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Cell-Autonomous Generation of Mitochondrial Superoxide is a Signal for Cell Death in Differentiated Neuronal Precursor Cells

Colin J. Scott¹, Emily A. Seidler¹, and Leonard A. Levin^{1,2}

¹Department of Ophthalmology and Visual Sciences University of Wisconsin School of Medicine and Public Health

²Department of Ophthalmology University of Montreal

Abstract

The vast majority of optic neuropathies result from retinal ganglion cell (RGC) axonal injury. This induces cell death and is associated with a burst of mitochondria-generated superoxide within the soma. It is unclear whether there is a clear causal relationship between superoxide generation and cell death. To determine whether mitochondrial-generated superoxide can cause cell-autonomous death signaling, we knocked down SOD2 in a pure population of RGC-5 cells, a neuronal precursor cell line that can be differentiated to resemble retinal ganglion cells. RGC-5 cells were differentiated and transfected with siRNA for SOD2 or a scramble control. Viability, superoxide production, cytotoxic RNA transfection efficiency, and measurement of SOD2 protein levels by immunoblotting were assayed at varying times after transfection. SOD2 knockdown increased intracellular superoxide levels and cell death was presumed triggered from knockdown. This was amplified when extramitochondrial superoxide was elevated with the redox cycling agent menadione. Dysregulation of mitochondrial superoxide in differentiated RGC-5 cells is likely a potent signal for cell death, consistent with a role of this reactive oxygen species in apoptosis signaling after axonal injury.

Keywords

Optic neuropathy; superoxide; neurons; superoxide dismutase; gene silencing

1. Introduction

Optic neuropathies are frequent causes of irreversible visual loss. The most common optic neuropathy, glaucoma, is the leading cause of irreversible visual loss worldwide. While the pathophysiology of the various optic neuropathies may differ, in all cases there is death or dysfunction of retinal ganglion cells (RGC). Most optic neuropathies are precipitated by axonal injury, which can be inflammatory (e.g. optic neuritis), ischemic, compressive, traumatic, or glaucomatous (Levin, 2007).

The process connecting injury of the axon to death of the retinal ganglion cell body is incompletely understood. We showed that optic nerve injury induces a burst of superoxide

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Correspondence: Leonard A. Levin, M.D., Ph.D. Department of Ophthalmology and Visual Sciences University of Wisconsin Medical School 600 Highland Avenue Madison, WI 53792 *Telephone:* (608) 265-6546 *Fax:* (608) 265-6021.

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anion in retinal ganglion cells, beginning hours after injury (Lieven et al., 2006). Superoxide, a reactive oxygen species, can serve as a redox-active signal transduction molecule (Finkel, 2000). It is dismutated by one of three forms of superoxide dismutase (SOD) to hydrogen peroxide: SOD1, which is cytoplasmic, SOD2, which is mitochondrial, or SOD3, which is extracellular. Intracellular scavenging of superoxide with pegylated superoxide dismutase-1 (PEG-SOD1) both decreases RGC superoxide levels and increases their viability after injury (Lieven et al., 2006; Schlieve et al., 2006). These findings are consistent with a model in which superoxide serves as an intracellular signaling molecule after axonal injury, similar to that seen when sympathetic neurons are deprived of nerve growth factor (Greenlund et al., 1995). On the other hand, it is also possible that increased superoxide is a byproduct of the cell death process, and is necessary but not sufficient for death to occur after axonal injury. This possibility was studied by testing whether down-regulation of constitutive superoxide scavengers leads to RGC death. SOD2 knockdown in the mouse with adeno-associated virus (AAV) delivery of a ribozyme targeted at murine SOD2 results in RGC death and pathological features of an optic neuropathy (Qi et al., 2003). This finding does not by itself prove that the death of RGCs is due to superoxide within the cell, i.e. is cell-autonomous, because the effect of SOD2 knockdown in other retinal neurons or glia could indirectly cause RGC death, e.g. by excitotoxicity, reduction in local neurotrophin concentrations, or release of cytotoxins.

To define the role of superoxide in signaling neuronal death, we utilized *in vitro* knockdown of SOD-2 in differentiated RGC-5 cells, a cell line which when exposed to low concentrations of staurosporine becomes differentiated, stops dividing, and develops a neuronal morphology (Frassetto et al., 2006; Lieven et al., 2007). These cells have many features of neuronal precursor cells and share some characteristics of RGCs. We used silencing RNA (siRNA) to achieve SOD-2 knockdown, and found that the latter induced increased superoxide levels and significant neuronal death, consistent with elevated intracellular superoxide being sufficient for autonomously signaling RGC death.

2. Results

Transfection and knockdown with superoxide dismutase 2 siRNA

Measurement of silencing was performed by quantifying SOD2 protein expression by Western blotting, compared to a scrambled siRNA and normalized to actin. There was progressive knockdown of SOD2 from 24 to 96 hours after transfection of SOD2 siRNA (Figure 2). At 72 and 96 hours there was substantial knockdown, with SOD2 levels of $22.1 \pm 11.5\%$ and $18.8 \pm 15.9\%$, respectively, compared to siScramble and normalized to actin. With these high silencing levels achievable, further studies could be performed to assess the functional effect of SOD2 knockdown in neuronally differentiated RGC-5 cells.

SOD2 knockdown increases superoxide levels

We measured relative intracellular superoxide levels in differentiated RGC-5 cells with HET, which reacts with superoxide to form the fluorescent product oxy-Et (Zhao et al., 2003). RGC-5 cells were differentiated at 24 hours, transfected with SOD2 or scrambled siRNA 24 hours later, and superoxide measured 96 hours later. RGC-5 cells transfected with SOD2 siRNA produced $3402 \pm 393\%$ fluorescence units, at baseline compared to $738 \pm 277\%$ for scrambled siRNA controls ($p = .005$). This difference persisted throughout the 64 minute period of recording (Figure 3A). This pattern is consistent with a constant excess of superoxide, presumably as a result of SOD2 knockdown coupled with decreased production over time. The increase of superoxide in the scrambled siRNA relative to baseline was likely due to stress placed on the cells when the media was removed and replaced by PBS, as cells would not be expected to survive for prolonged periods under these conditions.

To examine the effect of knocking down SOD2 in cells exposed to increased level of ROS, transfected and differentiated RGC-5 cells were incubated with xanthine (250 μ M) and xanthine oxidase (0.1 U/ml). Xanthine/xanthine oxidase leads to the production of both extracellular superoxide and hydrogen peroxide. We previously demonstrated that extracellular hydrogen peroxide increases intracellular mitochondrial RGC superoxide levels (Nguyen et al., 2003; Schlieve et al., 2006). Again at baseline there was a difference in the levels of superoxide between the SOD2 and scrambled siRNA conditions (the scale of the Y-axis is compressed in Figure 3B, compared to 3A). However, there was a much sharper rise in fluorescence over the first 30 minutes of recording in cells where SOD2 was knocked down, compared to scrambled siRNA. This difference diminished over time, and the difference in the level of superoxide in the SOD2 vs. scrambled knockdown cells eventually became the same as at baseline by the end of the recording period. This indicates that the knockdown of SOD2 resulted in a decrease in the ability of differentiated RGC-5 cells to handle an induced superoxide burst, the majority of which was generated within 30 minutes. The reason that the shape of the curves is different in the two panels reflects the kinetics of how quickly superoxide goes up with knockdown, compared to xanthine/xanthine oxidase.

SOD2 knockdown reduces cell viability

SOD2 is critical to the function of cells, including RGCs, as demonstrated by AAV ribozyme-mediated knockdown of retinal SOD2 *in vivo* (Qi et al., 2003). We wished to determine whether the effect of SOD2 knockdown *in vivo* reflected a cell autonomous effect, or was the result of effects on other retinal cells. To do this, we tested whether SOD2 knockdown affected survival of differentiated RGC-5 cells, measuring viability by MTT assay, in the presence or absence of endogenous generators of superoxide. We used menadione, which generates extramitochondrial intracellular superoxide by redox cycling (Meany et al., 2007). To control for off-target effects, we used pooled scrambled siRNA as a control. In preliminary experiments, there was an insignificant difference in cell viability between sham transfected and siScramble-transfected cells (1207 ± 478 vs. 1659 ± 562 ; $p = 0.64$).

SOD2 knockdown at 96 hours caused a moderate but significant reduction in viability ($75.7\% \pm 0.1\%$ of scramble control; $p=0.026$) (Figure 4). Cells treated for 24 hours with menadione (100 μ M), a redox-cycling agent that increases levels of superoxide within the mitochondrial matrix, demonstrated a much greater reduction in viability following SOD2 knockdown ($16.5 \pm 11.9\%$ of scramble control; $p=0.02$). To confirm the site specificity of the superoxide generator, we incubated differentiated RGC-5 cells with pegylated superoxide dismutase-1 (PEG-SOD1), which crosses cell membranes and depletes superoxide. We also used calcein/propidium iodide staining to ensure that the MTT results were not confounded by oxidative changes from superoxide. When cells were treated with menadione in the presence or absence of PEG-SOD1, the superoxide generator induced cell death (Figure 5). Together, these results were consistent with SOD2 knockdown decreasing cell viability.

3. Discussion

We demonstrated that gene silencing of SOD2 in differentiated RGC-5 cells resulted in increased superoxide levels which likely caused decreased viability. These results were seen in the absence of glia or other retinal neurons, i.e. were cell-type autonomous. Effects on viability were seen in cells exposed to menadione, which increases levels of extramitochondrial superoxide (Meany et al., 2007). The transfection efficiency of SOD2 siRNA was high, and the effects of SOD2 silencing began as early as 24 hours after transfection, with the most pronounced silencing at 72 hours after transfection.

Retinal ganglion cells are the primary cell type involved in optic neuropathies. They can be studied *in vitro* in primary cell cultures of either mixed retinal cells or affinity-purified RGCs

(Barres et al., 1988). Purities of 95-99% are achievable, but require the binding of an antibody to cell-surface Thy-1, which may have other biological effects (Leifer et al., 1984). If large numbers of RGCs are required, a correspondingly large number of animals need to be used. The RGC-5 cell line is an alternative (Krishnamoorthy et al., 2001). RGC-5 cells are neuronal precursor cells that are mitotically active and do not have a neuronal morphology in their basal state. We previously showed that nanomolar concentrations of the broad-spectrum kinase inhibitor staurosporine induces RGC-5 differentiation, manifested by arrest of cellular proliferation, extension of dendrites and axons, and development of ionic channels similar to those seen in mature RGCs (Frassetto et al., 2006; Lieven et al., 2007). Others have reproduced the ability of staurosporine to differentiate RGC-5 cells (Harvey and Chintala, 2007), although the question of whether they are truly RGCs or not is controversial, and therefore our results cannot necessarily be extrapolated to RGCs *in vivo* (Van Bergen et al., 2009). All experiments with RGC-5 cells were performed at a passage number of less than 8, to maintain their ability to differentiate into neurons. Although differentiated RGC-5 cells are not true RGCs, their homogeneity and purity make them useful for gene silencing experiments. They are also helpful for assess whether a biological mechanism in neurons is cell-type autonomous because they can be purified to homogeneity.

It is therefore possible that there are unrecognized differences between differentiated RGC-5 cells and RGCs. On the other hand, the fact that the current findings support the *in vivo* results of Qi and colleagues in RGCs (2003) is encouraging. Studying RGCs from mice in which SOD2 was inactivated by homologous recombination is not an alternative because homozygotes die within the first 10 days of life with a dilated cardiomyopathy, accumulation of lipid in liver and skeletal muscle, and metabolic acidosis (Li et al., 1995). This indicates that SOD2 is required for normal biologic function of tissues by maintaining the integrity of mitochondrial enzymes susceptible to direct inactivation by superoxide. Treatment with an SOD mimetic, MnTBAP, rescued SOD2^{-/-} mutant mice and dramatically prolonged their survival (Melov et al., 2001). Interestingly, animals developed a pronounced movement disorder progressing to total debilitation by 3 weeks of age. Future studies could include the use of purified RGC cultures from mice conditionally deficient in one or more SOD isoforms.

Two groups have used siRNA-mediated gene silencing in RGCs *in vivo* (Lingor et al., 2005) and in RGC-5 cells against c-Jun, Bax and Apaf-1 (Lingor et al., 2005), and PKR (Shimazawa et al., 2007). However, those studies were performed with either undifferentiated cells or cells treated with concanavalin A, which does not produce a true neuronal morphology. The study of Lingor and colleagues (2005) achieved RNA interference with custom single oligonucleotide duplexes, while we used pools of 4 duplexes. Advances in the field have now allowed for commercially available pooled sequences for greater reliability and efficiency, as described above, with less off-target effects. Their study used 50 nM staurosporine added to cells that had had proliferation halted with concanavalin A to induce apoptosis. We treated with 316 nM staurosporine without concanavalin A, which results in differentiation without obvious evidence of apoptosis (Frassetto et al., 2006).

Gene silencing with siRNA is a methodology prone to artifact. While we cannot be sure that no off-target effects were seen, our use of 4 pooled siRNA duplex species is less likely to affect other genes, and we did not see knockdown of our actin control. Although we did not measure RNA levels, the near complete inhibition of immunodetectable SOD2 at the protein level by immunoblotting is evidence that substantial SOD2 knockdown was achieved. Other controls included pooled scrambled RNA that was used to account for the known toxic effects of siRNA, particularly at higher concentrations. These methods employed to achieve knockdown of the SOD2 gene have not been previously described in RGC-5 cells or RGCs, and our results demonstrate high transfection efficiency and reliable and consistent knockdown, with our small sample size yielding a relatively low standard error at 96 hours post-transfection. There was

moderate loss of RGC-5 actin over time in both the SOD2 and scrambled knockdown. This is likely due to a combination of the effects of the transfection procedure, toxic effects of staurosporine, and the fact that neuronally differentiated RGC-5 cells were not supported with neurotrophic factors.

Brain-derived neurotrophic factor (BDNF) has been shown to increase survival of RGC-5 cells, and supplementation may result in increased viability (Harper et al., 2009). Our finding of increased RGC-5 cell death when there was preferential knockdown of SOD2 beyond that of actin appears consistent with a mechanism of action for superoxide as a signal for neuronal cell death. Our previous studies ruled out neurotrophin deprivation as the cause of elevated superoxide after RGC axotomy because a combination of neurotrophins did not prevent increases in H₂O₂-detectable superoxide (Lieven et al., 2006). Therefore, it is unlikely that the introduction of neurotrophic factors would have affected superoxide production or SOD2 knockdown. When PEG-SOD was added to menadione treated cells, there was a small yet significant increase in cell viability. In theory this would have been expected to be even greater, but it is likely that PEG-SOD does not enter the mitochondrial matrix as effectively as other mitochondrial or cytoplasmic compartments.

In summary, we found that SOD2 knockdown in differentiated RGC-5 cells is associated with increased levels of superoxide and cell death. Reduced SOD2 expression creates higher concentrations of superoxide that are likely the cause of the observed decreased viability. When oxidative stress was induced with pharmacological treatments that increase superoxide levels, greater superoxide production and cell death were observed. These results provide a model for the study of cell-autonomous superoxide signaling *in vitro* after optic retinal ganglion cell body or axonal injury, and also support prior studies showing that SOD2 is an endogenous scavenger for RGC survival *in vivo* (Qi et al., 2003). Our data also establish the use of siRNA in staurosporine-differentiated RGC-5 cells as a method for studying neuron-specific mechanisms of cell death and survival.

4. Methods

Cell Culture

RGC-5 cells were a kind gift of Dr. Neeraj Agarwal. They were cultured in Dulbecco's modified Eagle's medium (Mediatech, Inc., Manassas, VA) containing 1 g/L glucose with L-glutamine supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 µg/mL streptomycin. Cells were incubated at 37°C in humidified 5% CO₂. RGC-5 cells were then plated in Opti-MEM medium (Invitrogen, Carlsbad, CA) containing Eagles minimum essential medium, buffered with HEPES and sodium bicarbonate (2.4 g/L) and supplemented with hypoxanthine, thymidine, sodium pyruvate, L-glutamine, trace elements, and growth factors. Cells were plated at 5,000 cells/well in a 96-well format and incubated overnight to 80% confluence. Cells were then treated with staurosporine to a final concentration of 316 nM and incubated for 24 hours to induce differentiation as previously described (Frassetto et al., 2006). All cells were used at a passage number less than 8.

Transfection

siGENOME ON-TARGETplus SMARTpool silencing RNAs were obtained from Dharmacon (Lafayette, CO). Sequences are listed in Table 1. Transfection was performed according to the manufacturer's instructions. After incubation with staurosporine for 24 hours, media was replaced with new transfection reagent and media as described. Equal amounts of SOD2 or DY-547-labeled siGLO siRNA (2 µM) was added to Opti-MEM to a volume of 35 µL and incubated for 5 minutes. Then 1.2 µL of Dharmafect 4 was mixed with 33.8 µL of Opti-MEM and incubated for 5 minutes. The siRNA and lipofection solutions were then combined and

incubated for 20 minutes. The 70 μ L mixture was diluted with 280 μ L of Opti-MEM for a total volume of 350 μ L. Media was removed from cells and washed with 100 μ L of phosphate buffered saline. Finally, 100 μ L of the lipid-complex mixture was added to cells in triplicate for a final concentration of 100 nM. Control conditions were either a sham transfection with all described methods excluding siRNA, or siCONTROL scrambled non-targeting siRNA pool. Cells were incubated at 37°C in humidified 5% CO₂. The timelines for these procedures are depicted in Figure 1.

Immunoblot Analysis

At 24, 48, 72, and 96 hours post transfection, cells were lysed in RIPA buffer containing 1% Igepal CA-630, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (Fisher Scientific), 0.1 mg/mL phenylmethylsulfonyl fluoride (Sigma-Aldrich), and protease inhibitors (Complete Mini Protease Inhibitor Cocktail Tablet; 5 mg/mL; Roche Diagnostics, Mannheim, Germany) in PBS for 15 minutes at 4°C. Cell lysates from 3 wells were pooled for each sample, and incubated on ice for 60 minutes and centrifuged at 10,000g for 10 minutes at 4°C. The protein concentrations of the supernatants were determined by Bradford assay. Each sample was boiled in the presence of 4X lithium dodecyl sulfate (LDS) sample buffer (Invitrogen, Carlsbad, CA), and 10X reducing buffer resolved on a Bis-Tris 4% to 12% polyacrylamide gel (NuPAGE; Invitrogen) at 200 volts in 3-morpholinopropanesulfonic acid (MOPS) buffer, and transferred overnight at 50 mA to nitrocellulose membrane in a transfer apparatus (Mini Protean II; Bio-Rad Laboratories, Hercules, CA). After transfer, the membrane was blocked with 0.5% nonfat milk in TBS (pH 8.0) for 20 minutes and then probed with primary antibodies in blocking buffer using purified rabbit polyclonal anti-SOD2 (1:1000; Upstate, Charlottesville, VA) for 1 hour followed by secondary antibodies using purified horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (1:5000; Jackson ImmunoResearch Laboratories, West Grove, PA) for 1 hour. Blots were rinsed 3 times with TBS containing 0.05% Tween-20 (Fisher Scientific), and then washed 5 times for 10 minutes each at room temperature on an orbital shaker after each antibody exposure. Blots were treated with freshly prepared ECL solution of 100 mM Tris-HCl (pH 8.5), 1.25 mM luminol, 225 μ M p-coumaric acid (Sigma-Aldrich), and 1 mM H₂O₂ (Fisher Scientific) for 1 minute, and excess solution was allowed to drip off. Blots were then exposed to film (Blue Lite Autorad; ISC BioExpress, Kaysville, UT) and developed. Blots were performed once at 24 and 48 hours 5 times at 72 hours, and twice at 96 hours.

For anti-SOD2, the nitrocellulose membrane was then stripped with glycine stripping buffer (0.2 M glycine, pH 2.5, 0.05% Tween 20) at 56°C for 30 minutes. The Western blot procedure was then repeated using rabbit polyclonal anti-actin (1:1000) for 1 hour followed by secondary antibodies using HRP-conjugated goat anti-rabbit IgG. Blots at 72 and 96 hours were performed twice. Blots were treated with freshly prepared ECL solution then exposed to film as above. For anti-SOD2 blots, the anti-actin primary antibody was used in duplex because the molecular weight of SOD2 and actin are far apart. The films were scanned at 1200 dpi, converted to grayscale, and inverted. Band density was determined by comparing total intensity in an area containing the band of interest to the intensity of an equal size area of background using NIH ImageJ software. Band density readings are presented with respect to the density of the band from the control condition and each were normalized to respective actin bands.

Cell Viability Assessment

Cell viability was assessed with either 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) staining or calcein AM (Invitrogen, Carlsbad, CA) and propidium iodide (Invitrogen) staining. At 72 hours post-transfection, menadione (100 μ M) or vehicle was added to cells in triplicate and incubated for 24 hours. For MTT staining, At 96 hours post-transfection, media was removed and replaced with MTT at 500 μ g/mL in Opti-MEM media. Plates were incubated for 4 hours at 37°C, and medium replaced with 200 μ L of DMSO, which

was pipetted up and down to dissolve the formazan crystals. Absorbance was measured at 550 nm on a microplate reader (ThermoMax; Molecular Devices, Sunnyvale, CA). All readings were normalized to a standard curve derived from known numbers of RGC-5 cells. For individual cell counting, cells were stained with calcein-AM (10 µg/mL) and propidium iodide (1 mL/mL) in PBS for 30 minutes. The staining solution was removed and replaced with PBS. Cells were photographed on a Axiovert 135 microscope under epifluorescence, and live (calcein⁺) and dead (propidium iodide⁺) cells counted using ImageJ software.

Superoxide Measurements

Oxidation of dihydroethidium (HET) by superoxide converts HET, which exhibits weak blue fluorescence, to an ethidium derivative that exhibits peak fluorescence in the red spectrum (excitation 480 nm, emission 586 nm) (Zhao et al., 2003). At 96 hours post-transfection, cells were treated with 3.2 µM HET in PBS and incubated for 20 minutes. Cells were then exposed to 250 µM xanthine, and the reaction was started with addition of 0.1 U/mL xanthine oxidase. Background recordings were obtained by measurements of equal concentrations of xanthine/xanthine oxidase without cells. Control experiments were conducted in parallel without the addition of drugs. Fluorescence readings were obtained (1420 Victor 2 T Multilabel Counter; Wallac, Gaithersburg, MD) with excitation at 485 nm and emission at 580 nm. Readings were obtained every 8 minutes for 64 minutes. Background absorbances were subtracted from each condition.

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Figure 1. Time-line for experimental procedures

Low passage RGC-5 cells were freshly plated to begin an experiment on day 0. Staurosporine (316 nM) was added to cultures at 24 hours to differentiate RGC-5 cells to a neuronal (retinal ganglion cell-like) phenotype. Transfection with siRNA for SOD2 or scramble control was at 48 hours, and various assays (viability, superoxide production, measurement of SOD2 protein levels by immunoblotting) on subsequent days.

Figure 2. Protein levels of SOD2 decrease after knockdown

From 24 to 96 hours, there was increasing knockdown of SOD2 when differentiated RGC-5 cells were transfected with SOD2 siRNA. At 24 hours, knockdown was 44.4% reduced protein expression, compared to siScramble and normalized to actin. At 48 hours 89.3% knockdown was observed. Efficiency of knockdown (normalized to siScramble and to actin) remained high at 72 hours with 77.9% knockdown, and at 96 hours with 81.2% knockdown.

Figure 3. Superoxide production with SOD2 knockdown

Superoxide production was measured by incubating equal numbers of RGC-5 cells in each group with dihydroethidium (HEt), which when oxidized by superoxide produces the fluorescent product oxy-ethidium. Increased levels of superoxide were produced in SOD2 siRNA cells 96 hours post-transfection. In the control condition without drug treatment, there was a higher level of superoxide that rose over time. In xanthine (250 μ M)/xanthine oxidase (0.1 U/ml) treated cells, there was a sharp rise of superoxide produced in the SOD2 knockdown condition, which peaked at 32 minutes. Controls were conducted in parallel without cells to measure the background signal at each time point, and then subtracted from experimental values.

Figure 4. Viability assay of differentiated RGC-5 cells after SOD2 knockdown

Viability was compared between cells treated with the control scramble siRNA (siScramble) vs. SOD2 siRNA 96 hours post-transfection. The control condition without toxins demonstrated a 24% decrease in cell viability with SOD2 knockdown. Exposure for 24 hours with 100 µM menadione resulted in overall lower viability with relatively greater decrease of viability in knockdown cells at 84%. $p < 0.05$.

Figure 5. Viability assay of differentiated RGC-5 cells after treatment with cell permeable pegylated superoxide dismutase-1 (PEG-SOD1)

PEG-SOD1 had no effect on control cells, but increased viability of cells treated with menadione (which increases extramitochondrial superoxide). Asterisk: $p < 0.05$.