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Amplification of a Reactive Oxygen Species Signal in Axotomized Retinal Ganglion Cells

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ABSTRACT

Retinal ganglion cells (RGCs) undergo apoptosis after axonal injury. Elucidation of the sequence of intracellular events proximal to caspase activation may allow development of effective neuroprotective strategies. In this study, we explored the role that reactive oxygen species may have in signaling RGC apoptosis after axonal injury. Using the fluorescent probe dihydroethidium, we were able to measure intracellular superoxide anion production. We found that axotomized RGCs exposed to oxidative stress exhibited a secondary superoxide burst. The broad-spectrum caspase inhibitor Z-Val-Ala-DL-Asp-fluoromethyl ketone did not block the burst, suggesting it is proximal to caspase activation, but it was inhibited by cycloheximide, consistent with a requirement for protein synthesis. These results are consistent with RGC axotomy inducing synthesis of one or more proteins that mediate oxidative amplification. This could be an early event in signaling of RGC apoptosis after axonal injury. *Antioxid. Redox Signal.* 5, 629–634.

INTRODUCTION

AXONAL INJURY causes apoptosis in retinal ganglion cells (RGCs), a prototypical mammalian central neuron (2). Axotomy of RGCs induces activation of caspase 3, an enzyme distal in apoptosis signaling (14), mediated in part by activation of the proximal caspase 9 (15). Caspase 9 is activated by mitochondrial release of cytochrome *c* and other apoptosis-inducing factors, supporting the hypothesis that the mitochondrion plays an important role in RGC death after axotomy. However, it is unclear what events trigger mitochondrial signaling of apoptosis, and which mitochondrial processes are necessary or sufficient for apoptosis signaling to take place.

Results from our laboratory suggest that reactive oxygen species (ROS) are factors in the signaling of RGC death after axonal injury (10, 16, 18). Specific ROS scavengers and hypoxia reduce the death of cultured neonatal RGCs after axotomy, and RGC survival is dependent on redox state, with greatest survival under mildly reduced conditions (4, 5, 10). Further support for the role of ROS in axotomy signaling comes from experiments showing that RGCs in transgenic mice overexpressing copper/zinc superoxide dismutase (SOD) are significantly more likely to die after axotomy than wild type controls (19), consistent with the possibility that increased levels of hydrogen peroxide (H_2O_2) are detrimental to RGC survival. Also, nuclear factor-κB (NF–κB) translocates from the cytoplasm to the nucleus in axotomized RGCs (6). As NF–κB is activated by oxidative stress and serves to prevent apoptosis by increasing levels of other antiapoptotic genes, it is possible that it is induced as a result of axotomy-induced ROS signaling.

We have hypothesized that if ROS are involved in the signaling of apoptosis after axotomy, then their downstream effectors could be one or more proteins that participate in the regulation of apoptosis, and that when oxidized transduce apoptosis. Candidates include components of the mitochondrial permeability transition pore (7, 12, 17), nitric oxide (20, 21), or other targets. To study the mechanism by which ROS could induce apoptosis signaling in axotomized RGCs, we exposed them to oxidative stress using H_2O_2. We found that H_2O_2 induced a delayed superoxide burst in these cells, and we investigated possible mechanisms to explain this phenomenon.

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MATERIALS AND METHODS

Animals

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

Materials

Cell culture reagents were obtained from GIBCO (Grand Island, NY, U.S.A.). The fluorescent tracer 4',6-diamidino-2-phenylindole (DAPI), dihydroethidium (HEt), and Sytox Green were obtained from Molecular Probes (Eugene, OR, U.S.A.). Papain was obtained from Worthington Biochemical (Freehold, NJ, U.S.A.). Unless noted, all other reagents were obtained from Sigma (St. Louis, MO, U.S.A.).

RGC labeling and culture

RGCs were labeled and cultured using previously described methods (18). In brief, ganglion cells were retrogradely labeled by stereotactic injection of the fluorescent tracer DAPI (5 m*M*) dissolved in dimethylformamide into the superior colliculi of anesthetized postnatal day 2–4 Long–Evans rats. DAPI was chosen because its excitation and emission spectra do not overlap with those of HEt. Over several days, the DAPI is taken up at the RGC projection sites and retrogradely transported to the RGC somas, where it binds to and stains nuclear dsDNA. At postnatal day 7–8, the animals were killed by CO_2 gassing and decapitation, the eyes were enucleated, and the retinas were dissected free in Hank's balanced salt solution (HBSS; BioWhittaker). After two incubations in HBSS containing papain (12.5 U/ml), each for 7 min at 37°C, the retinas were gently triturated with a serological pipette and plated on poly-L-lysine–coated eight-well chambered coverglass (Nalge Nunc) to accommodate a 100× oil immersion lens, at a density of ~2,000 cells/mm^2. The cells were cultured for 24 h in Neurobasal A (GIBCO) with 0.7% methyl cellulose, 2% serum-free supplement B27 (GIBCO), and gentamicin (5 μg/ml) at 37°C and 5% CO_2.

Measurement of RGC superoxide anion in situ

Twenty-four hours after plating, the chambered coverglass cultures were placed on a Zeiss Axiovert 135 inverted microscope, with a stage heated to keep the temperature of the cultures constant at 37°C. RGCs were identified in mixed cultures by the presence of retrogradely transported DAPI, which appears blue with appropriate DAPI filters (excitation 330 nm, emission 450 nm) under epifluorescence.

The levels of intracellular superoxide anion (O_2^-) were measured by quantifying the oxidation of HEt. Oxidation by O_2^- converts HEt to ethidium (Et), which can be detected using appropriate filters (excitation 515 nm, emission 610 nm) (3). HEt is cell-permeant and gives off a weak blue fluorescence. Upon oxidation, the red-fluorescent Et accumulates in the nucleus. After placement of the coverglass on the microscope, the well medium was aspirated and replaced with HBSS without phenol red containing 3.2 μ*M* HEt and 1 μ*M* Sytox Green. Sytox Green was used to identify necrotic cells; it stains cells with compromised plasma membranes by binding to nucleic acids (excitation 504 nm, emission 523 nm). Using MetaFluor software, outlines of RGC nuclei were digitally traced for monitoring, and the intensity of the red fluorescence of Et in the selected region was measured using appropriate filters (excitation 515 nm, emission 610 nm). Images were acquired at a binning of 1, an exposure time of 200 ms, and 2× gain. Images were first taken every 20 s for 10 min to establish a baseline level of HEt conversion to Et. The cells were then typically treated with pharmacological agents or vehicle, and image acquisition continued every 20 s for an additional 30 min. For each image, a value for the average red fluorescence intensity within the selected region was recorded. Graphs of fluorescence intensity over time were created for each imaged cell.

Pharmacological treatments

Two different chemicals were used to directly or indirectly induce a burst of superoxide (Fig. 1). Menadione was used to generate intracellular superoxide anion by redox cycling (13) by the addition of 1 m*M* (final concentration) to the chambered coverglass culture 10 min into imaging. H_2O_2 was prepared as dilutions from a 30% stock; 9.5 m*M* (final concentration) was added to cells 10 min into imaging.

Several agents were used to analyze the apparent H_2O_2-induced burst in superoxide. To observe the effect of inhibiting protein synthesis, cells were treated with 100 μ*M* cycloheximide for either 1 h or 24 h prior to imaging. Tris(2-carboxyethyl) phosphine (TCEP) was used to inhibit sulfhydryl oxidation; cells were treated with 100 μ*M* TCEP 5 min before the addition of H_2O_2 during an imaging run. The multiple caspase inhibitor Z-Val-Ala-DL-Asp-fluoromethyl ketone (ZVAD-FMK) was added to cells at 50 μ*M* final concentration 5 min before H_2O_2 addition.

Statistical analysis

Due to variations in baselines and treatment responses, each graph of red fluorescence intensity versus time was scored with regard to the presence of a significant increase in intensity compared with baseline. Two observers masked to treatment group scored each graph; differences in scoring were adjudicated by consensus. Results were analyzed using Fisher's exact test.

RESULTS

HEt can be used for the quantitative measurement of superoxide levels in RGCs

For the purposes of this study, it was necessary to determine whether HEt can be used to measure the generation of superoxide anion within RGCs. First, to ensure that H_2O_2 alone did not cause oxidation of HEt, H_2O_2 at a final concentration of 175 m*M* was added to a 3.2 μ*M* solution of HEt in the absence of any cells. This did not result in any red fluorescence over 10 min (data not shown). Second, to ensure that superoxide indeed could oxidize HEt, a 1/10 volume of a saturated solution of KO_2 in dimethyl sulfoxide was added to a 3.2 μ*M* solution of HEt, again in the absence of cells. This resulted in

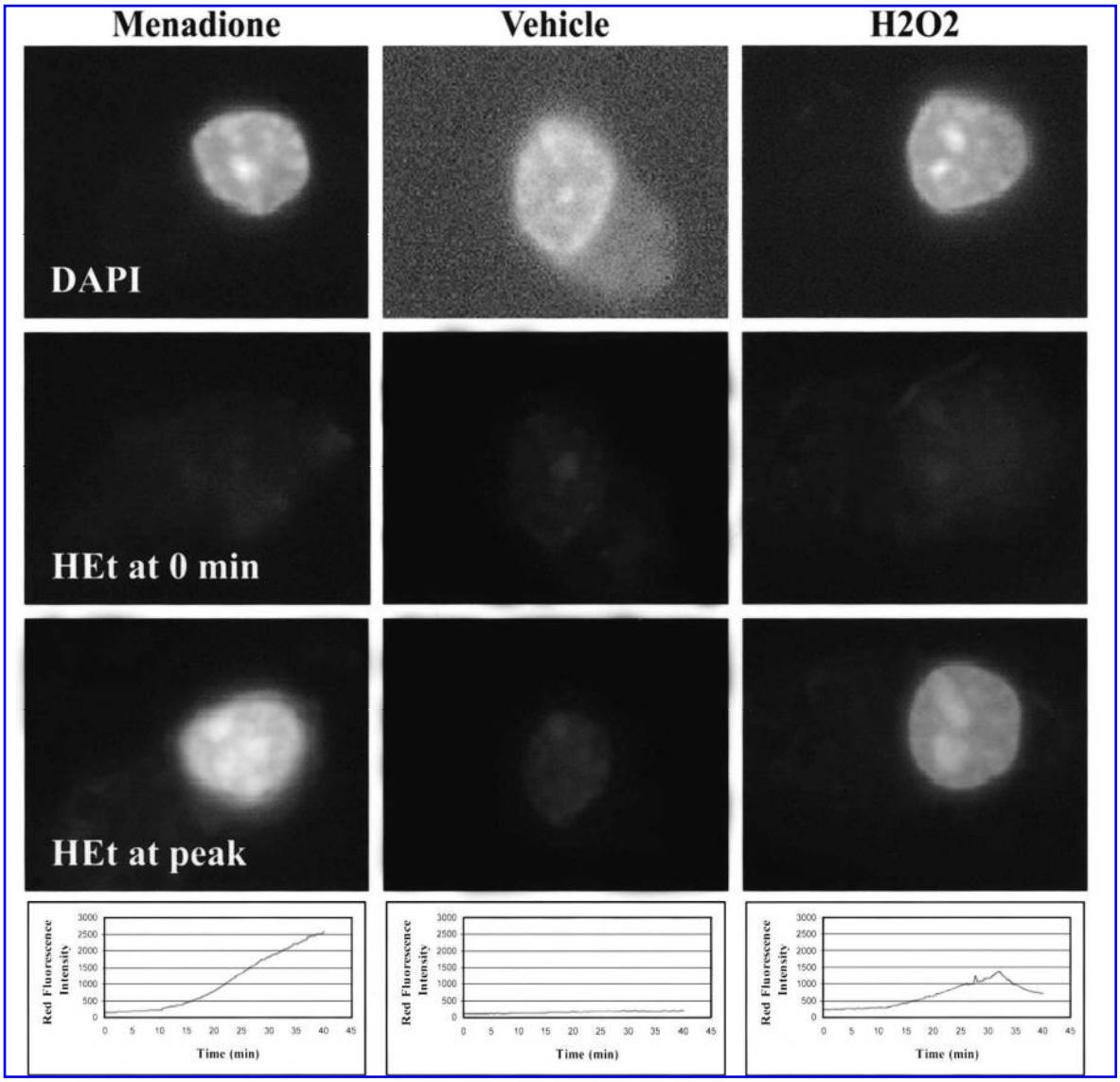

FIG. 1. Typical RGC responses to control and H_2O_2 treatments. All treatments were administered at 10 min into imaging. Menadione was added to a final concentration of 1 m*M*, and H_2O_2 was added to 9.5 m*M*. Each column depicts a single RGC. The first row of pictures displays the cells with DAPI filters (excitation 330 nm, emission 450 nm), whereas the second and third rows display the cells with HEt-specific filters (excitation 504 nm, emission 523 nm). All pictures were taken at the start of imaging, except for the third row, taken at the peak fluorescence reading (40 min for menadione and vehicle, 33 min for H_2O_2). All figures were normalized to reflect the actual fluorescence intensity, as measured by integrating fluorescence over the cell area. Graphs display red fluorescence intensity (arbitrary units) versus time (min).

an immediate short-lived increase in red fluorescence. Third, to test that superoxide in RGCs could be measured, DAPI-labeled RGCs within mixed retinal cultures were exposed to 1 m*M* menadione, which generates superoxide anion through redox cycling (13). We acquired red fluorescence intensities of the region coinciding with DAPI-stained nuclei for 10 min before and 30 min after treatment. One hundred percent of all graphs of imaging runs ($n = 16$) with menadione were scored as having an unambiguous increase in intensity compared with baseline. The rise in red fluorescence intensity after incubation with menadione demonstrates that HEt can be used to measure RGC superoxide levels, and together with the control experiments, suggests that it is not significantly affected by H_2O_2 levels.

H_2O_2-induced oxidative stress causes a secondary oxidative burst in axotomized RGCs

We had previously showed that apoptosis of axotomized RGCs is ROS-dependent. To study the mechanism by which ROS signaling could induce apoptosis in axotomized RGCs, we chemically induced oxidative stress in cultured RGCs using

9.5 m*M* H_2O_2. The high concentration of H_2O_2 was needed to mitigate the effects of cellular antioxidants (*e.g.,* reduced glutathione and several peroxidases) within the 30 min of imaging. The addition of 9.5 m*M* H_2O_2 to cultured RGCs caused a burst in superoxide in 76.2% of the cells. This effect is significantly higher than the 15.4% found with the addition of vehicle ($p = 0.0011$), but it is not significantly different from the 100% found with menadione ($p = 0.1329$). Therefore, oxidative stress in RGCs causes a secondary oxidative burst.

These experiments were also performed with freshly axotomized RGCs. When treated with 9.5 m*M* H_2O_2, RGCs incubated for 2 h did not exhibit a secondary oxidative burst (0% of RGCs showed a rise in superoxide). This result was significantly different from the effect of H_2O_2 seen with RGCs axotomized for 24 h ($p = 0.0001$) and not significantly different from that of vehicle applied to RGCs at 24 h after axotomy ($p = 0.4935$). This result suggests that RGCs do not amplify an oxidative stress 2 h after axotomy.

The rise in superoxide after H_2O_2 exposure could be due to other causes besides intracellular generation, *e.g.,* lipid peroxidation or reverse reaction of SOD. High concentrations of H_2O_2 can cause necrosis through lipid peroxidation; in the Fenton reaction, H_2O_2 and Fe^{2+} react to form hydroxyl radicals, which can destroy plasma membranes and cause necrosis. To test this, we used Sytox Green as an indicator of necrosis before and after H_2O_2 treatment in RGC cultures. We also added 100 μ*M* $FeSO_4$ in some assays to make sure that reduced iron was not a limiting reagent. We found that 9.5 m*M* H_2O_2 did not cause any Sytox Green positivity (necrosis) within 30 min of treatment ($n = 13$); the presence of Fe^{2+} did not affect this result ($n = 13$). In contrast, 95 m*M* H_2O_2 with or without the addition of Fe^{2+} caused all RGCs to become necrotic within 30 min of treatment ($n = 11$ without Fe^{2+}; $n = 13$ with Fe^{2+}). These results suggest that lipid peroxidation does not have a significant effect specific to the culture conditions, H_2O_2 concentration, and time frame used.

Another possibility was that the high H_2O_2 concentration might interfere with SOD activity. SOD catalyzes the conversion of superoxide anion to H_2O_2. By adding a large amount of H_2O_2, the forward reaction may be inhibited or even the reverse reaction rate may increase. The result in either scenario is an accumulation of superoxide anion, which would oxidize HEt and confound results. Because *t*-butyl hydroperoxide (TBHP) is not a substrate for the reverse SOD reaction due to its structure, but should generate a peroxide oxidative stress, we used it in place of H_2O_2 to treat cultured RGCs. Adding 9.5 m*M* TBHP to RGCs 24 h after axotomy, we found that 80% of the RGCs exhibited a burst in superoxide. This effect was significantly higher than that with vehicle ($p = 0.0217$) and not significantly different from the H_2O_2 effect ($p = 0.4334$). This result suggests that H_2O_2 does not affect SOD kinetics. As another test of the hypothesis that reverse SOD activity could induce superoxide production after H_2O_2 exposure, we treated RGCs with polyethylene glycol-SOD. If H_2O_2 was indeed causing an increase in reverse SOD activity, then in the presence of excess H_2O_2 the native SOD is rate-limiting, and increasing intracellular SOD would further increase superoxide levels. However, the reverse was true, with increased SOD levels resulting in decreased superoxide levels (Fig. 2). Together, these experiments suggest that H_2O_2 does not produce superoxide via a reverse SOD reaction.

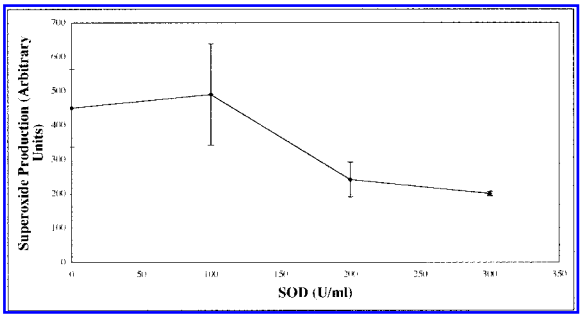

FIG. 2. Effect of polyethylene glycol-SOD addition on superoxide production in H_2O_2-exposed RGCs. Values were obtained by subtracting the theoretical 40-min time point intensity derived from the baseline (before treatment) from the actual intensity at 40 min (after H_2O_2 treatment). There was a trend for a linear inverse relationship ($p = 0.103$ by ANOVA for trend).

The secondary oxidative burst is not the downstream result of caspase-activated apoptosis

We wanted to determine where in the sequence of apoptotic signaling this secondary oxidative burst occurred, specifically, whether it was before or after the point of no return, *i.e.,* caspase activation. To examine this, we treated RGC cultures with 50 μ*M* ZVAD-FMK, a broad-spectrum caspase inhibitor. During superoxide imaging, ZVAD-FMK was added to cultures 5 min before the addition of H_2O_2. We found that 58.3% of treated RGCs exhibited a burst of superoxide. This effect was significantly higher than that of vehicle addition ($p = 0.04$) and was not significantly different from that of H_2O_2-only (no ZVAD-FMK) ($p = 0.43$). This result suggests that caspase inhibition does not block the secondary oxidative burst, implying that the burst occurs upstream from caspase activation.

The secondary oxidative burst is not caused by thiol oxidation

To determine whether the target of oxidation in the apoptosis signaling pathway involves a thiol, we treated RGC cultures with 100 μ*M* TCEP prior to H_2O_2 addition. TCEP is a non–thiol-containing reducing agent that should prevent thiol oxidation. If the latter is a prerequisite for the secondary oxidative burst, then TCEP should block the rise in superoxide. During superoxide imaging, TCEP was added to RGC cultures 5 min prior to H_2O_2 treatment. We found that 100% of treated RGCs exhibited the secondary oxidative burst. This result was significantly higher than that with vehicle ($p < 0.0001$) and not significantly different from that with H_2O_2 only ($p = 0.14$). The finding that TCEP does not block the secondary oxidative burst implies that one or more thiols is unlikely to be a target for oxidation by H_2O_2.

Oxidative amplification requires protein synthesis

We hypothesized that an oxidative stress in axotomized RGCs induces the oxidation of a protein target, which is responsible for a secondary burst in superoxide. To test whether

protein synthesis was necessary for the superoxide burst, we treated RGC cultures with 100 μ*M* cycloheximide, either 1 h or 24 h before superoxide imaging. After treatment with cycloheximide for 1 h, 85.7% of cultured RGCs produced a superoxide burst. This result is significantly different from that with vehicle ($p = 0.0044$) and not different from the H_2O_2-only effect ($p = 1.0$). Treating with cycloheximide for 24 h completely inhibited the superoxide burst (0% of RGCs showed the effect). This result was not significantly different from that with vehicle ($p = 0.24$), but significantly different from H_2O_2-only treatment ($p < 0.0001$). The fact that cycloheximide could block the secondary oxidative burst when present in the medium for 24 h, but not for 1 h, suggests that the oxidative amplification response to oxidative stress is protein-mediated and requires synthesis of one or more proteins.

DISCUSSION

These results demonstrate that induction of an oxidative stress in cultured RGCs induces a secondary burst of superoxide anion, resulting in amplification of the original oxidative signal. Importantly, the fact that it occurs only after a 24-h delay after axotomy suggests that it may be part of a ROS signaling mechanism for axonal damage. This is supported by our findings and those of other laboratories that there is an optimal redox level for RGC survival after axotomy. The secondary ROS burst could represent a means for amplifying an oxidative signal or, alternatively, generating a signal for inducing apoptosis. Other studies have demonstrated the ability of ROS to act as intracellular messengers; examples involve the activation of NF–κB and AP-1, stimulation of growth factors and cytokines, and cellular migration (8, 9, 11).

Although these data are consistent with oxidative stress inducing a secondary superoxide signal, there are other possible explanations for the rise in RGC HEt fluorescence after incubation with H_2O_2. First, the conversion of nonfluorescent HEt to fluorescent Et could actually reflect oxidation by H_2O_2, and not superoxide. However, incubation of HEt and H_2O_2 in cell-free medium did not cause a rise in fluorescence, implying that HEt is not oxidized by H_2O_2.

A second possibility is that reverse activity of SOD could enzymatically produce superoxide from H_2O_2. HEt itself has some SOD activity (1). However, if this were the case, then it would not explain why the rise was only seen 24 h after axotomy, nor why inhibition of protein synthesis would block the rise. Furthermore, inducing an oxidative stress with TBHP also induced a superoxide rise, even though TBHP cannot be directly converted to superoxide. Finally, artificially increased SOD activity (via polyethylene glycol-SOD) decreased the levels of superoxide, while if there were reversed SOD activity, it would be expected to increase.

A third possibility is that the rise in superoxide is not a signaling response, but a result of cellular damage from oxidative stress, *i.e.,* associated with necrotic cell death. However, we used Sytox Green positivity as a marker of plasma membrane disruption. RGCs did not become Sytox Green positive with 9.5 m*M* H_2O_2, but did become positive in the presence of 95 m*M* H_2O_2. Furthermore, this would not explain the increase in superoxide in RGCs 24 h after axotomy, but not 2 h after axotomy.

A fourth possibility is that photooxidation caused breakdown of HEt to Et. However, we accounted for this in acquisition of a baseline before addition of H_2O_2 or other agents, and there were clear differences in rate of increase in red fluorescence after addition, compared with before addition. Furthermore, photooxidation should have the same effect on RGCs, independent of the time after axotomy.

If indeed H_2O_2 induces superoxide as part of an intracellular signal transduction pathway, what does this contribute to our understanding of ROS generation after axotomy? Our previous (13) results demonstrating a dependence of axotomized RGCs on redox state, coupled with the present experiments' requirement for protein synthesis for the superoxide burst, suggest that axotomy induces the synthesis of one or more proteins that are likely involved in superoxide generation after axonal injury. H_2O_2, as an alternative form of oxidative stress, may activate some of the same mechanisms for superoxide generation in the cell, most likely mitochondrial. Alternatively, H_2O_2 itself may be an intracellular signaling molecule.

Although these data are supportive of oxidative stress inducing a secondary superoxide burst, consistent with amplification, there are several caveats. First, the intracellular targets for H_2O_2 or other molecules are not known. We have hypothesized that an attractive target is one or more protein sulfhydryls, which, when oxidized, result in induction of an apoptosis program. However, the fact that TCEP, a sulfhydryl reductant, failed to block the burst suggests that sulfhydryl oxidation may not be the relevant mechanism for this signal. To elucidate this, it will be necessary to study the oxidation state of sulfhydryls in several pertinent signaling proteins. Second, we used high doses of H_2O_2 for these experiments, primarily because we had previously showed that RGCs exhibit relatively robust levels of peroxidase activity (16). However, these high doses may have multiple effects on multiple intracellular molecules, and proof that oxidative stress activates a signaling pathway will require dissection of the target(s) that become oxidized. Third, although axotomy appears to be necessary for the amplification response to occur, we do not know the time course for an RGC to become competent to respond to oxidative stress by producing superoxide, other than that it is at least 2 h.

The optimal approach to demonstrating ROS generation after axotomy may be in the live animal. In this way, the effects of dissociation can be separated from axonal injury, and the normal cell–cell interactions within the retina can be preserved. Furthermore, this would also allow study of adult RGCs after optic nerve crush, which are difficult to study in culture. However, it is difficult to deliver adequate levels of HEt to the retina via an intravitreal or intravenous route, and until this is possible, our knowledge of ROS signaling after RGC axotomy will depend on *in vitro* experiments.

In summary, we demonstrated that an oxidative stress induced a secondary superoxide burst in RGCs that had been axotomized 24 h previously. Our interpretation is that axotomy makes the RGC competent to generate superoxide, perhaps as part of an intracellular signaling process, and that this generation can also be activated by oxidative stress. Agents that interfere with the ability to generate superoxide may also inhibit signaling of RGC death after axotomy, and may represent novel targets for neuroprotection in optic nerve disease.

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ABBREVIATIONS

DAPI, 4',6-diamidino-2-phenylindole; Et, ethidium; HBSS, Hanks' balanced salt solution; HEt, dihydroethidium; H_2O_2, hydrogen peroxide; NF-κB, nuclear factor-κB; RGC, retinal ganglion cell; ROS, reactive oxygen species; SOD, superoxide dismutase; TBHP, *tert*-butyl hydroperoxide; TCEP, tris(2-carboxyethyl)phosphine; ZVAD-FMK, Z-Val-Ala-DL-Asp-fluoromethyl ketone.

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