometric analysis was performed in a masked fashion as previously described.22 Briefly, images of toluidine blue-stained sections of the retina and optic nerve were captured with a video camera mounted on a light microscope, and then data were entered into the computer memory. Myelin fiber areas and ganglion cell counts were quantified with NIH Image software. For statistical analysis, AAV-SOD2 and RzSOD2-inoculated right eyes were compared with left eyes that received the control virus rAAV-GFP and with the uninoculated eyes of animals with EAE and the eyes of normal unsensitized animals. Statis- tical analysis was performed with the Student t test for unpaired data.

RESULTS

Mitochondrial Oxidative Stress Starts Early

As an initial gauge of ROS activity, we used the fluorescent probes DCFDA and DHE. DCFDA detects H2O2, and DHE is a probe for O2-•. The optic nerves of 10 mice humanely killed 3 days after antigenic sensitization and 10 mice humanely killed 6 days after antigenic sensitization and 10 normal unsensitized mice were excised and incubated in DCFDA and red dye or dihydroethidium and green dye (MitoTracker; Molecular Probes). Fluorescence microscopy of the cryosectioned EAE specimens revealed that relative to normal optic nerve in which superoxide anion was undetectable (Figs. 1A, 1C), superoxide detected by red labeling with dihydroethidium was seen as early as 3 days after sensitization for EAE (Fig. 1D). ROS activation was associated with some loss of optic nerve mitochon- drial membrane potential, as detected by diminished green labeling (MitoTracker; Molecular Probes; Fig. 1E) relative to the normal optic nerve (Fig. 1B). Because of the loss of membrane potential, we could not use colocalization shown in the merged panel (Fig. 1F) to prove that mitochondria were the source of superoxide anion in early EAE. We also found that red labeling (MitoTracker; Molecular Probes) of mitochondria (Fig. 1J) was associated with hydrogen peroxide detected by green labeling with 2’-7’-DCFDA (Fig. 1K). Colocalization (MitoTracker; Molecular Probes) sug- gested the source of hydrogen peroxide was mitochondria of the optic nerve (Fig. 1L). In general, the loss of membrane potential did not appear to be associated with the presence of hydrogen peroxide. However, loss of red labeling (Mito- Tracker; Molecular Probes) indicated that mitochondrial mem- brane potential was diminished at several perivascular foci where hydrogen peroxide was highly expressed (Fig. 1L).

RzSOD2 treatment in normal unsensitized mice, and SOD2-inoculated eyes compared with treatment with AAV-GFP. It shows the anti-SOD2 ribozyme exacerbated acute optic neuritis, decreasing myelin fiber area by 23% compared with treatment with AAV-GFP in mice sensitized for EAE (P < 0.01). Myelin fiber area in mice sensitized for EAE and injected with the anti-SOD2 ribozyme was reduced 41% compared with mice injected with RzSOD2 but not sensitized for EAE (P < 0.005). Treatment with AAV-SOD2 was beneficial, with a 46% protective effect on myelin fiber preservation relative to the contralateral eyes treated with AAV-GFP (P < 0.01). INL, inner nuclear layer.
DCFDA labeling was absent in the optic nerves of unsensitized control mice.

We examined the effect of SOD2 gene inoculation on dismutation of superoxide during early EAE. Ten mice received intravitreal injections of AAV-SOD2 into the right eyes. They were sensitized for EAE 2 weeks after intraocular injections to allow sufficient time for expression of the AAV-delivered transgene (SOD2) and then were humanely killed 3 days after antigenic sensitization. Examination of SOD2-inoculated optic nerves revealed diminished DHE (Fig. 1G) and green labeling (MitoTracker; Molecular Probes; Figs. 1H, 1I) compared with eyes not treated with AAV-SOD2. Clearly, SOD2 gene inoculation reduced the accumulation of superoxide anion.

**Inflammatory Cells Are Absent during Early EAE**

We excluded inflammatory cells as the source of superoxide anion and hydrogen peroxide in early EAE by light microscopic examination of toluidine blue-stained optic nerves (Fig. 2A) and by immunofluorescence labeling with an anti–macrophage antibody (Fig. 2B). As positive controls the presence of inflammatory cells is shown in the 1-month EAE optic nerve (Figs. 2C, 2D). Clearly, ROS activation began before CNS infiltration by inflammatory cells, long thought to be the source of mediators of tissue injury in EAE and MS.23

**Apoptosis Starts during Early EAE**

Given that ROS exposure has been linked to loss of mitochondrial membrane potential11,24 leading to the release of cytochrome c and to apoptosis,25-27 we examined the optic nerves and retinas of 10 mice—sensitized for EAE 3 days earlier—for apoptosis. Ten mice inoculated with only Freund adjuvant served as controls. We found TUNEL-positive cells in the ganglion cell layer of the retina in 3-day EAE animals (Fig. 3A) but not in controls inoculated with the adjuvant (Fig. 3B). In addition, we saw TUNEL-positive cells in the 3-day EAE optic nerve (Fig. 3C) but not in controls inoculated with the adjuvant (Fig. 3D). TUNEL-positive cells in the nerve were identified as oligodendrocytes by colocalization with an anti–oligodendrocyte antibody (Fig. 3E). Oligodendrocytes in the adjuvant-inoculated nerves were TUNEL negative (Fig. 3F).

**Acute Experimental Optic Neuritis**

One month after sensitization of 10 mice for EAE, we found electron-dense cerium perhydroxide reaction product formed by the reaction of perfused cerium chloride and endogenous hydrogen peroxide within mitochondria (Fig. 4R), some of which were swollen and showed dissolution of cristae (Fig. 4J). These mitochondrial findings were not limited to fibers that had lost their myelin sheaths, often considered the hallmark of MS and EAE,28 but were more widespread. Myelinated axons also contained swollen mitochondria that exhibited disorganization and dissolution of cristae, some to the point that only a double membrane sheath identified the organelle. At this stage of EAE, mononuclear inflammatory cells involved in active demyelination were prevalent in the retrolubar nerve (Fig. 4N). Quantitative comparisons with the optic nerves of five normal mice that were not sensitized for EAE showed that eyes from animals undergoing acute EAE lost half their optic nerve myelin fiber area compared with normal optic nerve (Fig. 4U).

**Lowering Mitochondrial Antioxidant Protection Exacerbates Acute Optic Neuritis**

To support the pathogenicity of mitochondrial ROS activity in EAE, we first suppressed antioxidant defenses in the organelle by using a ribozyme designed to target the SOD2 mRNA for destruction.29 This ribozyme, delivered by an AAV-2 vector, was injected into the vitreous cavity of the right eyes of 10 mice, whereas the left eyes were injected with AAV-GFP. One month later, immunoblots revealed the MnSOD level was reduced by half in the pooled optic nerves of AAV-RzSOD2-injected eyes relative to the left eyes injected with AAV-GFP (data not shown). Next, we tested the effect of lowered SOD2 levels on experimental optic neuritis by injecting the AAV-containing anti–SOD2 ribozyme into the right eyes of 10 animals sensitized for EAE and 10 normal mice that were not sensitized for EAE. The left eyes of both groups received control treatment by injection with AAV-GFP. Mice were humanely killed 1 month later. We found that reducing mitochondrial SOD2 activity with the anti–SOD2 ribozyme exacerbated mitochondrial and axonal swelling, RGC, and myelin fiber loss (Figs. 4C, 4G, 4K, 4O, 4S, 4U) compared with controls, normal mice injected with the AAV-RzSOD2 but not sensitized for EAE (not shown), and normal mice sensitized for EAE but treated by inoculation with AAV-GFP (Fig. 4U). In fact, the severe optic nerve head swelling induced by the anti–SOD2 ribozyme accompanied by EAE (Figure 4C) was worse than in any other animal examined in this study. Relative to AAV-RzSOD2-treated EAE eyes, the EAE-only optic nerve head (Figs. 4B, 4J), retina (Fig. 4F), and retrolubar nerve (Figs. 4N, 4U) showed less severe RGC, axonal and mitochondrial swelling, and demyelination. The normal optic nerve head (Figs. 4A, 4I), retrolubar nerve (Figs. 4M, 4Q), and retina (Fig. 4E) are shown for comparison with untreated EAE and ribozyme treatment (Fig. 4U).

**Increasing Mitochondrial Defenses Ameliorate Acute Optic Neuritis**

Next, we bolstered mitochondrial anti-ROS defenses. In vitro, inoculation of murine fibroblasts (3T3) with the human SOD2 AAV resulted in the expression of human SOD2 mRNA (Fig. 5A) and increased mitochondrial MnSOD protein approximately sixfold in rat retinal ganglion cells (RGC-5; Fig. 5B) and almost twofold in the murine optic nerve (Fig. 5C). Mitochondrial SOD activity in cultured retinal ganglion cells (RGC-5) was increased approximately 2.5-fold compared with cells treated with AAV-GFP (Fig. 5D). Injection of AAV containing the SOD2 gene into the mouse eye increased MnSOD immunogold in the optic nerve (Fig. 5F) compared with control AAV-GFP inoculation (Fig. 5E). The MnSOD immunogold was found predominantly in the mitochondria of axons of the optic nerve.

We compared the AAV-SOD2–inoculated right eyes of 10 mice sensitized for EAE and humanely killed 1 month later with the left eyes that received control treatment by injection with AAV-GFP. We found that AAV-SOD2–inoculated nerves (Figs. 4D, 4H, 4L, 4P, 4T, 4U) exhibited less mitochondrial and axonal swelling, with 46% more myelin fiber preservation than the contralateral nerves treated by inoculation with AAV-GFP (Fig. 4U). Suppression of mitochondrial injury by SOD2 was not limited to demyelinated axons but was also seen in myelinated axons that exhibited less hydropic degeneration, disorganization, and dissolution of cristae (Fig. 4T).

**Long-term Antioxidant Gene Transfer Suppresses Optic Neuritis**

Next, we tested the effects of the SOD2 construct on chronic EAE, focusing on long-term neuroprotection. The right eyes of 20 mice received intraocular injections of AAV-SOD2, and the left eyes were injected with a control AAV expressing GFP. To follow the effects of modulation of experimental optic neuritis in living animals, we used volume measurements of the optic nerve obtained by serial 3-D magnetic resonance imaging (MRI) performed at 2 weeks and then at 1, 3, 4, 6, 7, and 12 months after sensitization for EAE. Because ocular injections may be
Suppression of Mitochondrial Oxidative Stress

The protective effect of SOD2 seen in the optic nerve was mirrored in the retina, the site of retinal ganglion cell bodies. Unlike our observations in acute EAE, in which substantial degeneration of the nerve fiber layer and loss of RGCs was mainly evident in eyes inoculated with anti-SOD2 ribozyme, the RGC loss predominated in the chronic stages of EAE (Figs. 6I, 6K). AAV-SOD2 treatment helped to preserve the nerve fiber layer and RGCs (Figs. 6J, 6L). Quantitative analysis revealed a 32% loss of RGCs 1 year after sensitization for EAE compared with normal animals (Fig. 7C). We found that SOD2 suppressed ganglion cell loss fourfold, limiting it to 7% in EAE. Even at 1 year, RGCs of unprotected and AAV-GFP–inoculated EAE animals were still undergoing apoptosis (Fig. 6K), suggesting that the neurodegenerative process was ongoing and active even this late in the disease course.

**GFP Expression in EAE**

One month after sensitization for EAE, we found GFP expression in the retina exclusively in ganglion cells (Figs. 8A–C).
Although some GFP labeling was evident in the optic nerve (Figs. 8D, 8G), it did not appear to colocalize with inflammatory cells (Figs. 8E, 8F) or oligodendrocytes (Figs. 8H, 8I). 

Thus, the tropism of AAV2 for RGCs was not altered by EAE, suggesting that the protective effect of SOD2 was likely attributed to expression of this antioxidant enzyme in the mitochondria of RGCs and their axons (Figs. 5C, 5F).

**DISCUSSION**

We have provided evidence showing that the pathway leading to neurodegeneration in the EAE animal model of MS can be ameliorated by suppression of mitochondrial oxidative stress that began before the infiltration of inflammatory cells classically believed to be the mediators of disease activity. Although demyelination is the classic target of disease activity in EAE and MS, axonal and neuronal loss are becoming increasingly recognized as the major causes of persistent clinical disability, with mitochondria playing a substantial role in the neurodegenerative process.8,30,31 Loss of mitochondrial membrane potential can increase the release of cytochrome c, one pathway leading to neuronal apoptosis that is mediated by the Bcl-2 family of proteins.24,32,33 Bcl-2 increases in MS lesions further support a role for apoptosis in the axonal and neuronal degeneration of MS.34 We showed that increasing mitochondrial defenses against superoxide suppressed loss of mitochondrial membrane potential and protected RGCs and axons of the optic nerve. Our previous work focused on ROS released by inflammatory cells.10 Clearly, they were not the source of superoxide we found in early pre-inflammatory EAE.

As we have found here in the EAE optic nerve, a superoxide burst from mitochondria has been described with RGC injury.11 Generation of mitochondrial superoxide is predominantly mediated by complex I and III of the electron transport chain.11,35 Complex I deficiency in longstanding MS has been attributed to oxidative stress.36,37 Loss of oxidative phosphorylation activity in MS has been detected that is comparable to or exceeds the levels found in disorders of optic nerve degeneration associated with mutated mtDNA.12,38 Tajouri et al.38 also found that the expression of mitochondrial ATP synthase and cytochrome b was altered, thus implying deficits in oxidative phosphorylation induced by oxidative stress may contribute to axonal and mitochondrial injury in MS. Although superoxide may mediate injury directly, peroxynitrite, formed by the reaction of superoxide and nitric oxide, mediated nitrilation of key mitochondrial proteins in vitro and in the EAE nervous system, perhaps contributing to deficits in oxidative phosphorylation.39,40

**FIGURE 6.** Micrographs of chronic EAE. (A) Excavation of the optic nerve head (arrows) and atrophy of the AAV-GFP–inoculated retrobulbar nerve were seen 3 months after sensitization for EAE. (B) Protection with SOD2 ameliorated cupping of the optic nerve head and atrophy of the retrobulbar nerve. (C) In eyes control injected with AAV-GFP, 1 year after sensitization for EAE, excavation of the optic nerve head was advanced (arrows), extending to the lamina sclerae, and the retrobulbar nerve was markedly atrophic (double arrows). (D) Protection with SOD2 ameliorated cupping of the optic nerve head and atrophy of the retrobulbar nerve even at 1 year. Demyelinated plaques seen at 3 months in control-inoculated nerves (E) were suppressed with SOD2 (F). Cystic spaces (arrows) in the optic nerve seen 1 year after EAE sensitization in control-inoculated nerves (G) were ameliorated with SOD2 treatment (H). RGC loss predominated the chronic stages of EAE (I), contrasting with the preservation of RGCs by SOD2 at 1 year after sensitization for EAE (J). Transmision electron microscopy shows cystic spaces in the nerve fiber layer (NFL) left by degenerating RGCs and an apoptotic cell (K, arrow). SOD2 treatment preserved the NFL and RGCs (L). Axons with swollen mitochondria (arrow) in control (AAV-GFP–inoculated nerves (M) were ameliorated with SOD2 treatment showing normal axonal mitochondria (arrow), 3 months after sensitization for EAE (N). Degenerating axons, some with aggregation of mitochondria (asterisk), hydropic degeneration, and loss of cristae evidenced ongoing neurodegeneration 1 year after sensitization for EAE (O). These findings were suppressed by SOD2 1 year after sensitization for EAE (P).
were detected between SOD2-treated and normal optic nerve (P < 0.05). A one-third loss of RGCs was seen in uninoculated EAE relative to control treatment with AAV-GFP (P < 0.0001). The relatively lower levels of mitochondrial SOD in oligodendrogial cells and axons that we previously detected likely increased their vulnerability to the effects of oxidative stress. Here, by increasing MnSOD expression, we were able to suppress not only myelin loss of the optic nerve but also mitochondrial vacuolization, swelling, and dissolution of cristae of optic nerve axons. The localization of the GFP reporter gene to retinal ganglion cells and increased MnSOD in axons of the optic nerve suggested that this protective effect resulted predominantly from the neuroprotection of neurons and axons in EAE.

Suppression of axonal damage by AAV-SOD2 was apparent not only at foci of demyelination but also in the optic nerve head, which does not contain myelinated axons. At these sites, axons with normal-appearing myelin sheaths and unmyelinated axons of the ONH exhibited mitochondrial swelling with dissolution or disorganization of cristae. These abnormalities were substantially ameliorated by genetically increasing mitochondrial SOD2, thus protecting axons and myelin in the optic nerve. On the other hand, increasing oxidative stress by reducing SOD2 gene expression with the ribozyme increased our findings of cystic mitochondria, devoid of stainable contents and cristae. This is strong evidence supporting the role that the reduction of mitochondrial oxidative stress can play in modulating experimental optic neuritis.

Although inflammatory cells transect axons and cause neurodegeneration in MS, we31,32 showed here that mitochondrial ROS suppression with AAV-SOD2 was effective not only before and during inflammation but also after the inflammation subsided. This is not unlike MS, which eventually becomes a disease characterized by progressive neuronal and axonal degeneration. In EAE we found that the apoptosis of RGCs, together with mitochondrial and axonal degeneration, were still active 1 year after sensitization for EAE, long after the inflammatory phase had subsided. Increasing mitochondrial antioxidant defenses provided long-term neuroprotection against RGC and axonal loss.

At present, treatment for patients with optic neuritis who do not recover vision is limited.33 Recent OCT measurements showing loss of macular volume in MS and optic neuritis suggest this may be caused by RGC loss.34 Fortunately, visual function tests for most patients, followed up for 10 years after an initial attack of optic neuritis, show relatively mild impairment.35 However, for those with severe visual loss that has persisted for more than 6 months, there is no remedy. Intravenous immunoglobulin proved unsuccessful in restoring visual function in MS patients blinded by recurrent attacks of optic neuritis.46 Because the neurodegeneration of MS does not appear to be substantially driven by inflammation, it is refractory to immunomodulatory drugs.47,48 Although these drugs suppress the inflammatory phase of the disease, an additional approach is needed to tackle the neurodegenerative component.

We have shown here that the mitigation of ganglion cell death and the loss of axons and myelin in experimental optic neuritis may be achieved by genetically induced expansion of mitochondrial defenses against superoxide. Increasing antioxidant defenses in the optic nerve using the AAV viral vector offers some promise for the future. The optic nerve is a readily accessible site for gene transfer, particularly with the AAV2 vector that selectively infects RGCs. The optic nerve is rich in mitochondria, which are widely accepted as the major intracellular source of ROS,49 thus making the nerve more susceptible to mitochondrial perturbations. In addition, RGCs whose

Our previous work had detected endogenous increases in the expression of MnSOD in mitochondria, induced by cytokines released by infiltrating inflammatory cells, in the undulaterated optic nerves of EAE animals.41 However, this endoge-
axons constitute the optic nerve are highly dependent on oxidative phosphorylation, and the optic nerve is a frequent initial site of involvement in MS. Increasing mitochondrial SOD expression provided long-term neuroprotection against EAE in the optic nerve for most of the lifespan of a laboratory mouse. Still, the injury we detected in early EAE suggests that this approach may have the best chance of success if initiated at the earliest stages of disease, to reduce the cumulative injury beyond which loss of function becomes irreversible. Whether a similar strategy applied to patients may help avert the demise of axons, neurons, and oligodendroglia in optic neuritis and MS remains to be demonstrated.

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References


