Retinal Ganglion Cell Axotomy Induces an Increase in Intracellular Superoxide Anion

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PURPOSE. Retinal ganglion cells (RGCs) undergo apoptosis after axonal injury. The time course of cell death is variable and depends in part on the degree of injury sustained. Decreasing reactive oxygen species (ROS) levels or shifting the redox state to reduction promotes the survival of RGCs in tissue culture after axotomy. It was hypothesized that a specific ROS, superoxide anion, acts as an intracellular signaling molecule for RGC death after axotomy.

METHODS. Intracellular superoxide levels were measured after dissociation in retrograde-labeled rat RGCs with use of the superoxide-sensitive fluorophores hydroethidium and MitoSOX Red. Having found a significant increase, the effect of axotomy was determined on superoxide levels independent of dissociation with an optic nerve crush model.

RESULTS. Optic nerve crush caused RGCs to undergo a superoxide burst. The burst was asynchronous and was manifested in only a fraction of cells at any given time. Neurotrophin deprivation was not responsible for the superoxide burst because it was not prevented by incubation with the neurotrophic factors brain-derived neurotrophic factor, ciliary neurotrophic factor, forskolin, or insulin. Several inhibitors of intracellular superoxide generation were studied, but only antimycin A, which inhibits complex III of the mitochondrial electron transport chain, blocked the increase in superoxide.

CONCLUSIONS. These findings suggest that superoxide generated in the mitochondrial electron transport chain could be a parallel system to neurotrophic deprivation for signaling cell death after axonal injury. (*Invest Ophthalmol Vis Sci.* 2006;47: 1477–1485) DOI:10.1167/iovs.05-0921

Retinal ganglion cells (RGCs) undergo apoptosis after axonal injury.[1] After axotomy, a number of important steps in the apoptotic cascade are initiated, including release of cytochrome c[2] and subsequent activation of caspases 9 and 3.[3,4] The release of cytochrome c as an early step in this process implicates the mitochondria as having an important role in RGC death, but the mechanisms that trigger these changes after injury are not fully understood.

Reactive oxygen species (ROS) play many important roles in cells, including gene expression[5] and intracellular signaling.[6] They are made during the production of mitochondrial energy.[7] However, high levels of ROS play important roles in the signaling of cell death in several neuronal systems, including RGCs.[8] Our studies and those of others[8-10] have indicated that ROS scavengers, a mildly reduced cellular redox state, and hypoxic conditions all contribute to the survival of RGCs after axotomy.

Growth factor deprivation through the loss of retrograde transport is believed to be one of the initiators of the apoptotic pathway in RGCs after axotomy. Transport of brain-derived neurotrophic factor (BDNF) from targets such as the superior colliculus is blocked by acute intraocular hypertension,[11] and the intravitreal delivery of BDNF and other neurotrophins has been shown to promote the survival RGCs after axotomy in vivo[12] and in vitro.[13]

Previous work in our laboratory[14] has shown that axotomy causes RGCs to produce superoxide anion in response to external oxidative stress, but only 24 hours after axotomy. Given that scavenging of superoxide has been shown to slow apoptosis in sympathetic neurons after nerve growth factor deprivation,[15] we hypothesized that axotomy of RGCs should lead to the intracellular production of superoxide as an early step in signaling apoptosis and that the addition of neurotrophic factors to these cells should inhibit superoxide generation. Instead, we found that though axotomy increased superoxide levels, this increase was not synchronous, and neurotrophins, which prevent RGC death, did not prevent the increase. We also found that mitochondria are the likely source of the increase in superoxide level after axotomy and that complex III of the mitochondrial electron transport chain is necessary for the increase to occur.

METHODS

Animals

All experiments were performed in accordance with institutional, federal, state, and ARVO guidelines regarding animal research.

Materials

Cell culture reagents were obtained from Invitrogen (Carlsbad, CA). Some reagents (dihydroethidium [Het], MitoSOX Red, Sytox Green, and 4′,6-diamidino-2-phenylindole [DAPI]) were obtained from Molecular Probes (Eugene, OR). Papain was obtained from Worthington Biochemical (Freehold, NJ). BDNF and ciliary neurotrophic factor (CNTF) were obtained from Peprotech (Rocky Hill, NJ). All other reagents, unless noted, were obtained from Sigma (St. Louis, MO).

RGC Labeling and Culture

RGCs were labeled and cultured using previously described methods.[14] Briefly, ganglion cells were labeled by stereotactic injection of the fluorescence tracer DAPI (5 mM) into the superior colliculi of anesthetized postnatal day (P)4 and P5 Long-Evans rats. DAPI was chosen as the retrograde tracer for these experiments because its excitation and emission spectra do not overlap with those of HEt. Over several days, DAPI is taken up at RGC projection sites in the superior colliculi and flows retrogradely back to the RGC somas, where it binds to the nuclear DNA. At P11 to P13, animals were killed by decapitation, after which the eyes were enucleated and the retinas dissected in Hanks balanced salt solution (HBSS). After two incubations in HBSS containing papain (2.4 U/mL), each for 7 minutes at 37°C, the retinas

were gently triturated with a serologic pipette and plated on poly-L-lysine–coated, 8-well chambered coverglass wells at a density of approximately 4500 cells/mm². Cell cultures were incubated for 1, 3, 6, 9, 12, or 24 hours in medium (Neurobasal A; Invitrogen) with 0.7% methyl cellulose, 2% serum-free supplement B27 without antioxidants (Invitrogen) at 37°C, 5% CO_2.

Optic Nerve Crush Surgery

Before humane killing and retinal culture, the left optic nerve of some animals underwent crushing according to published methods.[13] Briefly, the left optic nerve was exposed by lateral orbitotomy and the nerve was gently crushed with blunt forceps, sparing the retinal and optic disc circulations. Animals were returned to their cages and killed later after 1, 4, 8, 16, 24, 72, or 168 hours had elapsed. Retinal cultures from eyes undergoing optic nerve crush were performed in parallel with cultures from the unoperated eyes. After plating, cultures were incubated for 1 hour before superoxide level measurement.

Measurement of RGC Superoxide Levels In Situ

Intracellular superoxide levels were measured by quantifying the fluorescence of the oxidation product of HEt. Oxidation of HEt by superoxide converts HEt, which exhibits weak blue fluorescence, to an ethidium derivative that exhibits peak fluorescence in the rhodamine spectrum (excitation 480 nm, emission 586 nm).[16] HEt, which is cell permeant, enters the cell and, after oxidation, accumulates in the nucleus, where it binds DNA with a small shift in its emission spectrum to 567 nm.[16] HEt was chosen for these experiments over other fluorophores because of its increased specificity for superoxide compared with other dyes, such as dichlorodihydrofluorescein, and its derivatives, which are more general reactive oxygen species probes.

At predetermined time points after plating, the chambered coverglass cultures were placed on an inverted stage microscope (Axiovert 135; Zeiss, Oberkochen, Germany) with a 100× oil immersion lens and a heated stage to maintain the culture at 37°C. RGCs were identified in mixed retinal cultures by the presence of DAPI staining, which appears bright blue with appropriate filters (excitation 330 nm, dichroic 400 nm, emission 450 nm) under epifluorescence. Cells were treated with 3.2 mM HEt in medium (Neurobasal A; Invitrogen) without phenol red for 15 minutes. After 15 minutes had elapsed, RGCs were located by scanning horizontally across the width of each well, then advancing one field of view in the vertical direction and repeating the horizontal scan, stopping to measure fluorescence of all DAPI-positive cells. Images were acquired with a cooled CCD camera (Roper Scientific, Trenton, NJ) using MetaFluor software and filters appropriate for the superoxide-HEt product (excitation 480 nm, dichroic 505 nm, emission 580 nm long-pass). Fluorescence levels of RGCs were measured and recorded for as many cells as could be identified and imaged in 15 minutes. Images were acquired at a binning of 2, an exposure time of 200 milliseconds, and 1× gain. Measurements for experiments in which the animals underwent optic nerve crush were normalized to the average fluorescence of RGCs from the eyes not undergoing the procedure. For experiments not involving optic nerve crush, results were normalized to the 1-hour time point for each experiment.

Measurement of Mitochondrial RGC Superoxide Levels In Situ

Some experiments used MitoSOX Red to measure superoxide levels in the cell. MitoSOX Red is a recently developed fluorescent probe targeted to the mitochondria and highly selective to oxidation by superoxide. MitoSOX Red exhibits fluorescence similar to HEt (excitation 510 nm, emission 580 nm) and binds to mitochondrial DNA after oxidation. Cells were treated with a 5 μM MitoSOX Red solution in medium (Neurobasal A; Invitrogen) without phenol red that was replaced after 10 minutes with medium alone. Imaging of cells proceeded for 15 minutes using the settings described above for HEt, and data were analyzed similarly.

Measurement of RGC Viability

RGCs were identified by the presence of DAPI, which appears blue when viewed with appropriate filters under epifluorescence. Cell viability was determined by metabolism of calcein-AM by intracellular esterases to calcein, which fluoresces green when viewed with fluorescein filters. Cells were incubated in a 1 μM solution of calcein-AM in phosphate-buffered saline (PBS) for 30 minutes, after which the medium was replaced with fresh PBS. RGC viability was assessed at 24 hours. Wells were counted in duplicate.

Pharmacologic Treatments

Cultures were prepared in medium alone or medium supplemented with 500 U/mL polyethylene glycol–conjugated superoxide dismutase (PEG-SOD) or a combination of 50 ng/mL BDNF, 10 ng/mL CNTF, 5 μg/mL insulin, and 5 μM forskolin[17] to prevent an increase in intracellular superoxide. Menadione (1 mM final concentration) was added to induce synchronous generation of superoxide by redox cycling within the mitochondrial electron transport chain.

Inhibition of Endogenous Superoxide Production

Rotenone (1 μM), diphenyleneiodonium (DPI; 100 μM), indomethacin (1 mM), allopurinol (1 mM), and antimycin A (1 μM) were also added in some experiments to determine the role of endogenous ROS-producing systems and the electron transport chain in the production of superoxide.

Cell-free Assay for HEt Interaction

To eliminate the possibility of a direct interaction between PEG-SOD or antimycin A and HEt oxidation by superoxide, we used a modification of the method of Zhao et al.[16] Xanthine oxidase (0.05 U/mL), HEt (1 mM), and either PBS, PEG-SOD (500 U/mL), or antimycin A (100, 10, or 1 μM) were added to a 96-well black plate with clear bottom. Reaction was initiated by the addition of 1 mM xanthine to a final volume of 100 μL. The plate was incubated for 30 minutes and protected from light. Fluorescence readings were obtained (1420 Victor 2 T Multilabel Counter; Wallac, Gaithersburg, MD) with excitation at 485 nm and emission at 580 nm. Wells were counted in duplicate or greater.

Statistical Analysis

Histogram and individual cell burst data were prepared as a weighted mean of the fraction of cells in each category, with weighting relative to the sample size from each experiment. Errors for weighted means were calculated by SPSS methodology. Comparison between 2 groups was by unpaired t test. Tests for changes in level over time were performed with ANOVA for trend. Tests for changes in frequency over time were performed with χ^2 for trend. Significant differences required $P < 0.05$.

RESULTS

RGCs Undergo an Increase in Intracellular Superoxide Levels after Axotomy

We previously showed that superoxide is produced as a secondary response to oxidative stress in RGCs 24 hours after axotomy.[14] To study RGC superoxide levels generated by axotomy alone, we measured fluorescence levels with the superoxide probe. HEt in individual RGCs underwent axotomy as a result of the dissociation that comes of preparing mixed retinal cultures. Mean superoxide levels in RGCs consistently increased by a small but significant amount after dissociation. When normalized to the baseline levels at 1 hour in culture, the increase was 18% at 6 hours in culture (1.00 ± 0.04 vs. 1.18 ± 0.05; $P = 0.006$) and 26% at 12 hours (1.26 ± 0.04; $P < 0.0001$; Fig. 1A). This increase was maintained for at least 24 hours after culture (Fig. 1B). Similar findings were seen in 8 independent experiments. Study of DAPI-negative (i.e., non-RGC) cells

FIGURE 1. Retinal ganglion cells in tissue culture undergo an increase in superoxide levels after dissociation. (A) Dissociation of retinal ganglion cells induces an increase in intracellular superoxide. Mixed retinal cultures were prepared from P11 to P13 rats and incubated for 1 hour to 24 hours. Superoxide levels from approximately 8 to 12 DAPI-labeled RGCs were measured with the fluorescent probe HEt and normalized to the values at 1 hour after dissociation. Results represent the mean of 8 independent experiments. *Asterisks* represent values significantly ($P < 0.05$) different from baseline. (B) Photomicrographs of RGCs (identified by DAPI positivity) with increasing superoxide levels over time. Fluorescence is primarily nuclear, reflecting the amplification of the fluorescent properties of oxy-Et when it binds DNA. Each photomicrograph depicts a different cell with representative fluorescence for the corresponding time point. (C) HEt measures superoxide levels induced by dissociation. RGCs in mixed retinal cultures were plated in the presence of HEt, and superoxide was measured 15 minutes later (0-hour condition). Parallel cultures were incubated for 1 hour in medium with or without PEG-SOD (500 U/mL). HEt was then added, and superoxide was measured (1-hour condition). All values were normalized to superoxide levels in the 1-hour

condition. The increase in HEt fluorescence after 1 hour of dissociation is significantly decreased by PEG-SOD, indicating that HEt is detecting superoxide. (D) The fraction of axotomized RGCs undergoing an increase in superoxide (defined as a 50% or greater increase in fluorescence above baseline) increased over 24 hours. This fraction approached its peak value at 6 hours and maintained this level to 24 hours. (E) Reactions of HEt and superoxide. HEt, which faintly fluoresces blue when excited in the ultraviolet spectrum, is a cell-permeable ethidium derivative. On oxidation by cellular superoxide, HEt is converted to oxy-Et, which binds nuclear DNA and then fluoresces strongly in the orange/red spectrum.
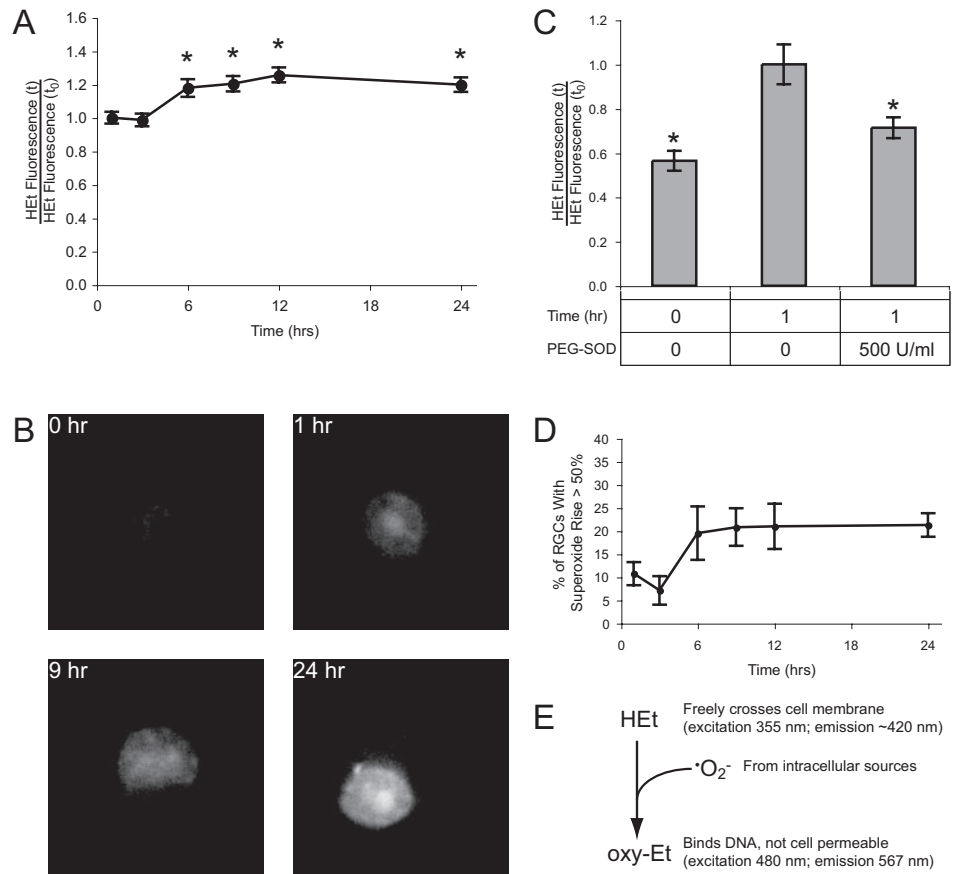
did not show a similar increase between 1 and 24 hours (1.00 ± 0.05 vs. 1.04 ± 0.05; $P =$ NS).

To verify that HEt was measuring superoxide and was not being oxidized by another ROS, such as peroxynitrite, cultures were plated in HEt staining solution, and superoxide was measured at 15 minutes, the minimum time to allow the cells to settle in the well. Parallel cultures were incubated for 1 hour in the presence or absence of 500 U/mL PEG-SOD. Superoxide levels from RGCs incubated for 1 hour were 79% higher than acutely dissociated RGCs (1.00 ± 0.09 vs. 0.56 ± 0.04; $P = 0.0001$), indicating that axotomy immediately adjacent to the cell body increases superoxide levels. The presence of 500 U/mL PEG-SOD resulted in a significant decrease in this rise (0.71 ± 0.05 vs. 1.00 ± 0.09; $P = 0.02$), indicating that HEt-associated fluorescence corresponds to superoxide levels (Fig. 1C).

Although the increase in mean fluorescence levels across RGCs was relatively small, it was apparent from examining the individual RGC fluorescence levels that a subset of RGCs had a much higher increase in superoxide. To study this, we calculated the fraction of RGCs undergoing a major increase in intracellular superoxide levels, arbitrarily defined as an increase of 50% compared with baseline at 1 hour after plating. The percentage of RGCs with a superoxide burst increased significantly from 10.7 ± 2.5 to 21.2 ± 2.5 over 24 hours ($P = 0.005$ by χ^2 for trend) (Fig. 1D). Again, non-RGCs did not show a similar increase ($P =$ NS by χ^2 for trend). These increases reflected oxidation of HEt to an oxy-Et intermediate with specific fluorescence characteristics (Fig. 1E).

Axotomy of RGCs Induces an Increase in Superoxide Levels Independent of Retinal Dissociation

The dissociation of whole retinas for mixed retinal cultures involves acute axotomy of the RGCs immediately adjacent to the soma when the retina is dissected from the optic nerve and gentle enzymatic digestion and mechanical trituration of the intact retina. To determine whether the increase in superoxide levels in RGCs resulted from dissociation alone or from axotomy, we separated the effects of the two using the technique of Shen et al.[13] The right optic nerves of P11 to P13 rats were crushed 2 mm behind the globe (Fig. 2A), and, at various times afterward, the animals were killed, RGCs were immediately cultured, and superoxide levels were measured. RGCs from retinas with crushed or uncrushed optic nerves were cultured in parallel. HEt fluorescence levels were compared with the mean baseline levels of the uncrushed group to assess the effects of dissociation.

Superoxide levels in RGCs from eyes undergoing optic nerve crush were minimally higher than RGCs from the unoperated eye when cultured 4 hours after crush. By 8 hours after crush, the crush group RGCs were significantly higher than the uncrushed group (1.17 ± 0.07 vs. 1.00 ± 0.04; $P = 0.03$) and were even higher at 24 hours (1.20 ± 0.05 vs. 1.00 ± 0.04; $P = 0.008$).

The relatively small increase in mean superoxide levels masks the much more dramatic changes in individual RGCs. Examination of the percentage of RGCs with a superoxide
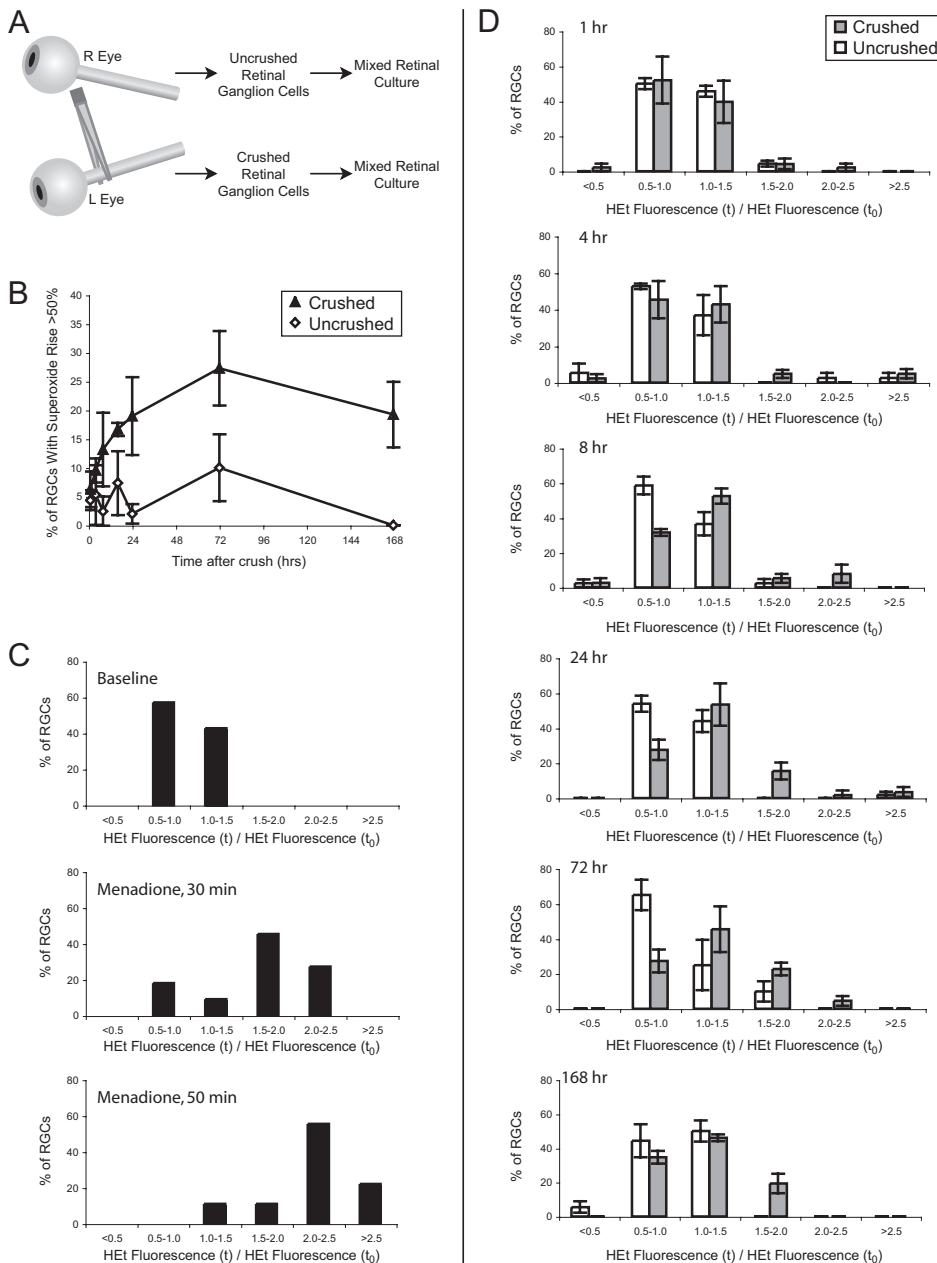
FIGURE 2. Retinal ganglion cell axotomy induces an increase in superoxide levels independent of retinal dissociation. (**A**) Axotomy of RGCs by optic nerve crush. RGCs underwent axotomy through exposure of the left optic nerve by lateral orbitotomy and by gentle crushing of the nerve with blunt forceps, sparing the retinal and optic disc circulations. Cultures were prepared at various times later, and superoxide level was measured 1 hour after dissociation with HEt. Retinas from the right (uncrushed) eye controlled for the effects of dissociation on superoxide generation. (**B**) The fraction of axotomized RGCs undergoing an increase in superoxide (defined as a 50% or greater increase in fluorescence above baseline) steadily increased over 24 hours after optic nerve crush and continued for 7 days. Negligible numbers of RGCs with intact axons had significant increases in fluorescence. *Filled triangles*: RGCs after optic nerve crush; *open diamonds*: RGCs without optic nerve crush. (**C**) Menadione induces a synchronous superoxide increase in RGCs. RGCs in mixed retinal cultures were incubated with the redox cycling agent menadione (1 mM), and superoxide levels were measured with HEt. A histogram was made of the per cell superoxide fluorescence readings, normalized to baseline (1 hour) mean. Menadione resulted in a rapid and synchronous increase in intracellular superoxide in virtually all RGCs. (**D**) In contrast, RGC axotomy produced a more gradual shift in distribution of superoxide levels, with only a subpopulation of cells undergoing increases at any given time. Histograms show the distribution of RGC superoxide levels in cultures after optic nerve crush (*gray bars*) and the nonaxotomized control retinas (*white bars*). The distribution shifted toward higher superoxide levels after longer periods of axotomy.

burst (as defined above) revealed that there was a significant ($P = 0.01$ by χ^2 for trend) increase in the number of RGCs with elevated superoxide levels (Fig. 2B). The percentage of cells exhibiting this increase increased steadily to $19.0\% \pm 6.7\%$ for cultures 24 hours after crush and peaked at $27.3\% \pm 6.5\%$ for cultures 72 hours after crush. Only 5% of RGCs from cultures of eyes without optic nerve crush showed a similar superoxide burst after dissociation, and it did not increase over time ($P =$ NS by χ^2 for trend).

## Increase in Superoxide Levels after Axotomy in RGCs Is Asynchronous

The substantial increase in superoxide levels in only a fraction of the RGCs at any one time suggested that axotomy induced an asynchronous signal for cell death, consistent with the relatively slow loss of RGCs after optic nerve crush in the rat.[18] We used histograms of HEt fluorescence levels over time to assess the distribution of superoxide increases in various conditions. As an example, exposure of RGCs to 1 mM menadione,

which rapidly increased superoxide by redox cycling within the electron transport chain, resulted in a series of histograms that corresponded to a synchronous increase in fluorescence (Fig. 2C). Nearly all cells exhibited an increase of at least 50% over baseline values within 1 hour of treatment. In contrast, the change in the distribution of RGC superoxide levels after optic nerve crush was neither rapid nor uniform. Histograms of superoxide levels in RGCs from retinas after axotomy showed a slow shift toward higher superoxide levels in a small subpopulation of cells at any given time (Fig. 2D). RGCs from the control that did not undergo axotomy did not demonstrate such a shift.

Addition of Neurotrophins Does Not Prevent an Increase in Superoxide after Axotomy

Neurotrophin deprivation is believed to be one of the mechanisms by which axotomy induces RGC death, but this is controversial. We therefore studied the effect of a combination of neurotrophic factors that support RGC survival on superoxide

FIGURE 3. The increase in superoxide levels in retinal ganglion cells after dissociation and axotomy was not inhibited by neurotrophic factors. (**A**) The addition of 50 ng/mL BDNF, 10 ng/mL CNTF, 5 μg/mL insulin, and 5 μM forskolin to the culture medium did not significantly reduce RGC superoxide levels compared with baseline at any time point after dissociation. *Filled circles*: RGCs cultured in the absence of neurotrophins; *open diamonds*: RGCs cultured in the presence of neurotrophins. The *asterisk* to the side of the line graphs represents a significant ($P = 0.02$ by ANOVA for trend) overall difference from baseline. (**B**) There was a significant increase during the 24 hours after dissociation in the proportion of RGCs with greater than 50% elevation of superoxide levels. *Filled circles*: RGCs cultured in the absence of neurotrophins; *open diamonds*: RGCs cultured in the presence of neurotrophins. (**C**) Culturing in MEM did not accentuate the effect of neurotrophic factors on preventing elevations of superoxide after dissociation. Addition of BDNF, CNTF, forskolin, or insulin to RGCs in Eagle MEM with 5% fetal calf serum did not significantly affect the increase in superoxide caused by dissociation. (**D**) Mechanisms of signal transduction for neurotrophins studied. BDNF bound to the TrkB receptor and eventually led to phosphorylation of Erk1 and Erk2, followed by activation of PI3-kinase, and Akt. Forskolin directly activated adenylate cyclase, increasing cAMP levels in the cell. This in turn recruited TrkB to the cell membrane, increasing the ability of BDNF to bind to the cell. CNTF bound the CNTFRα receptor and induced the JAK/Stat3 pathway. Insulin bound to the insulin and IGF-1 receptors and induced PI3-kinase and Akt.
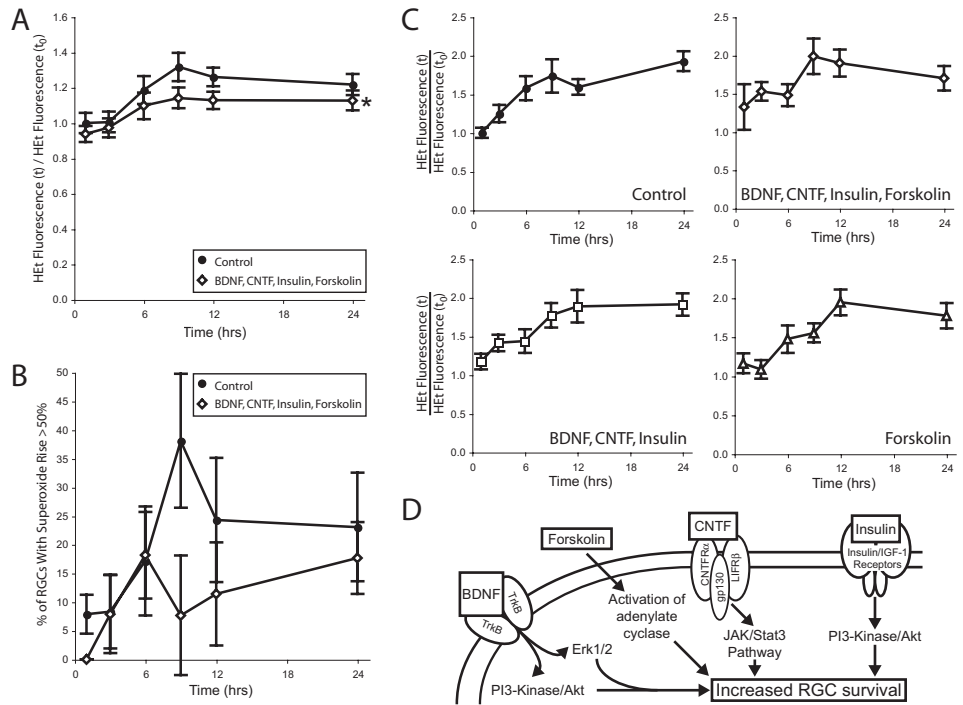
levels after dissociation.[17] RGCs were cultured in the presence of BDNF (50 ng/mL), ciliary neurotrophic factor (CNTF; 10 ng/mL), insulin (5 μg/mL), and forskolin (5 μM), and superoxide levels were measured at varying times after dissociation. Parallel cultures were treated with medium alone. The combination of neurotrophic factors minimally and nonsignificantly reduced superoxide levels (1.31 ± 0.08 vs. 1.14 ± 0.06 at 9 hours, 1.26 ± 0.05 vs. 1.14 ± 0.05 at 12 hours; $P = 0.08$ and $P = 0.07$ by multiple t test), though there was an overall significant difference across all time points by repeated-measure ANOVA ($P = 0.020$; Fig. 3A). Neurotrophins incompletely prevented an increase in the percentage of RGCs with a superoxide burst, defined as a greater than 50% increase in superoxide compared with baseline. After 24 hours in culture, 18% of RGCs cultured in the presence of neurotrophic factors had elevated superoxide levels compared with 23% for medium alone (Fig. 3B), and both the control cells and those in the presence of neurotrophic factors experienced significant increases over time in the percentage of cells with a superoxide burst ($P = 0.01$ and $P = 0.04$ by χ^2 for trend).

Barres et al.[17] have shown that depolarization or increased intracellular cAMP level improves survival in combination with neurotrophic factors. We had already shown that forskolin (which increases intracellular levels of cAMP) in combination with BDNF, CNTF, and insulin did not prevent axotomy induction of intracellular superoxide. To test whether depolarization in combination with neurotrophic factors could block superoxide generation, we incubated RGCs in 50 mM K^+, along with BDNF, CNTF, and insulin for 1, 3, 6, 9, 12, and 24 hours, and measured superoxide with HEt at each time point. Depolarization in combination with neurotrophic factors did not suppress the increase in superoxide at any time point ($P = $ NS by t test) or as a treatment effect across all time points ($P = $ NS by repeated-measure ANOVA).

We wondered whether the failure of neurotrophins and forskolin to block the increase in RGC superoxide was because the medium (Neurobasal A [Invitrogen] with B27 supplement) was already rich enough to mask small effects from neurotrophic factors. To magnify the effect that neurotrophic factors might have on superoxide levels, RGCs were cultured in minimal essential medium (MEM; 5% fetal calf serum, and antibiotics) in the presence or absence of factors. Although the magnitude of the increase in superoxide levels over time was higher in MEM than in complete medium, the presence of all four neurotrophic factors did not significantly affect the level of superoxide compared with control (Fig. 3C). A similar lack of effect was seen when RGCs were cultured in the presence of BDNF, CNTF, and insulin together or of forskolin. Combined, these results suggest either that the response to neurotrophic factors is downstream of superoxide or, more likely, that the induction of superoxide by axotomy is independent of a reduction in neurotrophin signaling (Fig. 3D).

Polyethylene Glycol–Conjugated Superoxide Dismutase Promotes RGC Survival after Dissociation

The use of ROS scavengers has been shown to increase the viability of RGCs in culture, and our data suggested a significant functional role for superoxide in signaling axotomy-induced death. We therefore examined the ability of pegylated SOD, which crosses cell membranes, to increase viability of RGCs after dissociation in culture. Typically, most RGCs die in 1 to 6 days in the absence of neurotrophic or other factors. PEG-SOD significantly improved viability in a dose-dependent manner ($P = 0.02$ by ANOVA for trend), with an increase of more than 80% in viable RGCs at 24 hours compared with control at 5 U/mL or greater (Fig. 4A). Similar results were not seen with cell impermeable nonpegylated SOD, implying that superoxide
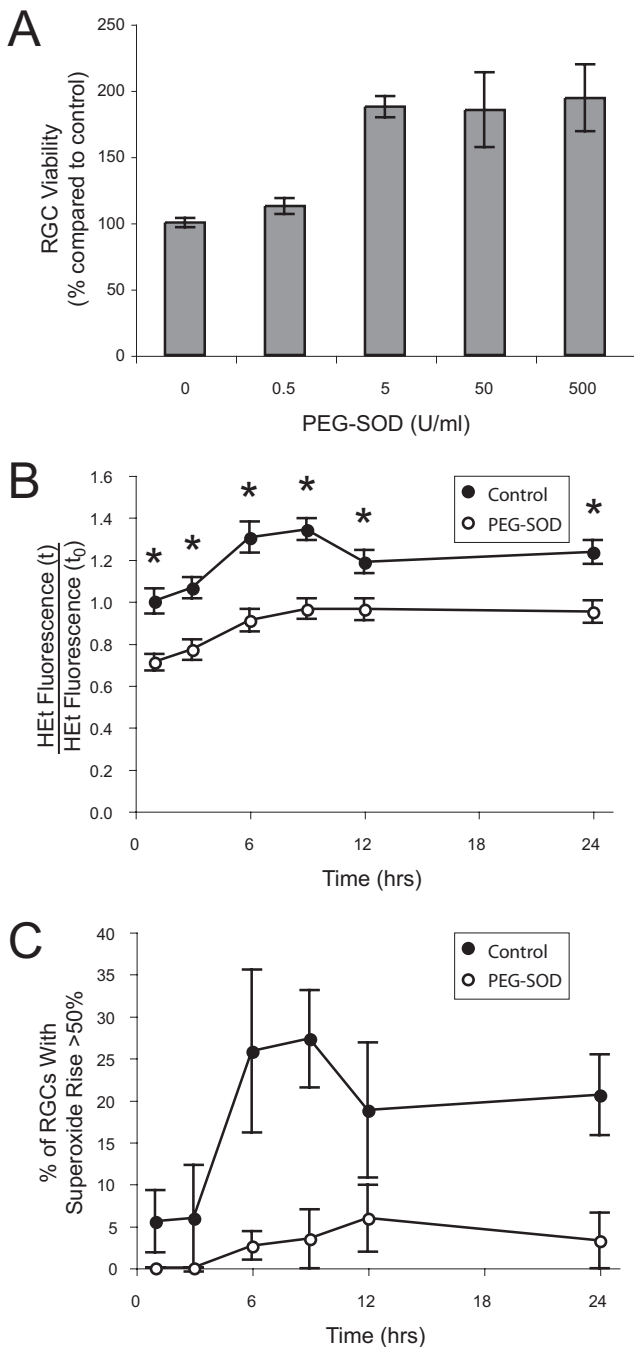
FIGURE 4. Incubation with PEG-SOD led to increased RGC viability and decreased superoxide levels after dissociation. (**A**) PEG-SOD rescued RGCs from death induced by acute axotomy in culture. Cells cultured for 24 hours in the presence of PEG-SOD at concentrations from 0.5 to 500 U/mL showed increased viability, with an approximate increase of 85% or more in viable RGCs after 24 hours in culture for doses of 5 U/mL and greater. The increase in viability was significantly dose dependent ($P = 0.02$ by ANOVA for trend). (**B**) PEG-SOD prevented the increase in superoxide brought on by dissociation. Superoxide levels were kept significantly lower at all time points ($P < 0.005$ for all time points by unpaired t-test) in the presence of 500 U/mL PEG-SOD. (**C**) PEG-SOD prevented virtually all cells from undergoing an increase in superoxide. The percentage of RGCs with an increase in superoxide levels of 50% over baseline was considerably lower for cells cultured in the presence of 500 U/mL PEG-SOD. No RGCs treated with PEG-SOD had superoxide levels exceeding 50% above baseline at 1 hour or 3 hours after plating, and there was an insignificant increase in the percentage exhibiting this increase for up to 24 hours.

acted intracellularly. In comparison, survival after dissociation and axotomy in cultures treated with BDNF, CNTF, insulin, and forskolin was not as great ($26\% \pm 22\%$ above control). Given that approximately 30% to 50% of RGCs are alive after dissociation and axotomy at 24 hours, the 80% increase in viability with PEG-SOD translates into an absolute increase in viability of 24% to 40%.

PEG-SOD Blocks the Increase in Superoxide Levels after Dissociation

If the increase in HEt fluorescence after axotomy is truly the result of intracellular superoxide, treatment of the cells with PEG-SOD should prevent that increase. RGCs cultured in the presence of 500 U/mL PEG-SOD had significantly lower superoxide levels than control at every time point after dissociation (Fig. 4B). This reduction in superoxide levels was also reflected in a lower percentage of cells with superoxide bursts over time (Fig. 4C), with at most 6% of RGCs having a greater than 50% increase in superoxide levels and a nonsignificant increase over time ($P = \mathrm{NS}$ by χ^2 for trend). To ensure that PEG-SOD was acting intracellularly, we compared its ability to scavenge HEt-detectable superoxide with nonpegylated SOD, which is not cell permeable. RGCs incubated for 1 hour after dissociation and treated with nonpegylated SOD (500 U/mL) had superoxide levels approximately the same as untreated RGCs (0.99 ± 0.04 vs. 1.00 ± 0.17; $P = \mathrm{NS}$ by t test), whereas RGCs treated with PEG-SOD (500 U/mL) had superoxide levels of 0.59 ± 0.10 of control. To ensure that PEG-SOD was not itself affecting HEt, we measured superoxide in cell-free assays. PEG-SOD (500 U/mL) did not affect background-oxidized HEt fluorescence levels in the absence of a superoxide-generating system (1095 ± 161 with PEG-SOD vs. 1071 ± 26 without PEG-SOD; $P = \mathrm{NS}$) but, as expected, significantly reduced HEt oxidation in the presence of superoxide anion (2258 ± 59 with PEG-SOD vs. 7983 ± 342 without PEG-SOD; $P < 0.001$).

Superoxide Produced after RGC Axotomy Arises from Mitochondria

Mitochondria are the principal site for superoxide generation in the cell, with superoxide resulting from leakage of electrons from reduced intermediates in the electron transport chain to molecular oxygen.[19] We suspected that mitochondria could be the source of superoxide generated after axotomy but could not use HEt to localize the source of production because it does not target intracellularly. Instead we used the mitochondria-selective superoxide probe MitoSOX Red to measure superoxide in RGC mitochondria after axotomy. We found that mean mitochondrial superoxide levels were significantly higher after 24 hours in culture than baseline fluorescence at 1 hour (1.00 ± 0.07 vs. 1.39 ± 0.14; $P = 0.03$). Baseline levels at 1 hour were not significantly different from those when cells were plated in medium containing MitoSOX Red and imaged after 10 minutes (1.00 ± 0.26 vs. 0.99 ± 0.21). Mitochondrial superoxide levels in cultures treated with 500 U/mL PEG-SOD were lower at 1 hour (0.91 ± 0.08) and 24 hours (1.19 ± 0.19), and the mean increase over time was not significant.

Inhibition of Complex III of the Mitochondrial Electron Transport Chain Blocks the Increase in Superoxide after Axotomy

Superoxide has several sites of generation within cells, including mitochondria. Our studies with MitoSOX Red suggested that mitochondrial superoxide levels increased after RGC axotomy. To determine the source of the superoxide, we incubated RGCs for 1 hour and 24 hours in the presence of several inhibitors known to block endogenous ROS-generating systems and electron transfers in the mitochondria. Rotenone (1 μM),
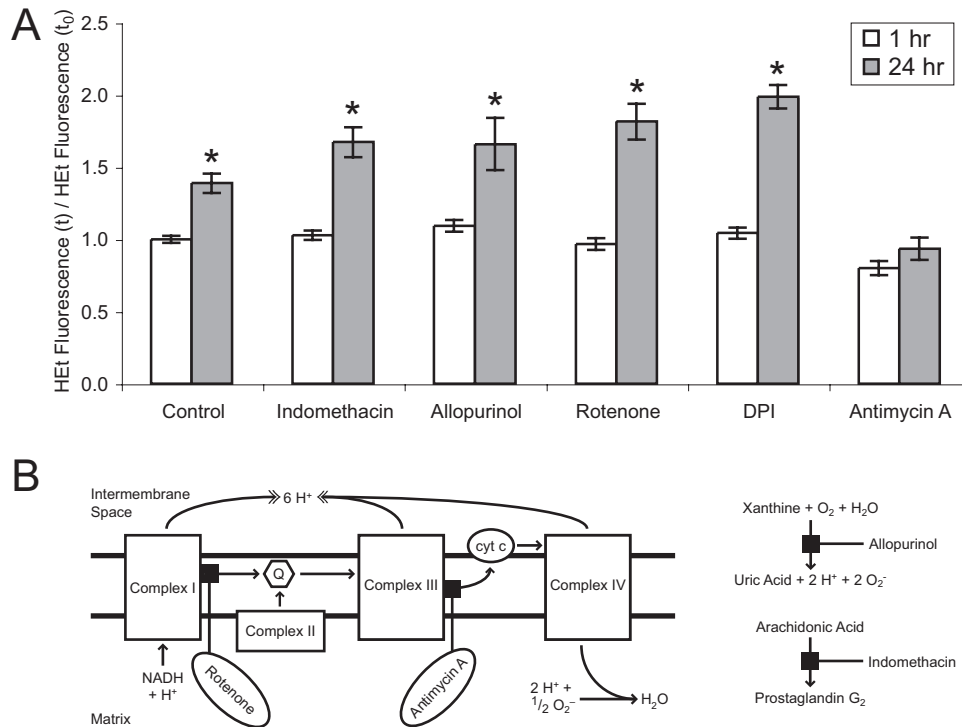
FIGURE 5. Inhibition of complex III of the mitochondrial electron transport chain prevented an increase in RGC superoxide levels after dissociation. (**A**) Antimycin A blocked the increase in superoxide levels after dissociation in RGCs. Cells were treated with various inhibitors of electron transport and other intracellular generators for 1 hour or 24 hours before measurement of superoxide levels. Conditions treated with indomethacin, allopurinol, rotenone, and DPI all showed significant increases in superoxide from 1 hour to 24 hours ($P < 0.0001$ for all conditions). Only antimycin A, which blocked electron transport in complex III of the respiratory chain, prevented the increase. (**B**) Endogenous sources of ROS and their inhibitors. The flow of electrons through the electron transport chain was a source of potential ROS because electrons flow through the system toward oxygen. Rotenone and antimycin A blocked the transfer of electrons to ubiquinone and between cytochrome b_H and the semiquinone intermediate, respectively. Allopurinol inhibited xanthine oxidase, which generated superoxide anion. Indomethacin inhibited cyclooxygenase, which converts fatty acids to peroxides (e.g., arachidonic acid to prostaglandin G_2).

an inhibitor of complex I of the mitochondrial electron transport chain, did not prevent the increase in superoxide level—that is, levels at 1 hour and 24 hours were significantly different (0.96 ± 0.03 vs. 1.82 ± 0.10; $P < 0.0001$). DPI (100 μM), a nonspecific inhibitor of NADPH oxidase and other flavoproteins, also proved ineffective in preventing the increase in superoxide levels (1.04 ± 0.04 vs. 1.99 ± 0.18; $P < 0.0001$). Inhibition of cyclooxygenase with indomethacin (1 mM) and inhibition of xanthine oxidase with allopurinol (1 mM) similarly failed to prevent an increase in superoxide levels (1.02 ± 0.04 vs. 1.67 ± 0.12, 1.09 ± 0.04 vs. 1.66 ± 0.08, respectively; $P < 0.0001$ for all comparisons). Only the inhibition of complex III of the electron transport chain with antimycin A (1 μM) successfully blocked the increase in superoxide level (0.80 ± 0.05 vs. 0.94 ± 0.08; $P = $ NS; Fig. 5A). The ability of a complex III inhibitor to prevent an oxidative burst after RGC axotomy was seen in 5 independent repetitions of the experiment and suggested that superoxide generation under these conditions was in the distal electron transport chain (Fig. 5B). Similar inhibition of superoxide production was seen with two other complex III inhibitors, stigmatellin and myxothiazol (which produce complex III inhibition at low concentrations and inhibit complex I and III at high concentrations). We found significant inhibition of RGC superoxide generation after dissociation with stigmatellin at 1 nM and myxothiazol at 1 μM (data not shown). Higher concentrations (3.2 μM and 10 μM) of myxothiazol led to increased generation of superoxide, consistent with the blocking of complex I. Finally, antimycin A caused decreased survival of some RGCs at 24 hours (28.2% ±

8.7% of control), as would be expected from a drug that blocks electron transport in mitochondria. To differentiate the effect of antimycin A inhibition on ROS generation from its effect on survival, we performed experiments in which we only imaged superoxide levels in live cells, as defined by their ability to exclude Sytox Green. We again found inhibition with antimycin A of superoxide generation induced by dissociation when live cells alone were imaged (data not shown). To control for any effects of antimycin A on the oxidation of HEt by superoxide, we performed a cell-free assay on a fluorescence plate reader using xanthine (1 mM) and xanthine oxidase (0.05 U/mL) in the presence of HEt (1 mM). Fluorescence readings after 30 minutes were not significantly different in the presence or absence (4546 ± 149 and 4574 ± 148, respectively; $P = $ NS) of antimycin A, even at 100 μM (4466 ± 66; $P = $ NS). Together, these studies suggest that complex III blockade inhibits superoxide generation in RGCs after axotomy.

DISCUSSION

We used superoxide-sensitive fluorescent dyes to measure intracellular levels of superoxide in RGCs after axotomy. We found that optic nerve crush approximately 2 mm from the optic disc induced an increase in superoxide that was asynchronous, affecting at most 27% of cells at any given time and peaking at 3 days after injury. Retinal dissociation, which axotomizes RGCs immediately adjacent to the soma, induced a more synchronous increase in superoxide, affecting up to 20%

of cells and peaking 12 hours after injury. In comparison, pharmacologic treatment with menadione, which directly elevates superoxide levels,[20] causes a highly synchronous superoxide burst, affecting virtually all RGCs and peaking within 60 minutes of exposure.

The asynchronous increase in superoxide levels after axonal injury that we observed could explain the delayed death of RGCs in rat optic nerves after intraorbital transection, when losses are approximately 20% at 5 days, 50% at 7 days, and 90% at 14 days.[12] In some cases, RGCs may persist despite extended periods after axotomy, and it has been suggested that distinctive metabolic features of these cells may underlie their prolonged survival. The variable onset of increase in superoxide could result from one such distinctive feature, namely differences in the generation or scavenging of superoxide after axonal injury.

It has been hypothesized that RGC death after axonal injury in the mature CNS results from lack of retrograde transport of neurotrophic factors[21] or from decreased levels of endogenous ocular neurotrophins.[22,23] Good evidence indicates that the normal developmental loss of RGCs partly results from the competition for target-derived molecules (particularly neurotrophins) during attempts to extend their axons and to form connections in target areas of the central nervous system. It is therefore reasonable to assume that adult RGCs, like other neurons,[24] are also dependent on neurotrophic agents for their survival.

Support for the role of neurotrophin dependence comes from experiments using identified neurotrophic factors to rescue neurons after axotomy. Purified neonatal RGCs (which are axotomized during dissociation) can be kept alive for significant periods with a cocktail of factors, including BDNF, CNTF, forskolin, and insulin.[17] Intraocular administration of certain neurotrophins (e.g., BDNF) delays RGC death after axotomy in adult rats[12,25] and cats.[26] Gene delivery of BDNF to the retina or to the RGC itself also increases survival in experimental glaucoma,[27,28] as does the inhibition of apoptosis.[29]

However, there is no direct evidence that RGC death after axotomy is only caused by deprivation of BDNF or other neurotrophic factors. It is possible that the increased survival seen after neurotrophin administration is not a specific rescue effect from neurotrophin deprivation but rather is a generalized prosurvival effect. RGCs maintain viability for long periods of time during temporary neurotrophin deprivation (e.g., axonal transport from a compressive optic neuropathy or papilledema is decreased) or even after axonal transection, suggesting that there are other mechanisms for signaling cell death and perhaps compensatory mechanisms for sustaining survival. Furthermore, retrograde axonal transport is rapid, and the subacute time-course by which RGCs die after axonal injury does not reflect the time-course of interrupted retrograde axonal transport. This probably explains why damage to the RGC axon in several different places results in similar rates of death after axonal injury. Finally, Barres et al.[13] demonstrate that RGC axotomy induces changes in responsiveness to neurotrophins independent of neurotrophin deprivation. Together, these findings suggest that axotomy can signal changes at the cell body independent of neurotrophin deprivation.

Our data are also inconsistent with neurotrophin deprivation as the cause of elevated superoxide after RGC axotomy. We found that neurotrophins, which maintain RGC viability in culture, did not prevent increases in HEt-detectable superoxide. Given that superoxide increases after RGC axotomy, reducing superoxide levels after axotomy with PEG-SOD increases viability, and neurotrophins do not block the increase in superoxide, it is likely either that neurotrophin deprivation does not signal RGC death after axotomy or that it does signal death but in parallel to a signal transduced by superoxide. The latter possibility is similar to the dual mechanisms for RGC

death after target ablation described by Cui and Harvey.[30] Together, these findings suggest that superoxide generated in the mitochondrial electron transport chain could be a parallel system to neurotrophic deprivation for signaling cell death after axonal injury.

PEG-SOD effectively prevented the asynchronous increase signaled by axotomy and dissociation. This confirms that the changes seen are actually caused by increased superoxide levels and suggests a specific physiologic response to axotomy that PEG-SOD inhibits to maintain cell viability. More important, the efficacy of PEG-SOD in promoting viability of RGCs after acute axotomy implies that the superoxide increase is not a secondary result of mitochondrial dysfunction signaled by axotomy as, for example, a result of cytochrome *c* release.[8,9] Our previous results showing failure of the broad spectrum caspase inhibitor to block superoxide release after axotomy[14] also support a role for superoxide, which is proximal or parallel to caspase activation. However, these results do not indicate whether the superoxide increase is proximal, distal, or parallel to Bax translocation to the mitochondria, another element of axotomy signaling.[31,32] Finally, the increase in MitoSOX Red fluorescence after dissociation strongly supports a model that would place the source of superoxide generation after injury in the mitochondria, as does the decrease in MitoSOX Red fluorescence in axotomized RGCs treated with PEG-SOD.

The involvement of mitochondria in signaling of RGC death is not surprising. The most common hereditary optic neuropathy, Leber hereditary optic neuropathy (LHON), is characterized by mitochondrial DNA mutations affecting components of complex I. These mutations should affect all cells in the body with respiring mitochondria, yet in most patients only RGCs are affected. Another example of an important role for mitochondria in signaling RGC death is the apparent idiosyncratic nature of their mitochondrial permeability transition pore.[33]

The most abundant site of superoxide generation within cells is through leakage from mitochondria, whereby reduced electron transport chain components transfer electrons to molecular oxygen.[19,34,35] Our studies indicate that, based on experiments with pharmacologic inhibitors, complex III inhibition (with antimycin A) but not complex I inhibition (with rotenone) decreases the rate of superoxide generation after RGC dissociation. However, this does not prove that complex III is a source. Typically, complex I or complex III inhibition leads to an increase in superoxide generation[36] because the upstream electron carriers in the electron transport chain become reduced and capable of reacting with O_2 to generate superoxide. One important difference between standard studies of superoxide generation and our experiments is that we looked under conditions in which the cell is perturbed (from axotomy), whereas other studies typically focus on the physiologic leak of superoxide from the electron transport chain. It is likely that the sources of superoxide generation differ greatly under these conditions. Therapeutic strategies addressing the specific sites of generation of superoxide and other ROS signals after axotomy may be attractive targets for neuroprotective therapies.

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