The Molecular Basis of Oxidative Stress–Induced Cell Death in an Immortalized Retinal Ganglion Cell Line

Pamela Maher¹ and Anne Hanneken²

PURPOSE. To characterize the molecular basis of oxidative stress–induced death, a process that has been implicated in several chronic eye diseases, in RGC-5 cells, an immortalized retinal ganglion cell (RGC) line.

METHODS. The responses of RGC-5 cells to oxidative stress induced by three different treatments—glutathione depletion, tert-buty1 peroxide addition, and hydrogen peroxide addition—were examined and compared. The level of cell death was monitored with the MTT assay. The effects of glutathione depletion on the intracellular levels of glutathione, reactive oxygen species, and calcium were determined. The type of cell death was assessed with assays for DNA fragmentation and caspase activation. Compounds that were shown to be protective of central nervous system–derived nerve cells exposed to oxidative stress were tested to see whether they could also protect the RGC-5 cells. In addition, several compounds that have been found to be protective in primary cultures of RGCs or in animal models of retinal dysfunction were tested against each of the inducers of oxidative stress.

RESULTS. The cell death triggered by all three inducers of oxidative stress shared several features, suggesting that there is a final common pathway of oxidative stress–induced death in the RGCs. In addition, several compounds were identified that protected RGCs from multiple forms of oxidative stress.

CONCLUSIONS. The RGC-5 line is an excellent model for studying mechanisms of RGC death in response to oxidative stress and for the identification of neuroprotective compounds. (Invest Ophthalmol Vis Sci. 2005;46:749–757) DOI:10.1167/iovs.04-0883

Recent advances in the field of neuroprotection indicate that healthy neurons can be protected from injury, and damaged neurons can be rescued from dying by blocking specific steps in the cell death cascade. These results suggest that it may be feasible to protect healthy cells and rescue damaged cells in glaucoma, optic neuropathies, and various retinovascular conditions, such as diabetic retinopathy and retinal vein occlusions. However, to design compounds that protect retinal neurons from cell death, it is important to have an understanding of the molecular pathways involved in the cell death cascade.

Oxidative stress, which can be defined as an imbalance between the production and removal of reactive oxygen species (ROS), has been implicated in many types of nerve cell death in the central nervous system (CNS) and in the eye.2–4 The presence of high concentrations of ROS can overwhelm the cell’s natural defense mechanisms and activate pathways that lead to programmed cell death. Although the precise mechanisms that give rise to the increases in ROS may vary from condition to condition, the cell death pathways appear to have several features in common.5 Among these features is that death proceeds by a series of steps and that the inhibition of any one of these steps can often rescue the nerve cells from death. Whereas there is argument about the precise forms of cell death that occur in nerve cells exposed to acute or chronic injury,6,7 there is agreement that certain agents can ameliorate the cell death process in various kinds of injury that involve oxidative stress.

One model of the induction of oxidative stress in nerve cells utilizes the amino acid glutamate. Glutamate is the major excitatory neurotransmitter in the CNS and in the eye, but it also has been implicated in nerve cell death after acute neurologic insults and in several different ocular diseases, including glaucoma, diabetic retinopathy, and various forms of retinal ischemia.7 Although glutamate is present in synaptic nerve terminals in millimolar concentrations, the extracellular concentrations are normally high only during the brief periods of synaptic transmission. However, certain forms of injury can result in extended periods of elevated extracellular glutamate levels. High levels of extracellular glutamate have been shown to be toxic to nerve cells in culture through two distinct processes: excitotoxicity,8,9 which occurs through the activation of ionotropic glutamate receptors, and a programmed cell death pathway called oxidative glutamate toxicity, or oxytosis.5,10 which is mediated by a series of disturbances to the intracellular redox system. Increases in the endogenous levels of ROS are key elements in the cell death cascade in both of these processes. In glutamate excitotoxicity, the activation of ionotropic glutamate receptors leads to an excessive influx of Ca²⁺, which activates a cell death cascade involving the accumulation of mitochondrially generated ROS.7,11,12 In oxidative glutamate toxicity, glutamate inhibits the uptake of cystine, which is essential for glutathione (GSH) biosynthesis, resulting in the depletion of GSH from the cells. GSH is the most abundant intracellular thiol and the major intracellular antioxidant. The glutamate-induced loss of GSH from cells leads to a biphasic increase in mitochondrial-derived ROS that can eventually reach levels many times higher than those in untreated cells.5 These high levels of ROS lead to Ca²⁺ influx, which is mediated by a cobalt-sensitive, cGMP-gated Ca²⁺ channel,13 and subsequently to cell death. The central role of ROS in the cell death cascade in oxidative glutamate toxicity is demonstrated by the protection provided by exogenous antioxidants such as vitamin E and propyl gallate. The importance of mitochondrial ROS production is further demonstrated by the observation that the mitochondrial uncoupler cyanide p-trifluoromethoxyphenylhydrazone (FCCP), as well as other mitochondrial inhibitors, blocks cell death in this model, by inhibiting ROS production.

From the Departments of ¹Cell Biology and ²Molecular and Experimental Medicine, The Scripps Research Institute, La Jolla, California. Supported by National Institute of Neurological Disorders and Stroke Grant NS28212 (PM) and funding from the Mericos-TSRI Neurobiology and Vision Science Program.

Submitted for publication July 26, 2004; revised September 27, 2004; accepted October 5, 2004.

Disclosure: P. Maher, None; A. Hanneken, None

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be marked "advertisement" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Corresponding author: Pamela Maher, The Salk Institute, 10010 N. Torrey Pines Road, La Jolla, CA 92037; pmaher@salk.edu.

Copyright © Association for Research in Vision and Ophthalmology 749
Our goal in the present study was to examine the response of retinal ganglion cells (RGCs) to oxidative stress to understand the molecular basis of oxidative-stress–induced RGC death. We used three models of oxidative stress in combination with an immortalized retinal ganglion cell line (RGC-5): two standard models that employ exogenous sources of ROS, H$_2$O$_2$, and tert-butyl peroxide (t-BOOH), and the model of oxidative glutamate toxicity, in which ROS are produced endogenously by mitochondria. The production of ROS in oxidative glutamate toxicity is likely to be more representative of that which occurs in injured nerve cells in vivo, and thus this model may offer unique advantages to investigators interested in understanding the molecular basis of oxidative-stress–induced RGC death.

Although the response of RGCs to oxidative stress can be studied in primary cultures of these cells, comprehensive biochemical and molecular studies with primary cultures are limited by the difficulty of obtaining a sufficient number of cells. Recently, an immortalized RGC line was described that retains many of the characteristics of primary RGCs. We used this cell line to characterize the response of the RGCs to oxidative stress and, in particular, their response to endogenously generated ROS, which is likely to be more relevant to the disease process. In the course of these studies, we also identified several molecules that can protect the cells from oxidative-stress–induced death and therefore may have potential benefits as therapeutic compounds for the treatment of glaucoma, optic neuropathies, and various retinovascular conditions, such as diabetic retinopathy and retinal vein occlusion.

**METHODS**

**Chemicals**

2′,7′-Dichlorodihydrofluorescein diacetate (H$_2$DCF-dA), indo-acetoxy-methylster (Indo-1), parironic 127, 5,5′,6,6′-tetracloro-1,1′,3,3′-tetraethyl-benzimidazolcarbocyanine iodide (JC-1), 4′,6′-diamino-2-phenyilindole (DAPI), and rhodamine-125 were purchased from Molecular Probes (Eugene, OR). SKF38393, quinpirole, and 2-(1-thienyl)ethyl 3,4-dihydroxybenzildienacyanacetate (TED) were from Tocris (Ellisville, MO). All other chemicals were from Sigma-Aldrich (St. Louis, MO).

**Cell Culture and Viability Assays**

RGC-5 cells were obtained from Neeraj Agarwal and were grown on tissue culture dishes in DMEM-low glucose supplemented with 10% FCS. Cell viability was determined by a modified version of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay, based on the standard procedure. To obtain consistent results with RGC-5 cells in the MTT assay, the cells were plated in 35-mm dishes at $7 \times 10^4$ cells/dish in complete medium and 18 hours later, the medium was replaced with DMEM-low glucose supplemented with 5% dialyzed FCS (DFCS), and the experimental agents were added. Twenty-four hours after the addition of the experimental agents, the cell culture medium in each dish was aspirated and replaced with 500 $\mu$L DMEM-low glucose with 5% DFCS containing 2.5 $\mu$g/mL MTT. After 4 hours of incubation at 37°C, the cells were solubilized by the addition of 500 $\mu$L of a solution containing 50% dimethylformamide and 20% SDS (pH 4.7). The absorbance at 560 nm was measured on the following day with a microplate reader (Flow Titertec Multiskan Plus, model Mk 11; ICN, Aurora, OH). Results obtained from the MTT assay correlated directly with the extent of cell death, as confirmed visually. Controls, including dishes without cells and cells without the experimental agents, were used to determine the effects of agents on the assay chemistry or cell viability, respectively.

**Microscopy**

A light microscope (Inverted Microscope Diaphot-TMD; Nikon, Tokyo, Japan) equipped with a phase-contrast condenser (Phase contrast-2 ELWD 0.3; Nikon), a 10× objective lens, and a digital camera (Coolpix 990; Nikon) were used to capture the images with the manual setting.

**Total Intracellular GSH and Oxidized Glutathione**

Cells were washed twice with ice-cold PBS, collected by scraping, and lysed with 3% sulfosalicylic acid. Lysates were incubated on ice for 10 minutes, and supernatants were collected after centrifugation in a microfuge (Eppendorf, Fremont, CA). On neutralization of the supernatant with triethanolamine, the concentration of total glutathione (reduced and oxidized; GSSG) was determined by the method of Tietze, with modifications. Briefly, neutralized supernatant (25 $\mu$L) was mixed with 175 $\mu$L of a reaction mixture containing 143 mM sodium phosphate (pH 7.5), 6.3 mM Na$_2$EDTA, 6 mM 5,5′-dithiobis(2-nitrobenzoic acid), and 0.25 mg/mL NADPH. The reaction was started by adding 1 U/mL of glutathione reductase. Color development was monitored at 405 nm in kinetic mode with a microplate reader. Pure GSH was used to obtain a standard curve. The protein content of each sample was determined using a bicinchoninic acid (BCA) protein assay kit from Pierce (Rockford, IL) with BSA as a standard.

**ROS Level**

The intracellular accumulation of ROS in the RGC-5 cells was determined with H$_2$DCF-dA. This nonfluorescent compound accumulates within cells on deacetylation. H$_2$DCF then reacts with ROS to form fluorescent dichlororofluorescein (DCF). RGC-5 cells were dissociated from tissue culture dishes with trypsin in DMEM in the presence of 5 $\mu$L H$_2$DCF-dA for 10 minutes at 37°C, washed once with room temperature DMEM (without phenol red), supplemented with 2% dialyzed FBS, and resuspended in 750 $\mu$L of the same solution containing 2 $\mu$g/mL propidium iodide (PI). Flow cytometric analysis was then performed (FACScan; BD Biosciences, San Jose, CA) with an excitation wavelength ($\lambda_{ex}$) of 475 nm and an emission wavelength ($\lambda_{em}$) of 525 nm. Data were collected on 10,000 live cells after gating for high-PI fluorescence to exclude dead cells. Median fluorescence intensities of control and test samples were determined with the accompanying software (CellQuest; BD Biosciences).

**Calcium Measurement**

The cytosolic accumulation of Ca$^{2+}$ in the RGC-5 cells was measured using the dye indo-acetoxy-methylster (Indo-1) and flow cytometry. This nonfluorescent compound is trapped within cells on deacetylation. The dye then binds Ca$^{2+}$ and on excitation by UV light, it emits fluorescent light at two wavelengths: 410 nm and 485 nm. The Ca$^{2+}$ concentration present in the cell is proportional to the ratio of FL485 to FL410, which reflects the fluorescence of Indo-1 bound to Ca$^{2+}$ versus the fluorescence of Indo-1 that is not bound to Ca$^{2+}$. The ratio of bound to unbound accounts for any changes that take place in the cell volume on exposure to any of the agents. RGC-5 cells were loaded with 1 $\mu$L Indo-1 in the presence of 0.005% pluronic F-127 for 30 minutes at 37°C. The cells were then dissociated from the dishes with trypsin in the presence of 5 $\mu$L DCF-dA, washed, and analyzed as described earlier. The Ca$^{2+}$ concentration is presented as the ratio of FL485 to FL410. Data were collected on 10,000 live cells after gating for high-PI fluorescence to exclude dead cells.

**Immunoblot Analysis**

For immunoblot analysis, RGC-5 cells from the same density cultures as used for the cell death assays were washed twice in cold phosphate-buffered saline (PBS) and scraped into lysis buffer containing 50 mM HEPES (pH 7.4), 150 mM NaCl, 50 mM NaF, 1.5 mM MgCl$_2$, 1 mM EGTA, 10% glyceral, 1% Triton X-100, 10 mM sodium pyrophosphate, 1 mM Na$_2$VO$_3$, 1 mM phenylmethylsulfonyl fluoride (PMSF), 15 $\mu$g/mL.
aprotinin, 1 μg/ml, pepstatin, and 5 μg/ml leupeptin. Lysates were incubated at 4°C for 30 minutes and cleared by centrifugation at 14,000 rpm for 10 minutes. Protein concentrations were determined with the BCA protein assay (Pierce). Equal amounts of protein were solubilized in 2.5 × SDS-sample buffer, separated on 10% SDS-polyacrylamide gels, and transferred to nitrocellulose. Transfers were blocked for 2 hours at room temperature with 5% nonfat milk in TBS/0.1% Tween 20. The immunoblots were then incubated for 1 hour at room temperature in horseradish peroxidase conjugated secondary antibodies (anti-rabbit or goat anti-mouse (Bio-Rad) diluted 1:5000 in 5% nonfat milk in TBS/0.1% Tween 20 and 0.05% Tween 20). The primary antibodies used were anti-poly(ADP-ribose) polymerase (PARP; catalog no. 9542) and anti-cleaved caspase 3 (catalog no. 9661; Cell Signaling, Beverly, MA). The transfers were rinsed with TBS and 0.05% Tween 20 and incubated for 2 hours at room temperature with 5% BSA in TBS and 0.05% Tween 20. The primary antibodies used were anti-poly(ADP-ribose) polymerase (PARP; catalog no. 9542) and anti-cleaved caspase 3 (catalog no. 9661; Cell Signaling, Beverly, MA). The transfers were rinsed with TBS and 0.05% Tween 20 and incubated for 1 hour at room temperature in horseradish peroxidase conjugated secondary antibodies (anti-rabbit or goat anti-mouse (Bio-Rad) diluted 1:5000 in 5% nonfat milk in TBS/0.1% Tween 20. The immunoblots were then developed (Super Signal; Pierce).

DNA Fragmentation

DNA fragmentation was measured by a cell death detection ELISA from Roche (Indianapolis, IN), according to the manufacturer’s instructions. This assay allows the specific detection of mono- and oligonucleosomes in the cytoplasm of cells.

Statistical Analysis

Experiments presented were repeated at least three times with triplicate samples. The data are presented as the mean ± SD. An unpaired Student’s t test was used to compare the data obtained. P < 0.005 was considered to be statistically significant.

RESULTS

In a review of a series of previous studies, we described how oxidative stress in CNS-derived nerve cells can be induced by high concentrations of extracellular glutamate through a pathway of oxidative glutamate toxicity that leads to the endogenous production of high levels of mitochondrially derived ROS. Because endogenous ROS production has been linked to the nerve cell death that occurs in multiple ocular disorders, we were interested in knowing whether this approach could also be used to induce oxidative stress and subsequent cell death in the RGC-5 cells. To this end, the cells were treated with concentrations of glutamate ranging from 1 to 25 mM and cell survival was determined after 24 hours by both visual inspection and the MTT assay. As shown in Figure 1A, the RGC-5 cells were resistant to glutamate treatment alone, showing significant cell death only at concentrations of 25 mM and higher. These results are consistent with previous studies of primary cultures of RGCs21 that showed a high level of resistance to oxidative stress. In CNS-derived nerve cells, glutamate can induce oxidative stress by several mechanisms, one of which is the depletion of GSH, the major intracellular antioxidant. Thus, we reasoned that this resistance to glutamate could be due to the ability of the RGC-5 cells to maintain high levels of GSH. Accordingly, we treated the cells with glutamate in combination with buthionine sulfoximine (BSO), which inhibits glutamate cysteine ligase, the rate-limiting enzyme in GSH biosynthesis. Under these conditions, the cells became significantly more sensitive to glutamate treatment. As shown in Figure 1A, 500 μM BSO alone was only slightly toxic to the cells, causing, at most, ~25% cell death. However, the combination of 500 μM BSO and 10 mM glutamate was very effective at killing the cells, resulting in the death of 85% to 90% of the cells. Consistent with these observations, glutamate alone (at concentrations below 25 mM) was not very effective at depleting cells of GSH, whereas BSO alone and the combination of glutamate and BSO reduced GSH to very low levels (Fig. 1B). The cause of the low level of cell death after treatment with BSO alone, despite its ability to reduce GSH levels significantly, appears to be that, whereas BSO blocks GSH biosynthesis, it does not block cys-

FIGURE 1. (A) RGC-5 cells were treated with increasing concentrations of glutamate or glutamate plus BSO. The cells were plated into 35-mm dishes at 7 × 10⁴ cells/dish in complete medium, and 18 hours later the medium was replaced with DMEM-low glucose supplemented with 5% FCS, and glutamate or glutamate plus BSO was added. Twenty-four hours later, cell survival was measured by the MTT assay. The results presented are the average ± SD of three to five independent experiments. (B) RGC-5 cells were treated with increasing concentrations of glutamate or glutamate plus BSO (BSO) for 8 hours, and cellular levels of total GSH were determined. The GSH level of the control sample (43.5 ± 8 nmoles/mg protein) was taken as 100%. The results are the means ± SD of duplicate determinations in three independent experiments. *Significant difference between the samples treated with glutamate alone and those treated with glutamate plus BSO.
tine uptake. Intracellularly, cystine is reduced to cysteine, which can function as an antioxidant and thereby prevent cell death in the presence of low GSH levels. Accordingly, reducing the cystine levels in the culture medium significantly enhanced BSO toxicity (data not shown).

To determine whether the combination of BSO plus glutamate also results in an increase in the levels of ROS, we used the dye H$_2$DCF-dA in combination with flow cytometry. This nonfluorescent compound accumulates within cells on deacetylation. H$_2$DCF then reacts with ROS to form a fluorescent dichlorofluorescein (DCF). As shown in Figure 2A, BSO plus glutamate treatment results in a 50- to 100-fold increase in DCF fluorescence, indicative of an accumulation of ROS, between 16 and 18 hours. At 18 hours, there was an unexpected significant increase in the number of cells with low DCF fluorescence relative to that at 16 hours. This peak of low DCF fluorescence was not seen in the presence of the soluble guanylate cyclase (sGC) inhibitor LY83583 which prevents Ca$^{2+}$ influx (data not shown; explained later). These results suggest that once cells reach a sufficiently high level of DCF fluorescence, they rapidly became permeable, allowing DCF to leak out of the cell in a Ca$^{2+}$-dependent process. However, these cells were not yet dead, since they did not label with PI. Consistent with the central role of ROS in glutamate-plus-BSO-induced death of the RGC-5 cells, the antioxidant propyl gallate promoted cell survival (Table 1, Fig. 3).

Using several nerve cell lines derived from the CNS, we and others have described a novel cell death pathway called oxidative glutamate toxicity, or oxytosis, which leads to the endogenous production of high levels of mitochondrially derived ROS and is seen in response to treatment with glutamate as well as other inducers of oxidative stress (for review, see Ref. 5). We have identified several inhibitors that specifically block the death mediated by this pathway. We used these inhibitors to determine whether cell death induced by treatment of the RGC-5 cells with glutamate plus BSO proceeds by a pathway similar to that in nerve cells derived from the CNS, such as the hippocampal cell line, HT22. Several compounds that block glutamate-induced cell death in HT22 cells inhibit ROS production either directly, by acting on mitochondria, or indirectly, by mechanisms that have yet to be established. Both the mitochondrial uncoupler FCCP and clorgyline act on mitochondria, preventing the exponential rise of ROS production that leads to death in the HT22 cells. As shown in Table 1, both of these compounds also blocked glutamate-plus-BSO-induced death in RGC-5 cells. Inhibitors of both RNA and protein synthesis block both ROS production and cell death in HT22 cells. They were also effective at preventing the glutamate-plus-BSO-induced death of the RGC-5 cells (Table 1). In addition, inhibitors of 12-lipoxygenase such as 2-(1-thienyl)-ethyl 3,4-dihydroxybenzylidenecyanoacetate (TED) block both ROS production and cell death in the HT22 cells. TED was equally effective at inhibiting glutamate-plus-BSO-induced death in the RGC-5 cells, consistent with the activation of 12-lipoxygenase by GSH depletion.

Calcium entry into cells is a necessary and final step in the cell death caused by oxidative glutamate toxicity in CNS-derived nerve cells. Ca$^{2+}$ influx into the HT22 cells can be blocked by inhibitors of soluble guanylate cyclase (sGC) and by the calcium channel blocker, cobalt. In RGC-5 cells, both cobalt (Fig. 3) and the sGC inhibitor LY83583 blocked glutamate-plus-BSO-induced cell death at concentrations similar to those that are effective in HT22 cells (Table 1). To substantiate a role for calcium influx in the death of the RGC-5 cells, we looked at calcium influx using the dye Indo-1 in combination with flow cytometry. This dye binds Ca$^{2+}$ and on excitation by UV light, it fluoresces light at two wavelengths: 410 nm and 485 nm. The Ca$^{2+}$ concentration present in the cell is proportional to the ratio of FL485 to FL410. As shown in Figure 2B, treatment of the RGC-5 cells for 19 hours with glutamate plus...
cells were treated with increasing concentrations of either H2O2 or t-BOOH and cell survival assayed after 24 hours by both visual inspection and the MTT assay. As shown in Figure 5, both H2O2 and t-BOOH killed the RGC-5 cells in a dose-dependent fashion. Similar to their response to glutamate, the RGC-5 cells are less sensitive to both t-BOOH (EC50 of 25 μM vs. 8 μM) and H2O2 (EC50 of 200 μM vs. 400 μM) than were the HT22 cells. To determine whether these agents kill the RGC-5 cells by a pathway similar to that activated by the combination of glutamate plus BSO, the ability of the inhibitors listed in Table 1 to block both H2O2 and t-BOOH-induced cell death was tested. The antioxidant propyl gallate was effective at preventing cell death induced by both H2O2 and t-BOOH (Table 1, Fig. 3). However, inhibitors that block the production of ROS (FCCP, clorgyline, protein, and RNA synthesis inhibitors) after treatment with glutamate plus BSO had little or no effect on peroxide-induced cell death. In contrast, cobalt, which blocks Ca2+ influx also prevented peroxide-induced death (Table 1, Fig. 3). Curiously, LY83583 which blocks Ca2+ influx induced by glutamate plus BSO by inhibiting soluble guanylate cyclase does not prevent peroxide-induced death, suggesting that the peroxides may induce Ca2+ influx by a somewhat different mechanism. However, both apomorphine and the selective dopamine D1 receptor agonist SKF38393 also protected the cells against H2O2 and t-BOOH (Figs. 3, 6), consistent with the idea that dopamine receptor activation can block Ca2+ influx. Furthermore, both H2O2 and t-BOOH failed to induce the cleavage of either caspase 3 or PARP (Fig. 4B) or significant DNA fragmentation (Fig. 4A). Taken together, these results suggest that there is a final common pathway of cell death that is initiated by various forms of oxidative stress in nerve cells, which culminates in Ca2+ influx and is distinct from classic apoptosis. This finding is a key element in our study, as it suggests that compounds that protect against Ca2+ influx may be useful neuroprotective agents for preventing RGC death in ocular diseases associated with oxidative stress caused by a variety of conditions.

Table 1. Half-maximum Effective Concentrations for Protection of RGCs

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Glu/BSO</th>
<th>t-BOOH</th>
<th>H2O2</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHX</td>
<td>150 ng/mL</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Actin D</td>
<td>25 nM</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Clorgyline</td>
<td>30 μM</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>FCCP</td>
<td>1 μM</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>TED</td>
<td>0.5 μM</td>
<td>2 μM</td>
<td>5 μM</td>
</tr>
<tr>
<td>LYS8583</td>
<td>0.25 μM</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>CoCl2</td>
<td>40 μM</td>
<td>50 μM</td>
<td>50 μM</td>
</tr>
<tr>
<td>Propyl gallate</td>
<td>5 μM</td>
<td>10 μM</td>
<td>20 μM</td>
</tr>
</tbody>
</table>

Concentrations were determined by exposing the RGC-5 line to 10 mM glutamate plus 500 μM BSO, 0.05 mM t-BOOH or 650 μM H2O2 in the absence or presence of various concentrations (ranging from 1 to 100 μM) of the macromolecule synthesis inhibitors CHX and actinomycin D, the MAO inhibitor clorgyline, the mitochondrial uncoupler FCCP, the lipoygenase inhibitor TED, the soluble guanylate cyclase inhibitor LYS8583, the calcium channel blocker CoCl2, or the antioxidant propyl gallate and assessing cell survival 24 hours later by MTT assay. Similar results were obtained in three to five independent experiments. No indications of protection.

In the HT22 cells, dopamine and the dopamine receptor agonists apomorphine and apocadene prevent Ca2+ influx in response to glutamate treatment through a mechanism that appears to involve the D4 dopamine receptor.24 However, in the RGC-5 cells, whereas the nonselective dopamine agonist apomorphine protected the cells from glutamate plus BSO (Figs. 3 and 6), the selective dopamine D4 receptor agonist PD168077 was not effective at protecting the cells from death (Fig. 6). Similarly, the selective D2 receptor agonist quinpirol had little protective effect. In contrast, the selective D1 receptor agonist SKF38393 was highly effective at protecting the cells from glutamate plus BSO treatment with an EC50 of ~1 μM (Fig. 6). These data are consistent with studies on primary cultures of fetal rat retinal cells,25 which demonstrated specific protection from glutamate treatment by SKF38393.

In contrast to classic apoptosis, oxytosis, as defined in the HT22 cells, does not involve DNA fragmentation or caspase 3 activation. To determine whether this is also the case with the RGC-5 cells, DNA fragmentation was assayed with an ELISA. As shown in Figure 4A, significant levels of DNA fragmentation occurred after treatment with glutamate plus BSO as well as with the positive control, staurosporine, a known inducer of classic apoptosis.26 Similarly, staurosporine induced significant activation of caspase 3 and PARP cleavage (Fig. 4B), whereas no caspase 3 activation and only a very low level of PARP cleavage occurred after treatment of the RGC-5 cells with glutamate plus BSO. Taken together, these data indicate that glutamate plus BSO initiates a form of oxidative stress-induced cell death in RGC-5 cells that has characteristics of both classic apoptosis and oxytosis.

To understand further the response of RGCs to oxidative stress, we exposed the RGC-5 cells to other sources of ROS including the hydroperoxides H2O2 and t-BOOH. To determine whether these hydroperoxides can also kill RGC-5 cells, the cells were treated with increasing concentrations of either H2O2 or t-BOOH and cell survival assayed after 24 hours by both visual inspection and the MTT assay. As shown in Figure 5, both H2O2 and t-BOOH killed the RGC-5 cells in a dose-dependent fashion. Similar to their response to glutamate, the RGC-5 cells are less sensitive to both t-BOOH (EC50 of 25 μM vs. 8 μM) and H2O2 (EC50 of 200 μM vs. 400 μM) than were the HT22 cells. To determine whether these agents kill the RGC-5 cells by a pathway similar to that activated by the combination of glutamate plus BSO, the ability of the inhibitors listed in Table 1 to block both H2O2 and t-BOOH-induced cell death was tested. The antioxidant propyl gallate was effective at preventing cell death induced by both H2O2 and t-BOOH (Table 1, Fig. 3). However, inhibitors that block the production of ROS (FCCP, clorgyline, protein, and RNA synthesis inhibitors) after treatment with glutamate plus BSO had little or no effect on peroxide-induced cell death. In contrast, cobalt, which blocks Ca2+ influx also prevented peroxide-induced death (Table 1, Fig. 3). Curiously, LYS8583 which blocks Ca2+ influx induced by glutamate plus BSO by inhibiting soluble guanylate cyclase does not prevent peroxide-induced death, suggesting that the peroxides may induce Ca2+ influx by a somewhat different mechanism. However, both apomorphine and the selective dopamine D1 receptor agonist SKF38393 also protected the cells against H2O2 and t-BOOH (Figs. 3, 6), consistent with the idea that dopamine receptor activation can block Ca2+ influx. Furthermore, both H2O2 and t-BOOH failed to induce the cleavage of either caspase 3 or PARP (Fig. 4B) or significant DNA fragmentation (Fig. 4A). Taken together, these results suggest that there is a final common pathway of cell death that is initiated by various forms of oxidative stress in nerve cells, which culminates in Ca2+ influx and is distinct from classic apoptosis. This finding is a key element in our study, as it suggests that compounds that protect against Ca2+ influx may be useful neuroprotective agents for preventing RGC death in ocular diseases associated with oxidative stress caused by a variety of conditions.

Figure 3. RGC-5 cells were left untreated (A) or were treated with 10 mM glutamate plus 500 μM BSO (B, F, J, N) or 0.05 mM t-BOOH (C, G, K, O), or 650 μM H2O2 (D, H, L, P), alone (B-D) or in the presence of 20 μM propyl gallate (E-H), 10 μM apomorphine (I-L), or 100 μM CoCl2 (M-P). The cells were photographed 24 hours later.

Downloaded from iovs.arvojournals.org on 10/05/2019
DISCUSSION

In this study, we used a recently described RGC line (RGC-5) to characterize the cell death pathways induced by three different types of oxidative stress: one involving the endogenous production of ROS and the other two involving direct addition of either ROS or of a ROS generator. Much of this study focused on the induction of oxidative stress by the exposure of the cells to high concentrations of glutamate in combination with an inhibitor of GSH biosynthesis, BSO, which leads to the endogenous production of ROS. This combination causes both the depletion of GSH from the cells within 8 hours of treatment (results not shown) and then, subsequent to the decrease in GSH, a gradual increase in ROS to levels at least 50 times greater than control levels. The peak in ROS is followed rapidly by an increase in intracellular Ca\(^{2+}\) and cell death. Although the basic events in the RGC-5 cell death pathway are very similar to those in the HT22 cells, a cell line derived from mouse hippocampus, there are some differences that may provide insight into the specific response of RGCs to oxidative stress. First, the RGCs are much more resistant to glutamate-mediated GSH depletion than the HT22 cells. One possible explanation for this is that cystine uptake is approximately two times higher in the RGC-5 cells than in the HT22 cells (data not shown). Second, the depletion of GSH in the RGC-5 cells leads to a much slower increase in the levels of ROS than in the HT22 cells, although the final levels of ROS approach those in the HT22 cells. These results suggest that the maintenance of GSH levels in the RGCs plays an important role in cell survival. This conclusion is supported by an earlier study that showed that a glutathione peroxidase mimic could protect primary cultures of RGCs from death. Third, unlike the HT22 cells, the RGCs cannot tolerate high levels of ROS for an extended time. Instead, the data suggest that shortly after the cells reach a threshold level of ROS, the plasma membrane becomes leaky and the cells rapidly die. Thus, while the RGCs are initially more resistant to oxidative stress than the HT22 cells, they are less tolerant of a high level of stress. A high level of resistance of RGCs to oxidative stress was noted previously in a study of primary cultures of RGCs. The lack of tolerance to high levels of oxidative stress appears to be due to either an increased rate of Ca\(^{2+}\) influx or an increased sensitivity to Ca\(^{2+}\), because if Ca\(^{2+}\) influx is blocked by treatment with cobalt or the soluble guanylate cyclase inhibitor, LY83583, which we previously showed to block Ca\(^{2+}\) influx in the HT22 cells, then the RGC-5 cells would not die but instead would continue to
average cell survival was measured by the MTT assay. The results presented are with 5% DFCS and the experimental agents were added. 24 hours later stress could have beneficial effects on RGC survival in vivo. survive in the presence of high levels of ROS. Taken together, these data suggest that even a partial decrease in oxidative stress could have beneficial effects on RGC survival in vivo.

The RGCs are also sensitive to oxidative stress induced by treatment with either \( \text{H}_2\text{O}_2 \) or \( \text{t-BOOH} \). The cell death induced by both of these compounds shares some characteristics with that induced by glutamate plus BSO, suggesting that there is a common final pathway of oxidative stress–induced death in the RGCs. Of note, under none of these conditions did we see activation of the classic apoptotic pathway, although the classic pathway can be readily activated in the RGCs by treatment with staurosporine. Thus, no activation of caspase 3 was observed under any of the conditions of oxidative stress tested. However, glutamate plus BSO induced DNA fragmentation and low levels of PARP cleavage. In contrast, no PARP cleavage and little DNA fragmentation were noted with either \( \text{H}_2\text{O}_2 \) or \( \text{t-BOOH} \). These data suggest that compounds that target some of the steps of classic apoptosis may not be particularly useful in protecting RGCs from oxidative stress–induced death.

In our studies on HT22 cells, we identified several compounds that could block glutamate-induced cell death because they inhibited key steps in the cell death process. All these compounds were also found to inhibit glutamate-plus-BSO-induced death in the RGCs. These data suggest that the treatments induce a very similar process of cell death in the two different types of nerve cells—a programmed cell death pathway called oxytosis. Among the features of oxytosis is the generation of high levels of ROS from mitochondria once GSH levels decline below \( \sim 20\% \) of control levels. Similar to the HT22 cells, in the RGC-5 cells, cell death was blocked by both FCCP, a mitochondrial uncoupler, and clorgyline, an MAO inhibitor that we have shown can also block ROS production by mitochondria. Although most of these compounds may not be useful in the clinic, they have been useful for sorting out key steps in the cell death pathway. However, we have also identified additional compounds that act at each of the key steps in cell death and that may be more useful therapeutically. These compounds are flavonoids, and their neuroprotective actions on RGCs will be described in a future article.

The glutamate-induced cell death model used in this study was selected because it relies on the endogenous production of ROS, which have been linked to RGC death in multiple clinical conditions. Although the glutamate-induced cell death in this study was not directly mediated by ionotropic glutamate receptors, it has many features in common with excitotoxicity. For example, in both cases, the accumulation of mitochondrial derived ROS and \( \text{Ca}^{2+} \) influx plays a critical role in the cell death process. Similarly, both types of cell death appear to require gene transcription and protein synthesis, since both were blocked by actinomycin D and cycloheximide. Furthermore, there is evidence that the delayed cell death that occurs in a variety of excitotoxicity paradigms could be due to oxidative glutamate toxicity. Increases in glutamate to levels that could activate the oxidative pathway may be exacerbated by the decreased levels of glutamate transporters present in glaucoma.

One of the more interesting observations in these studies was the protective effect of dopamine receptor activation on oxidative-stress–induced RGC death. Both the nonspecific dopamine receptor agonist apomorphine and the specific dopamine D1 receptor agonist SKF38393 were very effective at protecting the cells not only from glutamate plus BSO but also from cell death induced by \( \text{t-BOOH} \) and \( \text{H}_2\text{O}_2 \). In the HT22 cells, it was shown that the selective dopamine D4 receptor agonist SKF38393 protected the cells by inhibiting \( \text{Ca}^{2+} \) uptake. In the RGC-5 cells, a D4 agonist had no effect on cell death, whereas the selective D1 agonist SKF38393 was extremely effective at preventing oxidative stress–induced death. Because the D1 agonist also protected against \( \text{t-BOOH} \) and \( \text{H}_2\text{O}_2 \) toxicity, it is probable that it acts at the same step in all cases. These data are in agreement with an earlier study that showed that selective dopamine receptor D1 agonists could protect primary cultures of fetal rat retinal cells from excitotoxic damage and further demonstrate the similarities between excitotoxicity and oxidative glutamate toxicity.

In summary, using a recently described immortalized RGC line, we characterized the oxidative stress–induced cell death pathways resulting from either the endogenous generation of ROS or the addition of exogenous ROS. The cell death path-

---

**Figure 5.** RGC-5 cells or HT22 cells were treated with increasing concentrations of \( \text{t-BOOH} \) (A) or \( \text{H}_2\text{O}_2 \) (B). The cells were plated into 35-mm dishes at \( 7 \times 10^4 \) cells/dish in complete medium and 18 hours later the medium was replaced with DMEM-low glucose supplemented with 5% DFCS and the experimental agents were added. 24 hours later cell survival was measured by the MTT assay. The results presented are the average \( \pm SD \) of three to five independent experiments.
ways induced by all three of these inducers of oxidative stress have several features in common and share several characteristics with cell death models developed with primary RGCs. The data also suggest sites where therapeutic intervention may be possible, even after oxidative stress is initiated.

References


10. Murphy TH, Miyamoto M, Sastre A, Schnaar RL, Coyle JT. Glutamate toxicity in a neuronal cell line involves inhibition of cystine transport leading to oxidative stress. *Neuron.* 1989;2:1547–1558.


12. Schinder AF, Olson EC, Spitzer NC, Montal M. Mitochondrial dysfunction is a primary event in glutamate neurotoxicity. *J Neurosci.* 1996;16:6125–6135.


