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The Effects of Oxidative Stress on Mitochondrial Transmembrane Potential in Retinal Ganglion Cells

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ABSTRACT

Retinal ganglion cells (RGCs) are central neurons that undergo apoptosis after axonal injury. As the relationship between mitochondrial and oxidative signaling of apoptosis in neuronal systems is unclear, we sought to achieve a better understanding of the interplay of these two pathways by investigating the effect of direct oxidative stress on mitochondrial membrane potential in cultured RGCs, as measured with the dual-emission probe JC-1. Treatment with hydrogen peroxide caused RGC mitochondrial depolarization. Several pharmacological treatments were used to define the mechanism. Whereas cycloheximide, tris(2-carboxyethyl)phosphine, and cyclosporin A were unable to prevent the depolarization, bongkrekic acid significantly reduced the severity of the depolarization. This suggests that the hydrogen peroxide-induced depolarization may act through mitochondrial permeability transition pore opening independent of thiol oxidation, and may be preventable under certain conditions. *Antioxid. Redox Signal.* 5, 641–646.

INTRODUCTION

It is now widely accepted that mitochondria play an important role in the signaling of apoptosis, typically via the release of cytochrome *c* and other apoptosis-inducing factors (17, 34). It has also been proposed that apoptosis is signaled through the opening of the mitochondrial permeability transition pore (PTP) (10, 13, 26, 27, 29). Opening of the PTP causes a loss of the mitochondrial transmembrane potential ($\Delta\Psi_m$) and allows for molecules smaller than 1,500 Da to pass between the mitochondrial matrix and the intermembrane space, and then into the cytoplasm (2, 3, 19, 21). The importance of PTP opening and its necessity in neuronal apoptosis are still under debate (13, 22), and it is uncertain whether mitochondrial depolarization alone is sufficient to induce apoptosis (33) in neurons.

Reactive oxygen species (ROS) have been implicated in a variety of normal cellular processes (7, 8, 12, 16, 20), and are also generated from disruptions in the mitochondrial electron transport system (9). In the nervous system, ROS are involved in the signaling pathways for cell death. This has been demonstrated in several systems, including sympathetic neurons (18), hippocampal neurons (5), cerebellar granule cells (30), and retinal ganglion cells (RGCs) (15).

RGCs are central neurons, undergoing apoptosis after axonal injury (1, 14, 23, 28). Our previous studies have shown that RGC apoptosis is critically dependent on oxidative redox state (15), and that PTP modulation can cause massive RGC death accompanied by mitochondrial depolarization (31). By imaging axotomized RGCs in real time, we hoped to clarify the interplay between oxidative stress and opening of the PTP. We hypothesized that treatment with hydrogen peroxide (H_2O_2) would lead to mitochondrial depolarization, and that modulation of the PTP would prevent the loss of $\Delta\Psi_m$. We found that when the adenine nucleotide translocase (ANT) was bound by bongkrekic acid (BA), mitochondrial depolarization was attenuated, suggesting a specific mechanism by which oxidative stress could cause mitochondrial depolarization in central neurons.

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MATERIALS AND METHODS

Animals

All experiments were performed in accordance with the U.S. Public Health Service Policy on Humane Care and Use of Laboratory Animals and the National Institutes of Health Guide for the Care and Use of Laboratory Animals, as well as the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and institutional guidelines regarding animal research.

Materials

Cell culture reagents and media were obtained from GIBCO (Grand Island, NY, U.S.A.). The retrograde fluorescent tracer 4',6-diamidino-2-phenylindole (DAPI) was obtained from Molecular Probes (Eugene, OR, U.S.A.) and prepared as a 5 m*M* solution in dimethylformamide (Acros, Fair Lawn, NJ, U.S.A.). 5,5',6,6'-Tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) was obtained from Molecular Probes and prepared as a 4 m*M* stock solution in dimethylformamide. Papain was acquired from Worthington Biochemical (Freehold, NJ, U.S.A.). Chambered cover glasses for imaging were acquired from Nalgene–Nunc (Naperville, IL, 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 (24). In brief, ganglion cells were labeled by stereotactic injection of the fluorescent tracer DAPI (5 m*M*) into the superior colliculi of anesthetized postnatal day 4 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 JC-1. Over a period of 3–4 days, DAPI flows via retrograde transport from RGC projection sites in the superior colliculi to the RGC somas in the retina, where it binds to nuclear DNA. At postnatal day 7–8, the animals were killed by decapitation, the eyes enucleated, and the retinas dissected free in Hanks' balanced salt solution (HBSS). 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 Pasteur pipette and plated on poly-L-lysine-coated eight-well chambered cover glass wells at a density of ~2,000 cells/mm^2. The cell cultures incubated (37°C, 5% CO_2, 80% H_2O) for 24 h in serum-free medium [Neurobasal-A with 0.7% methylcellulose, 5.0 μg/ml gentamicin, and 2% B-27 without antioxidants defined serum-free supplement; GIBCO].

Measurement of $\Delta\Psi_m$

The $\Delta\Psi_m$ was measured using the voltage-sensitive dye JC-1 (32), which associates with the mitochondrial inner membrane. JC-1 exists in two forms: monomer and J-aggregate. In its base state and when $\Delta\Psi_m$ is low, it associates with the mitochondrial inner membrane as a monomer, and emits green fluorescence (535 nm) when excited at 480 nm. When $\Delta\Psi_m$ is high, the dye forms J-aggregates near areas of high membrane potential, and emits red fluorescence (580 nm) when excited at 480 nm. As the total mitochondrial mass will vary from cell to cell, these absolute fluorescence values will also be quite variable. Therefore, the ratio of red to green fluorescence [$F(\lambda_{580})/F(\lambda_{535})$] is a more accurate method for comparing mitochondrial $\Delta\Psi_m$ status in different cells. Typically, cells with a healthy population of mitochondria with a high $\Delta\Psi_m$ have a high ratio of $F(\lambda_{580})/F(\lambda_{535})$ fluorescence, whereas cells whose mitochondria are in the process of losing their $\Delta\Psi_m$ (depolarization) have a low $F(\lambda_{580})/F(\lambda_{535})$ ratio.

Twenty-four hours after plating, mixed retinal cultures were treated with JC-1 (1 μg/ml) in Neurobasal-A for 15 min at 37°C, washed once with plain culture medium, and then imaged in Neurobasal-A, 2% B-27 without antioxidants, 21 m*M* HEPES, and 5 μg/ml gentamicin. RGCs were located in each well using a DAPI filter set (330 nm excitation, 450 nm emission). Filters used to detect JC-1 were 480 nm for excitation, 505 nm dichroic, and dual 535 nm and 580 nm emission filters mounted in a computer-controlled filter wheel (Sutter Instruments, Novato, CA, U.S.A.). A cooled CCD camera (Roper Scientific, Trenton, NJ, U.S.A.) mounted on a Zeiss Axiovert 135 inverted microscope collected the images, with image processing performed in real time. Images were acquired using Metafluor software at a binning of 2, exposure time 100 ms, 2× gain.

Initial measurements of $\Delta\Psi_m$ were acquired once every 2.5 min for 10 min to establish baseline fluorescence levels. Pharmacological treatments were applied immediately after the 10-min baseline period by adding reagents directly to the well. Background fluorescence was subtracted from the initial (t = 2.5 min) fluorescent measurements, and the ratio of fluorescence at 580 nm and 535 nm [$F(\lambda_{580})/F(\lambda_{535})$] was calculated. The point of maximal depolarization was chosen as that time after treatment where the ratio reached a minimum, and was used for subsequent analyses. Because of changes in fluorescence due to photobleaching and dye leakage, the effect of pharmacological agents on maximal $\Delta\Psi_m$ depolarization from baseline was determined with respect to that of media alone.

Pharmacological treatments

Components of the mitochondrial PTP pore were perturbed in an attempt to prevent the depolarization caused by oxidative stress. Tris(2-carboxyethyl)phosphine (TCEP) was used to reduce vicinal thiols located within the PTP complex. BA and cyclosporin A (CsA) were used to maintain the PTP in a closed state. Valinomycin, a mitochondrial inner membrane-specific K^+ ionophore, was used as a positive control in imaging experiments for baseline depolarization events. Cycloheximide (CHx) was used to inhibit protein synthesis. All concentrations were chosen based on initial dose-response experiments and methods established in the literature (4, 22, 25).

Statistical analysis

Means were compared with Student's unpaired t test.

RESULTS

H_2O_2 causes mitochondrial depolarization in RGCs in a concentration-dependent manner

We and others have hypothesized that a rise in ROS could act as a trigger for the apoptotic pathway, in conjunction with mitochondrial depolarization. To probe the relationship between

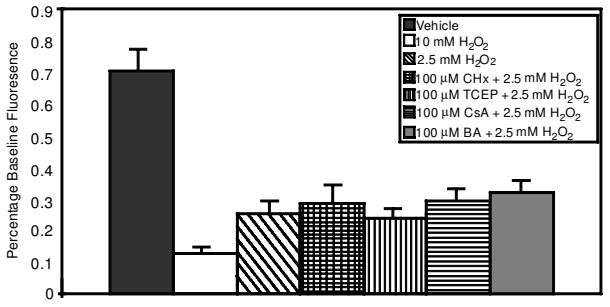

FIG. 1. Mitochondrial depolarization in RGCs treated with H_2O_2. Vehicle, H_2O_2 (10 m*M*), H_2O_2 (2.5 m*M*), H_2O_2 (2.5 m*M*) + CHx (100 µ*M*), H_2O_2 (2.5 m*M*) + TCEP (100 µ*M*), H_2O_2 (2.5 m*M*) + CsA (10 µ*M*), or H_2O_2 (2.5 m*M*) + BA (10 µ*M*) was added to cultures after 10 min of baseline acquisition. The point of maximal depolarization was determined as where the fluorescence ratio $F(\lambda_{580})/F(\lambda_{535})$ was at a minimum, and compared with the baseline fluorescence ratio. The decrease in $F(\lambda_{580})/F(\lambda_{535})$ was significant compared with vehicle for all conditions. Only treatment with H_2O_2 + BA had a significantly smaller decrease in ratio compared with treatment with H_2O_2 alone ($p = 0.007$).

ROS and mitochondrial depolarization in axotomized RGCs, mixed retinal cultures containing RGCs retrogradely labeled with DAPI were incubated for 24 h, and then imaged with the dual-emission probe JC-1 after application of several concentrations of H_2O_2. At high concentrations (10 m*M*), H_2O_2 creates an oxidative stress in RGCs. This led to a significant decrease in $\Delta\Psi_m$, as reflected by a decrease in $F(\lambda_{580})/F(\lambda_{535})$ to 12.7 ± 0.8% of baseline levels, compared with 68.7 ± 8.0% for vehicle alone ($p = 0.0002$) (Fig. 1). At this high concentration of H_2O_2, there is so much oxidation of cellular constituents that RGC mitochondrial depolarization proceeded in the presence of all pharmacological inhibitors tested (TCEP, BA, CsA, CHx; data not shown). To examine more physiologically relevant oxidative stresses, we progressively decreased the H_2O_2 dosage until reaching the minimum concentration capable of producing a reliable loss of $\Delta\Psi_m$. Concentrations of H_2O_2 lower than 2.5 m*M* showed less consistent and generally later depolarization (data not shown). Treatment with 2.5 m*M* H_2O_2 caused a significant decrease in $F(\lambda_{580})/F(\lambda_{535})$ to 24.8 ± 3.2% of baseline ($p = 0.0005$) (Figs. 1 and 2), and was therefore chosen as the concentration to evaluate mechanisms of mitochondrial depolarization from oxidative stress.

The depolarization caused by oxidative stress in RGCs is not dependent on protein synthesis

To determine the likelihood of a requirement for recently synthesized protein(s) mediating the decrease in $\Delta\Psi_m$, cultured RGCs were treated with 100 µ*M* CHx during plating and the 24-h incubation prior to imaging $\Delta\Psi_m$. RGCs in which

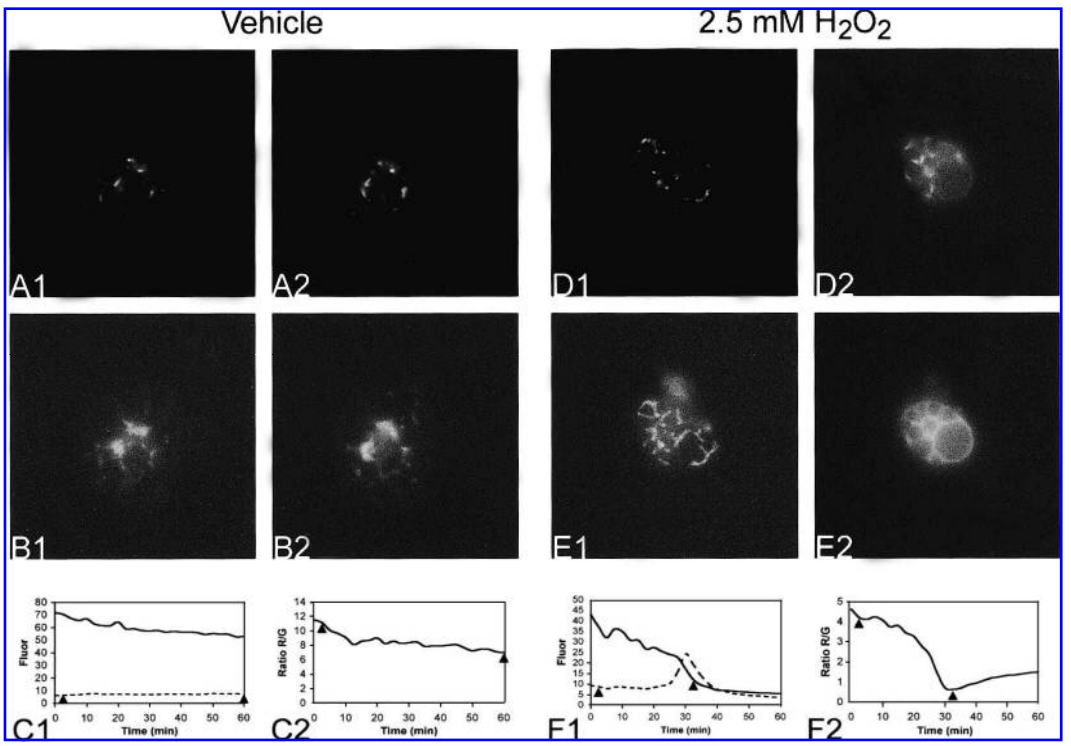

FIG. 2. H_2O_2-induced mitochondrial depolarization. Cells preincubated with JC-1 were imaged for 10 min to establish baseline fluorescence, then treated with either vehicle (**A, B, C**) or 2.5 m*M* H_2O_2 (**D, E, F**), and imaged for 50 min. Cells are shown at the initial point (A1, B1, D1, E1) and at the point of maximum depolarization (A2, B2, D2, E2) as defined by the ratio of fluorescence at 580 nm (A1, A2, D1, D2) to the fluorescence at 535 nm (B1, B2, E1, E2). The tracings below each micrograph demonstrate the change in fluorescence at λ = 580 nm (———) and λ= 535 nm (— — —) over the course of the treatment (C1, F1), and fluorescent ratio [$F(\lambda_{580})/F(\lambda_{535})$] (C2, F2). Arrows on the tracings indicate the time points when the displayed images were acquired.

protein synthesis had been inhibited over 24 h demonstrated a decrease in $F(\lambda_{580})/F(\lambda_{535})$ after treatment with 2.5 m*M* H_2O_2 commensurate with that seen in H_2O_2-treated RGCs with uninhibited protein synthesis (27.4 ± 5.7% versus 24.8 ± 3.2%; $p = 0.40$) (Fig. 1). The inability of CHx to block the decrease in $\Delta\Psi_m$ suggests that the mechanism by which the depolarization occurs is not mediated by a recently synthesized protein.

The decrease in membrane potential after oxidative stress is not caused by thiol oxidation

Previous studies in our laboratory have shown that one of the mechanisms underlying ROS signaling of apoptosis could be the critical dependence of RGC survival after axotomy on cellular redox state (15, 24). To determine if the loss in $\Delta\Psi_m$ caused by H_2O_2 is due to thiol oxidation by peroxide intermediates, mixed retinal cultures were pretreated with TCEP (100 μ*M*) for 24 h before imaging the $\Delta\Psi_m$. This concentration was chosen based on previous studies showing that it significantly prolongs RGC survival after axotomy in the absence of neurotrophic factors or serum. The presence of 100 μ*M* TCEP prior to, and during, exposure to H_2O_2 (2.5 m*M*) did not prevent the decrease in $\Delta\Psi_m$ (24.0 ± 2.9% versus 24.8 ± 3.2%; $p = 0.42$) (Fig. 1), suggesting that the mechanism of the H_2O_2-induced depolarization is independent of thiol oxidation of PTP components.

The depolarization is attenuated by pore modulation with BA, but not CsA

The ability of CsA to bind to the mitochondrial matrix protein cyclophilin D and prevent PTP opening and apoptosis has been demonstrated in other neuronal systems (6). BA has also been shown to prevent pore opening through binding to the ANT, thereby blocking apoptosis caused by *N*-methyl-D-aspartate excitotoxicity in cerebral cortical neurons (4). To explore the mechanism by which oxidative stress-induced depolarization occurred in our system, we attempted to block pore opening by pretreating cultures with CsA (10 μ*M*) or BA (10 μ*M*), followed by incubation with H_2O_2 (2.5 m*M*). CsA did not significantly affect the depolarization when compared with control (29.1 ± 2.8% versus 24.8 ± 3.2%; $p = 0.16$) (Fig. 1). In contrast, BA significantly inhibited the reduction in $\Delta\Psi_m$, compared with treatment with H_2O_2 alone (33.8 ± 3.5% versus 24.8 ± 3.2%; $p = 0.007$) (Fig. 1), or when compared with CsA ($p = 0.034$). The reduction in $\Delta\Psi_m$ with BA was still lower than treatment with vehicle alone, suggesting that ANT binding reduces the effect of oxidative stress without inhibiting it completely.

DISCUSSION

The relation between ROS formation and opening of the PTP in neurons is not well understood. Our experiments demonstrated that although H_2O_2 caused mitochondrial depolarization in RGCs, the mechanism of depolarization cannot be entirely explained by PTP opening. H_2O_2 is a potent oxidant, and in high doses reliably produced mitochondrial depolarization in RGCs. Lower doses also perturbed the $\Delta\Psi_m$, but in a less dramatic fashion. Whether or not the depolarization is a result of opening of the PTP alone or multiple factors is unclear. We used pharmacological agents affecting protein synthesis (CHx) and PTP components (CsA, BA, and TCEP) to understand better the mechanism behind the mitochondrial depolarization after treatment with low doses of H_2O_2. We found that the H_2O_2-induced depolarization was not dependent on protein synthesis, as evidenced by the nonprotective effect of CHx.

We pretreated RGC cultures with CsA, BA, and TCEP to prevent H_2O_2-induced mitochondrial depolarization via different mechanisms. CsA and TCEP did not significantly modify the mitochondrial depolarization, but pretreatment with BA was able to significantly attenuate, but not entirely prevent depolarization. As BA is known to inhibit the ANT, a component of the PTP complex, it is likely that it is in fact preventing pore opening signaled by the rise in H_2O_2. This is supported by another study showing that BA prevents H_2O_2-induced apoptosis and PTP-mediated mitochondrial depolarization in other cell types (11). Although BA was able to significantly alter the depolarization response to H_2O_2 treatment, it did not entirely prevent it. If H_2O_2 simply signaled PTP opening, then one would expect BA to completely disrupt H_2O_2-induced depolarization. It is therefore likely that H_2O_2 disturbs $\Delta\Psi_m$ via one or more other pathways in addition to action on the pore complex itself. The attenuation of H_2O_2 depolarization by BA raises the prospect that this or similarly acting agents could be neuroprotective in RGCs.

The lack of protection from oxidative stress by CsA is not entirely surprising. Vrabec *et al.* (31) showed that axotomized RGCs were not protected by treatments with CsA in culture, which in fact paradoxically increased the rate of apoptosis and dramatically increased PTP opening when combined with a peripheral benzodiazepine antagonist. Combining those observations with the data presented here, it is likely that CsA (or its complex with cyclophilin D) does not interact with the RGC PTP in the same manner as other cell types, and thus would not have a role in preventing mitochondrial depolarization in these cells.

We used the non–thiol-containing reducing agent TCEP to prevent the oxidation of cellular sulfhydryls, including the PTP-associated sulfhydryls that cause PTP opening when oxidized. The fact that 100 μ*M* TCEP was unable to prevent H_2O_2-induced mitochondrial depolarization implies that oxidative stress does not directly cause oxidation of pore-associated thiols. As we chose the concentration of TCEP based on survival studies where RGCs were incubated for 24–72 h, it is possible that the dose used was too low to prevent depolarization caused by a short-term, high-dose H_2O_2 challenge. However, the dose of H_2O_2 was within the range we have previously used to kill RGCs over 24–72-h time periods, suggesting that the TCEP dose was not too low. Thus, because H_2O_2 was able to cause depolarization independently of sulfhydryl oxidation, oxidative stress may have a role mediating signaling in RGCs via another oxidative target. We also cannot directly implicate H_2O_2 as a signaling molecule, as interconversion between H_2O_2 and other intracellular ROS (*e.g.,* superoxide and hydroxyl radical) could occur.

In summary, we showed that H_2O_2 can cause mitochondrial depolarization in axotomized RGCs, presumably via opening

of the PTP. We found that this depolarization can be attenuated by pretreatment with BA, suggesting that the PTP opening in these RGCs may be reversible and could possibly be prevented under the proper conditions. A better understanding of the relationship between ROS signaling of apoptosis and PTP opening may lead to methods for preventing RGC apoptosis from occurring in diseases of the optic nerve.

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ABBREVIATIONS

ANT, adenine nucleotide translocase; BA, bongkrekic acid; CHx, cycloheximide; CsA, cyclosporin A; DAPI, 4',6-diamidino-2-phenylindole; HBSS, Hanks' balanced salt solution; H_2O_2, hydrogen peroxide; JC-1, 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide; PTP, permeability transition pore; RGC, retinal ganglion cell; ROS, reactive oxygen species; TCEP, tris(2-carboxyethyl)phosphine; $\Delta\Psi_m$, mitochondrial membrane potential.

REFERENCES

1. Berkelaar M, Clarke DB, Wang YC, Bray GM, and Aguayo AJ. Axotomy results in delayed death and apoptosis of retinal ganglion cells in adult rats. *J Neurosci* 14: 4368–4374, 1994.
2. Bernardi P. The permeability transition pore. Control points of a cyclosporin A-sensitive mitochondrial channel involved in cell death. *Biochim Biophys Acta* 1275: 5–9, 1996.
3. Bernardi P, Broekemeier KM, and Pfeiffer DR. Recent progress on regulation of the mitochondrial permeability transition pore; a cyclosporin-sensitive pore in the inner mitochondrial membrane. *J Bioenerg Biomembr* 26: 509–517, 1994.
4. Budd SL, Tenneti L, Lishnak T, and Lipton SA. Mitochondrial and extramitochondrial apoptotic signaling pathways in cerebrocortical neurons. *Proc Natl Acad Sci U S A* 97: 6161–6166, 2000.
5. Chan SL, Tammariello SP, Estus S, and Mattson MP. Prostate apoptosis response-4 mediates trophic factor withdrawal-induced apoptosis of hippocampal neurons: actions prior to mitochondrial dysfunction and caspase activation. *J Neurochem* 73: 502–512, 1999.
6. Chang LK and Johnson EM Jr. Cyclosporin A inhibits caspase-independent death of NGF-deprived sympathetic neurons: a potential role for mitochondrial permeability transition. *J Cell Biol* 157: 771–781, 2002.
7. Clark RA. Activation of the neutrophil respiratory burst oxidase. *J Infect Dis* 179 Suppl 2: S309–S317, 1999.
8. Dalton TP, Shertzer HG, and Puga A. Regulation of gene expression by reactive oxygen. *Annu Rev Pharmacol Toxicol* 39: 67–101, 1999.
9. Du G, Mouithys-Mickalad A, and Sluse FE. Generation of superoxide anion by mitochondria and impairment of their functions during anoxia and reoxygenation in vitro. *Free Radic Biol Med* 25: 1066–1074, 1998.
10. Dubinsky JM and Levi Y. Calcium-induced activation of the mitochondrial permeability transition in hippocampal neurons. *J Neurosci Res* 53: 728–741, 1998.
11. Dumont A, Hehner SP, Hofmann TG, Ueffing M, Droge W, and Schmitz ML. Hydrogen peroxide-induced apoptosis is CD95-independent, requires the release of mitochondria-derived reactive oxygen species and the activation of NF-kappaB. *Oncogene* 18: 747–757, 1999.
12. Finkel T. Oxygen radicals and signaling. *Curr Opin Cell Biol* 10: 248–253, 1998.
13. Friberg H, Ferrand-Drake M, Bengtsson F, Halestrap AP, and Wieloch T. Cyclosporin A, but not FK 506, protects mitochondria and neurons against hypoglycemic damage and implicates the mitochondrial permeability transition in cell death. *J Neurosci* 18: 5151–5159, 1998.
14. Garcia-Valenzuela E, Gorczyca W, Darzynkiewicz Z, and Sharma SC. Apoptosis in adult retinal ganglion cells after axotomy. *J Neurobiol* 25: 431–438, 1994.
15. Geiger LK, Kortuem KR, Alexejun C, and Levin LA. Reduced redox state allows prolonged survival of axotomized neonatal retinal ganglion cells. *Neuroscience* 109: 635–642, 2002.
16. Goldschmidt-Clermont PJ and Moldovan L. Stress, superoxide, and signal transduction. *Gene Expr* 7: 255–260, 1999.
17. Granville DJ, Cassidy BA, Ruehlmann DO, Choy JC, Brenner C, Kroemer G, van Breemen C, Margaron P, Hunt DW, and McManus BM. Mitochondrial release of apoptosis-inducing factor and cytochrome *c* during smooth muscle cell apoptosis. *Am J Pathol* 159: 305–311, 2001.
18. Greenlund LJ, Deckwerth TL, and Johnson EM. Superoxide dismutase delays neuronal apoptosis: a role for reactive oxygen species in programmed neuronal death. *Neuron* 14: 303–315, 1995.
19. Hirsch T, Marzo I, and Kroemer G. Role of the mitochondrial permeability transition pore in apoptosis. *Biosci Rep* 17: 67–76, 1997.
20. Jabs T. Reactive oxygen intermediates as mediators of programmed cell death in plants and animals. *Biochem Pharmacol* 57: 231–245, 1999.
21. Kroemer G, Zamzami N, and Susin SA. Mitochondrial control of apoptosis. *Immunol Today* 18: 44–51, 1997.
22. Krohn AJ, Wahlbrink T, and Prehn JH. Mitochondrial depolarization is not required for neuronal apoptosis. *J Neurosci* 19: 7394–7404, 1999.
23. Levin LA and Louhab A. Apoptosis of retinal ganglion cells in anterior ischemic optic neuropathy. *Arch Ophthalmol* 114: 488–491, 1996.
24. Levin LA, Clark JA, and Johns LK. Effect of lipid peroxidation inhibition on retinal ganglion cell death. *Invest Ophthalmol Vis Sci* 37: 2744–2749, 1996.
25. Meyer-Franke A, Wilkinson GA, Kruttgen A, Hu M, Munro E, Hanson MG Jr, Reichardt LF, and Barres BA. Depolarization and cAMP elevation rapidly recruit TrkB to

the plasma membrane of CNS neurons. *Neuron* 21: 681–693, 1998.
26. Nicholls DG and Budd SL. Mitochondria and neuronal glutamate excitotoxicity. *Biochim Biophys Acta* 1366: 97–112, 1998.
27. Nieminen AL, Petrie TG, Lemasters JJ, and Selman WR. Cyclosporin A delays mitochondrial depolarization induced by *N*-methyl-D- aspartate in cortical neurons: evidence of the mitochondrial permeability transition. *Neuroscience* 75: 993–997, 1996.
28. Quigley HA, Nickells RW, Kerrigan LA, Pease ME, Thibault DJ, and Zack DJ. Retinal ganglion cell death in experimental glaucoma and after axotomy occurs by apoptosis. *Invest Ophthalmol Vis Sci* 36: 774–786, 1995.
29. Scanlon JM and Reynolds IJ. Effects of oxidants and glutamate receptor activation on mitochondrial membrane potential in rat forebrain neurons. *J Neurochem* 71: 2392–2400, 1998.
30. Schulz JB, Weller M, and Klockgether T. Potassium deprivation-induced apoptosis of cerebellar granule neurons: a sequential requirement for new mRNA and protein synthesis, ICE-like protease activity, and reactive oxygen species. *J Neurosci* 16: 4696–4706, 1996.
31. Vrabec JP, Lieven CJ, and Levin LA. Cell-type specific opening of the retinal ganglion cell mitochondrial permeability transition pore. *Invest Ophthalmol Vis Sci* 44: 2774–2782, 2003.
32. White RJ and Reynolds IJ. Mitochondrial depolarization in glutamate-stimulated neurons: an early signal specific to excitotoxin exposure. *J Neurosci* 16: 5688–5697, 1996.
33. Wigdal SS, Kirkland RA, Franklin JL, and Haak-Frendscho M. Cytochrome *c* release precedes mitochondrial membrane potential loss in cerebellar granule neuron apoptosis: lack of mitochondrial swelling. *J Neurochem* 82: 1029–1038, 2002.
34. Yang J, Liu X, Bhalla K, Kim CN, Ibrado AM, Cai J, Peng TI, Jones DP, and Wang X. Prevention of apoptosis by Bcl-2: release of cytochrome c from mitochondria blocked. *Science* 275: 1129–1132, 1997.

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2. Elena Radi, Patrizia Formichi, Giuseppe Di Maio, Carla Battisti, Antonio Federico. 2012. Altered apoptosis regulation in Kufor-Rakeb syndrome patients with mutations in the ATP13A2 gene. *Journal of Cellular and Molecular Medicine* **16**:8, 1916-1923. [CrossRef]
3. Matthew G. Field, Dongli Yang, Zong-Mei Bian, Howard R. Petty, Victor M. Elner. 2011. Retinal flavoprotein fluorescence correlates with mitochondrial stress, apoptosis, and chemokine expression. *Experimental Eye Research* . [CrossRef]
4. Anna G. Barsukova, Dennis Bourdette, Michael Forte. 2011. Mitochondrial calcium and its regulation in neurodegeneration induced by oxidative stress. *European Journal of Neuroscience* no-no. [CrossRef]
5. A. Kanamori, M.-M. Catrinescu, N. Kanamori, K. A. Mears, R. Beaubien, L. A. Levin. 2010. Superoxide is an associated signal for apoptosis in axonal injury. *Brain* **133**:9, 2612-2625. [CrossRef]
6. Akiyasu Kanamori, Maria-Magdalena Catrinescu, Atif Mahammed, Zeev Gross, Leonard A. Levin. 2010. Neuroprotection against superoxide anion radical by metallocorroles in cellular and murine models of optic neuropathy. *Journal of Neurochemistry* no-no. [CrossRef]
7. Allison Loh, Majda Hadziahmetovic, Joshua L. Dunaief. 2009. Iron homeostasis and eye disease. *Biochimica et Biophysica Acta (BBA) - General Subjects* **1790**:7, 637-649. [CrossRef]
8. Patrizia Formichi, Elena Radi, Carla Battisti, Giuseppe Di Maio, Ermelinda Tarquini, Alessandra Leonini, Anna Di Stefano, Maria Teresa Dotti, Antonio Federico. 2009. Apoptosis in CADASIL: An in vitro study of lymphocytes and fibroblasts from a cohort of Italian patients. *Journal of Cellular Physiology* **219**:2, 494-502. [CrossRef]
9. Vicente Zanon-Moreno, Pilar Marco-Ventura, Antonio Lleo-Perez, Sheila Pons-Vazquez, Jose J. Garcia-Medina, Ignacio Vinuesa-Silva, Maria A. Moreno-Nadal, Maria Dolores Pinazo-Duran. 2008. Oxidative Stress in Primary Open-angle Glaucoma. *Journal of Glaucoma* **17**:4, 263-268. [CrossRef]
10. C BATTISTI, P FORMICHI, E RADI, A FEDERICO. 2008. Oxidative-stress-induced apoptosis in PBLs of two patients with Parkinson disease secondary to alpha-synuclein mutation. *Journal of the Neurological Sciences* **267**:1-2, 120-124. [CrossRef]
11. Patrizia Formichi, Elena Radi, Carla Battisti, Annalaura Pasqui, Gerarda Pompella, Pietro Enea Lazzerini, Franco Laghi-Pasini, Alessandra Leonini, Anna Di Stefano, Antonio Federico. 2007. Psychosine-induced apoptosis and cytokine activation in immune peripheral cells of Krabbe patients. *Journal of Cellular Physiology* **212**:3, 737-743. [CrossRef]
12. L DIWAKAR, R KENCHAPPA, J ANNEPU, V RAVINDRANATH. 2007. Downregulation of glutaredoxin but not glutathione loss leads to mitochondrial dysfunction in female mice CNS: Implications in excitotoxicity. *Neurochemistry International* **51**:1, 37-46. [CrossRef]
13. A LAABICH, C MANMOTO, V KUKSA, D LEUNG, G VISSVESVARAN, I KARLIGA, M KAMAT, I SCOTT, A FAWZI, R KUBOTA. 2007. Protective effects of myricetin and related flavonols against A2E and light mediated-cell death in bovine retinal primary cell culture. *Experimental Eye Research* **85**:1, 154-165. [CrossRef]
14. Domalapalli Maneesh Kumar, Neeraj Agarwal. 2007. Oxidative Stress in Glaucoma: A Burden of Evidence. *Journal of Glaucoma* **16**:3, 334-343. [CrossRef]
15. Christopher R. Schlieve, Annie Tam, Bradley L. Nilsson, Christopher J. Lieven, Ronald T. Raines, Leonard A. Levin. 2006. Synthesis and characterization of a novel class of reducing agents that are highly neuroprotective for retinal ganglion cells. *Experimental Eye Research* **83**:5, 1252-1259. [CrossRef]

16. Cathrin Dressler, Juergen Beuthan, Gerhard Mueller, Urszula Zabarylo, Olaf Minet. 2006. Fluorescence Imaging of Heat-Stress Induced Mitochondrial Long-Term Depolarization in Breast Cancer Cells. *Journal of Fluorescence* **16**:5, 689-695. [CrossRef]
17. P. Formichi, E. Radi, C. Battisti, E. Tarquini, A. Leonini, A. Di Stefano, A. Federico. 2006. Human fibroblasts undergo oxidative stress-induced apoptosis without internucleosomal DNA fragmentation. *Journal of Cellular Physiology* **208**:2, 289-297. [CrossRef]
18. Laurie Conley, Theresa L. Geurs, Leonard A. Levin. 2005. Transcriptional regulation of ceruloplasmin by an IL-6 response element pathway. *Molecular Brain Research* **139**:2, 235-241. [CrossRef]
19. James L. Franklin . 2003. Programmed Neuronal Death. *Antioxidants & Redox Signaling* **5**:5, 583-587. [Citation] [Full Text PDF] [Full Text PDF with Links]