A Glaucoma-Associated Mutant of Optineurin Selectively Induces Death of Retinal Ganglion Cells Which Is Inhibited by Antioxidants

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Purpose. Mutations in the coding region of the *OPTN* gene are associated with certain glaucomas. Although the function of the optineurin protein is yet to be elucidated, the most common mutation, E50K, is associated with a severe phenotype. This study explores some functional features of optineurin and its mutants.

Methods. Plasmids expressing normal or wild-type (WT) and E50K, R545Q, H26D, and H486R mutant optineurin were transfected into HeLa, Cos-1, IMR32, and the rat retinal ganglion cell (RGC) line RGC-5, and their effects on cell survival monitored by morphologic observation of cells were studied. Expression of optineurin and its mutants was monitored by immunofluorescence staining of cells and by Western blotting.

Results. The E50K mutant of optineurin selectively induced the death of retinal ganglion cells but not of the other cell lines tested. Although the expression of optineurin and E50K mutant suppressed cell death induced by tumor necrosis factor-α in HeLa cells, they potentiated this cell death in retinal ganglion cells. Cell death induced by the optineurin mutant in retinal ganglion cells was inhibited by the antioxidants *N*-acetylcysteine and Trolox. Reactive oxygen species (ROS) were produced upon expression of E50K, which were reduced by antioxidants. Coexpression of manganese superoxide dismutase with the E50K mutant abolished ROS production and inhibited cell death.

Conclusions. The E50K mutation of optineurin acquired the ability to induce cell death selectively in retinal ganglion cells. This cell death was mediated by oxidative stress. The present findings raise the possibility of antioxidant use for delaying or controlling some forms of glaucoma. (*Invest Ophthalmol Vis Sci.* 2007;48:1607–1614) DOI:10.1167/iovs.06-0834

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Glaucoma is the second major cause of permanent blindness worldwide.[1] Blindness occurs as a gradual loss of visual field resulting from the death of retinal ganglion cells (RGCs). A number of genetic susceptibility factors have been suggested to contribute to glaucoma.[2] One of the genes associated with normal-tension glaucoma (NTG) and primary open-angle glaucoma (POAG) is *OPTN*, which codes for the protein optineurin.[3] Certain missense mutations in the coding region of *OPTN* are associated with adult-onset and juvenile open-angle glaucoma. The mutation E50K (in which glutamic acid in position 50 is replaced by lysine in the coded protein) is the most common disease-causing mutation of optineurin reported thus far in the families studied.[3–11] However, little is known about the molecular mechanisms responsible for the pathogenesis of glaucoma caused by such mutations. Optineurin is a cytoplasmic protein that is preferentially expressed in RGCs.[12,13] This 577 residue–long protein has been reported to interact with several proteins with diverse functions such as Rab8, Huntingtin, adenovirus E3 14.7-kDa protein, myosin VI, and transcription factor IIIA.[14–18] Expression of optineurin mRNA and protein is induced by treatment of cells with tumor necrosis factor (TNF)-α and interferons.[14,16]

Several functions have been proposed for optineurin primarily on the basis of its interaction with other cellular proteins. For example, optineurin links myosin VI to the Golgi complex and plays a central role in Golgi ribbon formation and exocytosis.[17] Optineurin also links Huntingtin to Rab8 and modulates cellular morphogenesis.[15] A role for optineurin in cell survival has been suggested in NIH3T3 cells under conditions of high oxidative stress (25 mM H_2O_2).[13] Optineurin can reverse the protective effect of adenovirus E3 14.7-kDa protein on cell killing induced by the overexpression of the intracellular domain of 55-kDa TNF-α receptor in HeLa cells.[14] However, none of these studies have explored the role of optineurin or its mutants in RGCs. It has been speculated that optineurin has a neuroprotective role in the eye and optic nerve,[3] but this is yet to be demonstrated experimentally.

We explored the possibility that the disease-associated mutants of optineurin may induce the death of RGCs, a cell type relevant for glaucoma. One of the mutants of optineurin, E50K, was found to induce the death of RGCs but not of the other cell lines tested. We have attempted to characterize this cell death pathway because understanding it is likely to help in designing strategies for preventing this cell death. Our results suggest that antioxidants inhibit E50K-induced cell death.

Methods

Expression Plasmids and Antibodies

The coding region of human optineurin cDNA was amplified by PCR using the placental cDNA library as the template. The nucleotide sequence of the cloned optineurin was identical with that reported in the database (accession number NM_021980). The PCR product was cloned in a pcDNA3 vector with HA tag at the 5′-end of cDNA.

Mutations in optineurin cDNA were created by using a PCR-based, site-directed mutagenesis strategy to make the E50K, R545Q, H26D, and H486R mutants. Nucleotide sequences of mutants and wild-type (WT) cDNA constructs were confirmed by automated DNA sequencing. Protein expression was confirmed by Western blotting and also by immunostaining of transfected cells using the HA-tag antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Expression plasmids for mutant caspases have been described.[19] Expression plasmid for human manganese superoxide dismutase (MnSOD) was kindly provided by Joseph J. Cullen (University of Iowa College of Medicine).[20] A rabbit polyclonal antibody (Ab 23,666) raised using a synthetic peptide corresponding to amino acids 575 to 591 of human optineurin was obtained (Abcam Ltd., Cambridge, UK). This antibody, which recognizes rat, mouse, and human optineurin, was used to detect endogenous optineurin in various cell lines by Western blotting.

Cell Culture, Transfections, and Immunofluorescence Staining

The rat RGC line RGC-5[21] and the cell lines IMR-32, Cos-1, and HeLa were grown as monolayers in DMEM containing 10% fetal calf serum and antibiotics (penicillin, streptomycin) in a humidified 37°C incubator with 5% CO_2. Transient transfections were performed using column-purified plasmids (Qiagen, Valencia, CA) and Lipofectamine PLUS reagent (Invitrogen, Carlsbad, CA) according to the manufacturers' instructions. For immunofluorescence staining,[22] cells grown on coverslips were transfected with the required plasmids and fixed after 32 to 48 hours (or as indicated) and were stained with the anti–HA-tag antibodies to visualize the overexpressed optineurin and its mutants.

Cell Death Assays

Quantitative analysis of dead or apoptotic cells was carried out essentially as described.[19] Cells grown on coverslips were transfected with 150 ng or 300 ng plasmids expressing normal optineurin (WT) or its mutants. After 32 hours of transfection, the cells were fixed and stained for optineurin (HA-tag antibody) to determine cell death. Cells were mounted in 90% glycerol containing 1 mg/mL paraphenylenediamine (antifade) and 0.5 μg/mL DAPI (4,6-diamidino-2-phenylindole) to stain the DNA. Cells showing immunofluorescence staining were counted, and those cells that showed loss of refractility, condensed chromatin, and cell shrinkage were scored as dead or apoptotic. At least 200 expressing cells were counted in each coverslip to determine the percentage of dead cells. Data represent mean ± SD from at least three independent experiments. Cells not expressing the transfected protein were also counted in each coverslip. Generally, 1% to 3% of nonexpressing cells showed apoptosis. When testing for the effect of caspase inhibitors and Bcl2 on cell death, the cells were cotransfected with 150 ng E50K expression plasmid along with 150 ng each of plasmids expressing mutant caspase (mCasp)-1, caspase (Casp)-9s, and Bcl2. After 32 hours of transfection, cells were fixed and stained with HA-tag antibody, and cell death was determined.

TNFα-Induced Cell Death Assays

Cells grown on coverslips were transfected with 300 ng plasmids expressing WT or E50K mutant optineurin. After 24 hours of transfection, the cells were treated with TNF-α (10 ng/mL) for 24 hours or with TNF-α and cycloheximide for 8 hours. Then they were fixed and stained for optineurin. The percentage of cells undergoing cell death was quantitated in optineurin-expressing and nonexpressing cells.

Detection of Intracellular ROS Levels

The intracellular accumulation of ROS (reactive oxygen species) in the E50K-transfected RGC-5 cells was assessed using 5- (and -6-)-chloromethyl-2′,-7′-dichlorodihydrofluorescein diacetate (CM-H_2DCFDA; Molecular Probes, Eugene, OR). This nonfluorescent compound accumulates within cells, and, on deacetylation., H_2DCF then reacts with ROS to form fluorescent dichlorofluorescein (DCF). RGC-5 cells transfected with wild-type optineurin or E50K mutant were washed twice with Hanks balanced salt solution (HBSS) and incubated with 10 μM CM-H_2DCFDA in HBSS for 1 hour at 37°C in the dark. Cells were then washed three times with HBSS and finally examined in HBSS supplemented with 10% fetal calf serum through live cell microscopy (Axiovert inverted microscope; Carl Zeiss, Oberkochen, Germany). Untransfected RGC-5 cells were taken as negative control, and cells treated with 600 μM H_2O_2 for 1 hour were used as a positive control.

Statistical Analysis

The unpaired Student's *t* test was used for data analysis with $n \geq 4$ for each experiment. $P < 0.05$ was considered statistically significant.

Results

Because the primary defect in glaucoma is the death of RGCs, we used the rat RGC line RGC-5, which is a useful model for RGCs, to analyze the effect of optineurin mutant expression on the survival of RGCs.[21] RGC-5 cells grown on coverslips were transfected with the plasmids expressing WT optineurin or its mutants. Transfected cells were stained with antibodies and examined by microscopy. Expression of the mutant E50K resulted in the death of 22.6% ± 3.3% cells, as revealed by the loss of refractility, condensation of chromatin, and decrease in cell size caused by the shrinkage of cytoplasm (Figs. 1A, 2A). These morphologic features of E50K-induced cell death are similar to those of apoptosis. Interestingly, cells expressing other mutants, namely R545Q, or H26D and H486R, which too have been linked to POAG,[4–6] did not display any more cell death than the basal value shown by WT optineurin (Fig. 1A). That the induction of cell death by E50K was not caused by its higher level of expression was verified by Western blot analysis (Fig. 1B). A mutant protein can induce stress in the endoplasmic reticulum (ER) because of improper folding, but the effect of E50K did not seem to be caused by ER stress because the level of calnexin (a chaperone protein induced by ER stress) did not increase on the expression of E50K (Fig. 1B).

The ability of the E50K mutant to induce cell death appears to be selective to RGCs; neither this mutant nor WT optineurin was able to induce cell death in IMR32 (a neuronal cell line), HeLa, or Cos-1 cells (Figs. 1C, 2B). The level of E50K expression in these cell lines (RGC-5, HeLa, Cos-1, and IMR-32) was compared by Western blot analysis. The differences in the levels of E50K expression were small (Fig. 1D) and were not likely to explain the selectivity of E50K to induce cell death in RGC-5 cells. The level of endogenous optineurin was higher in RGC-5 cells than in other cell lines (Fig. 1E). In RGC-5 cells, the optineurin band showed faster mobility (Fig. 1E). Cell death induced by E50K in RGC-5 cells was inhibited by the antiapoptotic protein Bcl2 ($P < 0.05$; Fig. 3A). Expression of caspase-9s (an inactive variant of caspase-9 that inhibits caspase-9 function) and mutant caspase-1 significantly reduced the effect of E50K on cell death ($P < 0.05$). The inhibitory effect of Bcl2, mutant caspase-1, and caspase-9s on E50K-induced cell death was not caused by their effect on the expression of E50K protein, as determined by Western blotting (Fig. 3B). These results suggest that caspases are required for E50K-induced cell death. TUNEL assay of DNA fragmentation did not reveal any significant labeling of DNA in E50K-expressing cells over the control (data not shown). Little activation of caspase-3 was observed on E50K mutant expression (data not shown).

TNF-α is a cytokine that induces many signaling pathways and induces cell death in many types of cells. The expression of TNF-α and TNF-α receptor-1 is upregulated in the retina and optic nerve head in persons with glaucoma.[23–25] Optineurin gene expression is induced by TNF-α in many cells.[14,16] An interaction between polymorphism in the optineurin and the

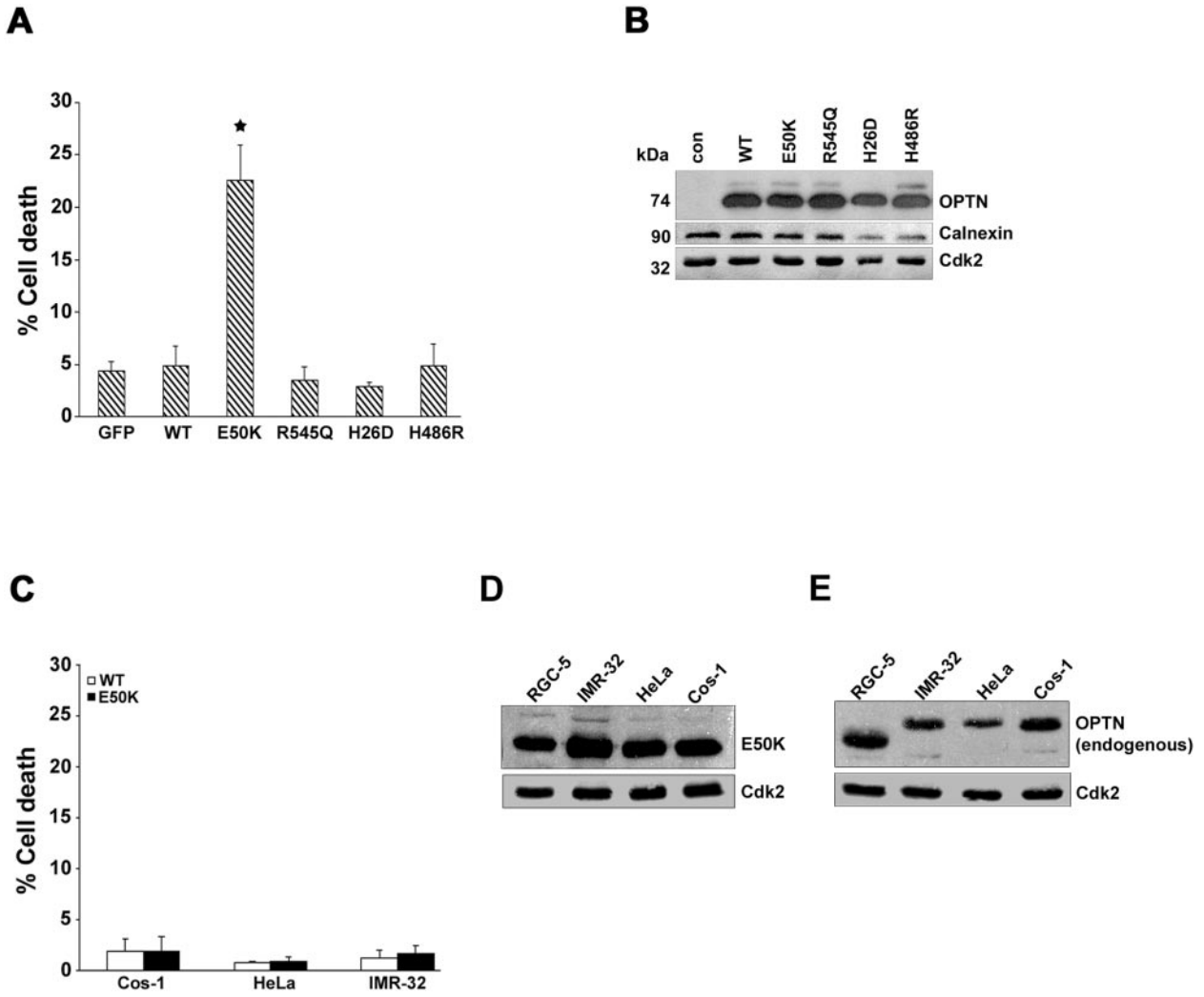

FIGURE 1. E50K mutant of optineurin selectively induces the death of RGCs. (**A**) Effect of expression of various mutants of optineurin on the induction of cell death in RGC-5 cells. Cells grown on coverslips were transfected with 150 ng plasmids expressing normal optineurin (WT) or its mutants. After 32 hours of transfection, cells were fixed and stained for optineurin (HA-tag antibody) to determine cell death. Data represent cell death in expressing cells (mean ± SD of at least 4 experiments) after subtracting the background cell death observed in nonexpressing cells, which was generally 1% to 3%. GFP was used as an additional control. $^*P < 0.01$ compared with WT optineurin–expressing cells (Student's *t* test). (**B**) Western blot showing expression of various mutants of optineurin and the level of expression of calnexin. Cdk2 was used as a loading control. (**C**) E50K mutant does not induce cell death in HeLa, IMR-32, or Cos-1 cells. Cells grown on coverslips were transfected with 300 ng indicated plasmids and processed as described in (**A**). (**D**) Western blot showing expression of E50K mutant in indicated cell lines. Cdk2 was used as loading control. Transfection conditions of E50K in various cell lines, grown in 24-well plates, were the same as those used for cell death assays. (**E**) Western blot showing expression of endogenous optineurin using optineurin antibody.

TNF-α genes has been suggested to increase the risk for glaucoma.[8,26] Therefore, we examined the effect of expression of optineurin and E50K mutant on TNF-α–induced cell death. Cells were transfected with E50K or WT optineurin, and 24 hours later these were treated with TNF-α for 24 hours. In HeLa cells, E50K and WT optineurin strongly inhibited TNF-α–induced cell death (Figs. 4A, 4B). Cell death induced by TNF-α and cycloheximide treatment was also inhibited significantly ($P < 0.05$; Fig. 4A). In contrast, TNF-α–induced cell death in RGC-5 cells was not inhibited either by E50K or by WT optineurin (Figs. 5A, 5B); here, E50K-expressing cells showed significantly more cell death than those expressing normal optineurin. Surprisingly, even WT optineurin expression increased TNF-α–induced death of RGC-5 cells ($P < 0.05$). These results suggest that optineurin is likely to be a component of the TNF-α–induced signaling pathway leading to cell death.

To understand the mechanism by which the E50K mutant induces cell death in RGC-5 cells, we investigated the possibility of E50K causing oxidative stress, which is known to lead to pathologic cell death.[27] We tested the ability of antioxidants to inhibit E50K-induced cell death. *N*-acetylcysteine (NAC), a precursor of glutathione, was added to the cells expressing E50K mutant because glutathione is a major antioxidant in mammalian cells. This resulted in an inhibition of E50K-induced cell death (Figs. 6A, 6B). Another antioxidant, Trolox (a water-soluble homolog of vitamin E), was also able to reduce this cell death. Cotransfection of a plasmid-expressing manganese superoxide dismutase (MnSOD), a mitochondrial enzyme, resulted in greater than 75% inhibition of E50K-induced cell death (Figs. 6A, 6B). The inhibition of cell death by NAC, Trolox, and MnSOD was significant ($P < 0.05$). The inhibitory effect of antioxidants on cell death was not caused by the reduced expression of E50K protein, as determined by Western blotting (Fig. 6C). Cell death induced by E50K in the presence of TNF-α was also inhibited significantly by antioxidants NAC and Trolox ($P < 0.05$; Fig. 6D). These results

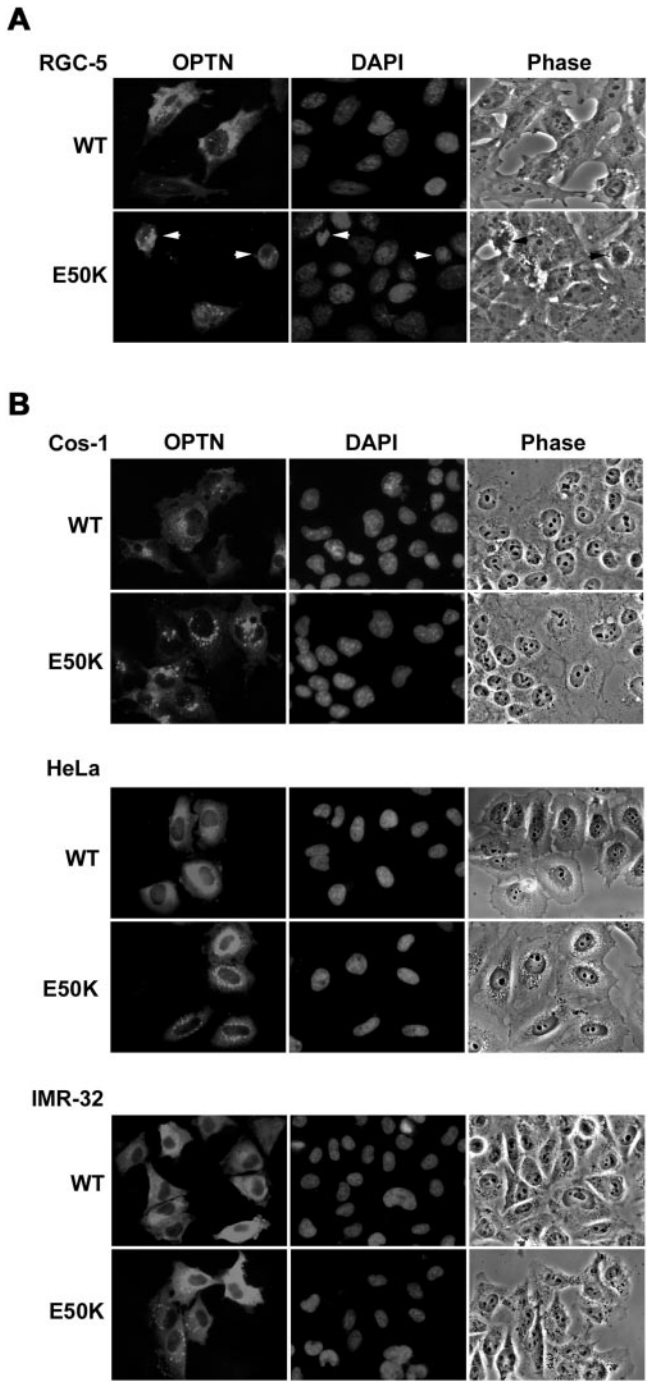

FIGURE 2. (**A**) Images of RGC-5 cells expressing normal or E50K mutant of optineurin, showing the induction of cell death by this mutant. *Arrowheads*: E50K-expressing dead cells. (**B**) Images of Cos-1, HeLa, and IMR-32 cells expressing normal or E50K mutant of optineurin.

suggest that oxidative stress induced by E50K plays an important role in cell death.

To investigate further the involvement of ROS in E50K-induced cell death, we determined the level of ROS in RGC-5 cells transfected with E50K mutant or normal optineurin using an ROS-sensitive probe, CM-H_2DCFDA. Expression of E50K resulted in an increase in ROS production, as shown by increased DCF fluorescence (Fig. 7A). Treatment of E50K-transfected cells with antioxidants resulted in reduced ROS production (Fig. 7B). Cotransfection of a plasmid expressing MnSOD with E50K resulted in nearly complete loss of ROS (Fig. 7B). These results suggest that E50K expression induces ROS production which is partly inhibited by NAC and Trolox.

DISCUSSION

It has been suggested that optineurin has a cytoprotective function that is disrupted by mutations leading to glaucoma.[3] Although this function of optineurin in RGCs is yet to be established, our results suggest that the E50K mutant has acquired the ability to induce cell death selectively in RGCs but not in other cells, a point of relevance to the disease condition. The ability of E50K to potentiate the TNF-α–induced death of RGCs points to the role of environmental, as well as genetic, factors in causing glaucoma. The finding that E50K mutant-induced cell death is mediated by oxidative stress indicates the potential of antioxidants in preventing or delaying glaucoma.

Interestingly, only the E50K mutant of optineurin was able to induce the death of RGC-5 cells; three other mutants were

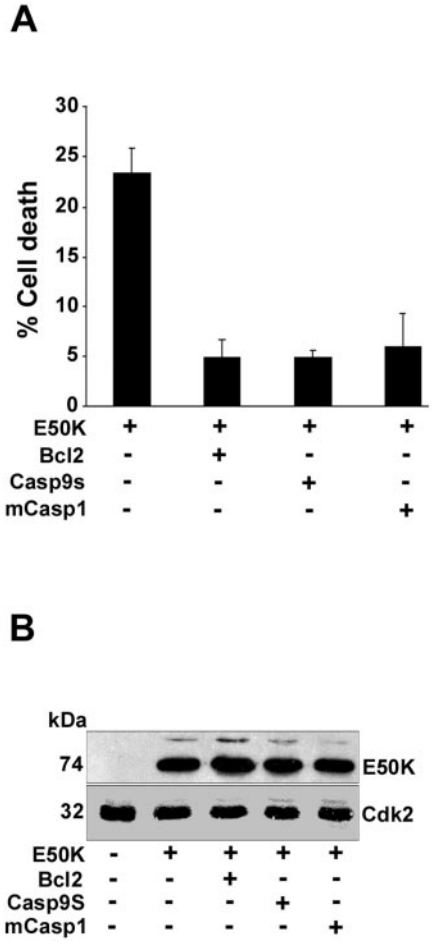

FIGURE 3. Effect of caspase inhibitors and Bcl2 on E50K-induced death of RGC-5 cells. (**A**) Cells grown on coverslips were cotransfected with E50K expression plasmid (150 ng) along with plasmids expressing mutant caspase (mCasp)-1, caspase (Casp)-9s, or Bcl2 (150 ng each). After 32 hours of transfection, cells were fixed and stained with HA-tag antibody, and cell death was determined. Expression of mutant caspase-1, caspase-9s, and Bcl2 resulted in significant inhibition ($P < 0.05$) of cell death. (**B**) Cells grown in 24-well plates were transfected as described in (**A**), and, after 32 hours, cell lysates were made for Western blotting. E50K protein level was determined using HA antibody.

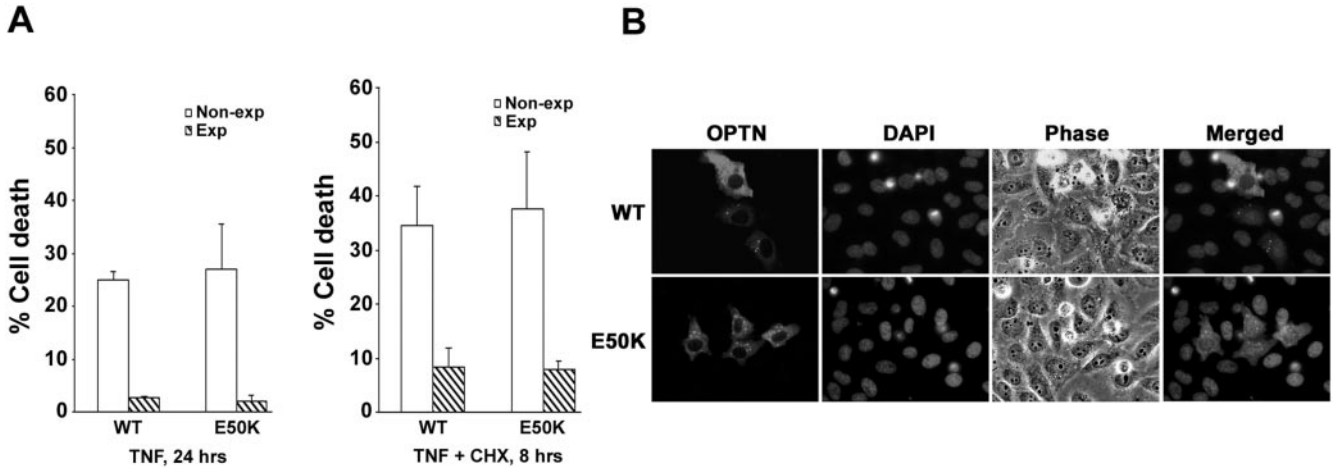

FIGURE 4. Inhibition of TNF-α–induced cell death of HeLa cells by optineurin. (**A**) HeLa cells grown on coverslips were transfected with 300 ng plasmids expressing WT or E50K mutant optineurin. After 24 hours of transfection, cells were treated with human TNF-α (10 ng/mL) for 24 hours (*left*) or with TNF-α and cycloheximide for 8 hours (*right*). Cells were then fixed and stained for optineurin. The percentage of cells undergoing cell death was quantitated in optineurin-expressing and nonexpressing cells. Data represent mean ± SD from three experiments. (**B**) Images of HeLa cells showing inhibition of TNF-α–induced cell death by optineurin and its mutant.

unable to do so. E50K is a unique mutation that has been found only in British patients or in patients of British descent. It has been suggested by Alward et al.[7] that a founder effect accounts for the E50K mutation frequency in British patients. It appears that E50K mutation alone is able to induce cell death when expressed at elevated levels. Glaucomas are generally multifactorial and are perhaps affected by multiple interacting loci.[2] It is likely, therefore, that other mutants (H26D, H486R) of optineurin may require interaction with genetic modifiers for inducing cell death in RGCs.

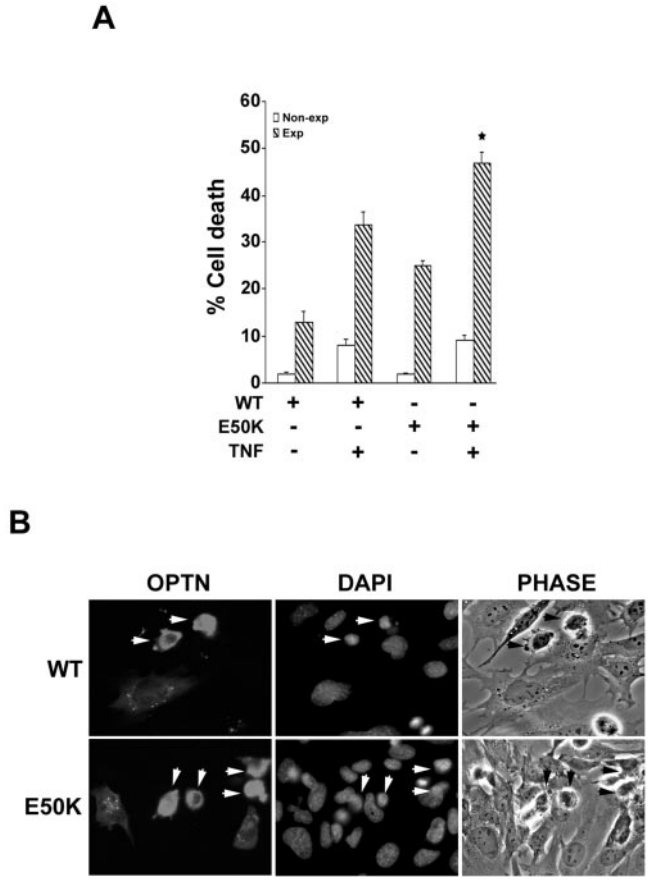

FIGURE 5. (**A**) Effect of WT and E50K mutant optineurin on TNF-α–induced killing of RGC-5 cells. This experiment was carried out as described for Figure 4A except that murine TNF-α was used. $^*P < 0.05$ compared with WT optineurin expressing TNF-treated cells (Student's *t* test). (**B**) Images of RGC-5 cells showing effect of optineurin and E50K mutant on the TNF-induced death of RGC-5 cells.

Oxidative stress in the cell may occur because of an imbalance between the production and removal of ROS, and it has been implicated in nerve cell death in the eye.[27,28] Cell death induced by oxidative stress can be prevented or reduced by blocking specific steps in the cell death cascade. Cell death induced by E50K in RGC-5 cells was inhibited by two structurally unrelated antioxidants (NAC and Trolox), which act by different mechanisms, suggesting that this cell death was mediated by oxidative stress. This suggestion is further supported by the observations that E50K expression results in increased production of ROS, which is reduced by NAC and Trolox. MnSOD completely abolished ROS production by E50K and inhibited cell death. These results suggest that the inhibition of E50K-induced cell death by NAC and Trolox is mediated, at least in part, by their ability to reduce ROS level. Cell death induced by oxidative glutamate toxicity in RGC-5 cells has features of classic apoptosis and oxytosis.[27] This type of cell death has morphologic features of apoptosis (rounding of cells, shrinkage of cytoplasm) but does not involve DNA fragmentation or caspase-3 activation.[27] E50K-induced cell death is morphologically similar to apoptosis but does not show any significant increase in DNA fragmentation (data not shown). Only a small increase in caspase-3 activation was seen. Taken together, these results suggest that the E50K mutant induces a type of cell death in RGC-5 cells that has characteristics of classic apoptosis and oxytosis.

TNF-α–induced cell death in HeLa cells was strongly inhibited by optineurin. It has been reported that optineurin can inhibit the protective effect of adenovirus E3 14.7-kDa protein on the killing of HeLa cells induced by overexpression of the intracellular domain of TNF receptor (TR55) or by RIP.[14] This implies that optineurin by itself has no effect on TR55-induced killing of HeLa cells. The cell death pathway induced by the intracellular domain of TNF receptor is believed to mimic the TNF-α–induced cell death pathway.[14] Therefore, it is likely that

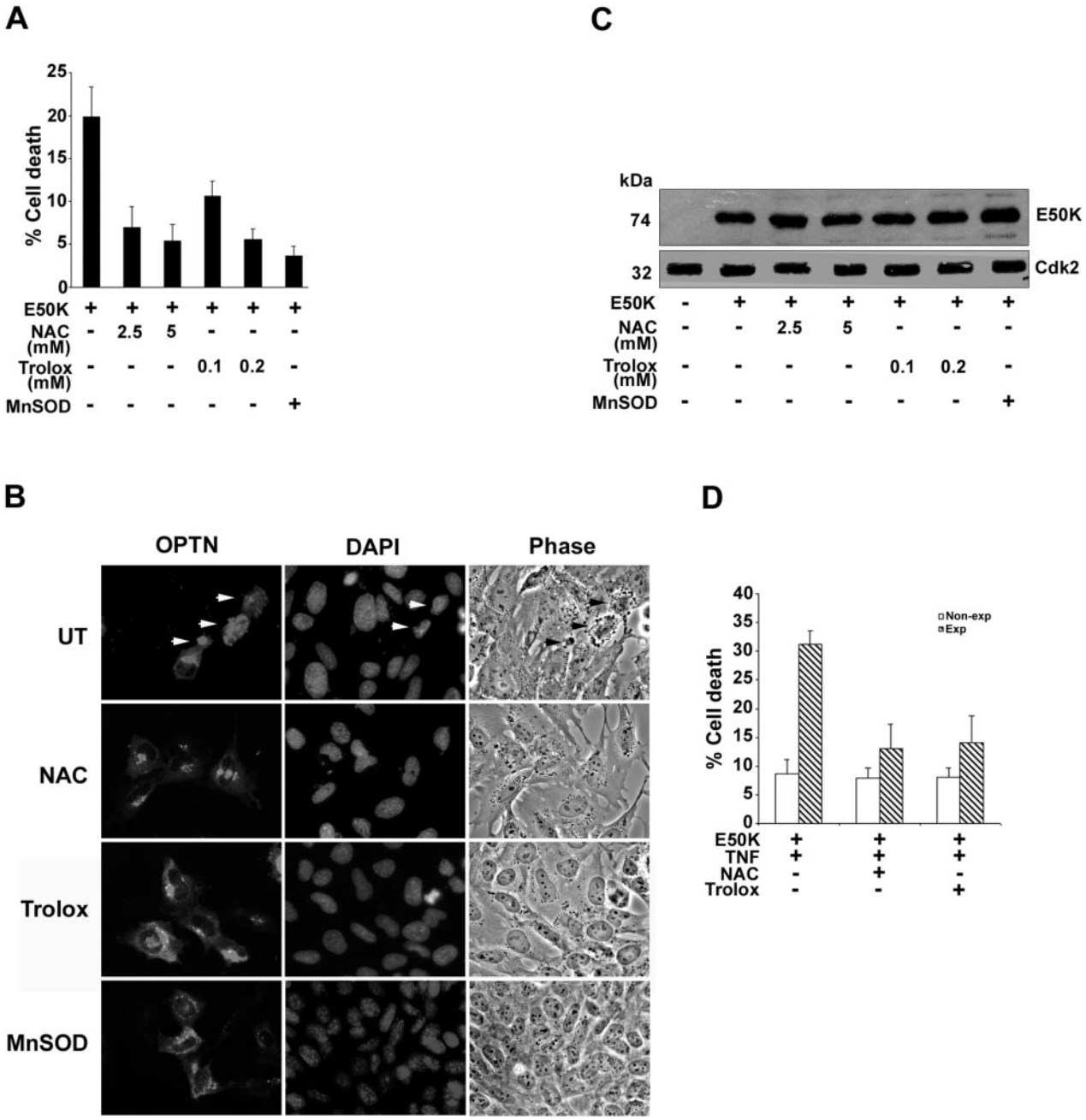

FIGURE 6. Effect of antioxidants on E50K-induced cell death of RGC-5 cells. (**A**, **B**) NAC or Trolox was added to the medium after 6 hours of cell transfection with E50K expression plasmid. After 22 hours of transfection, medium was replaced with fresh medium containing antioxidants. Cells were fixed and stained after 32 hours of transfection. (**C**) Western blot showing the level of E50K mutant in the presence of antioxidants. Cells grown in 24-well plates were transfected with E50K plasmid alone or with MnSOD. Cells were then treated with NAC or Trolox after 6 hours of transfection as in (**A**, **B**). Cell lysates were prepared after 32 hours of transfection for Western blotting. (**D**) Effect of antioxidants on E50K-induced cell death in presence of TNF-α. This experiment was performed as in (**A**) except that after 24 hours of transfection, murine TNF-α was added.

optineurin inhibits an upstream event in TNF signaling during inhibition of the TNF-α–induced cell death observed in HeLa cells.

How does the E50K mutant induce cell death in RGC-5 cells? Optineurin interacts with many proteins, and the Rab8 interaction domain (amino acids 58–209)[15] is present in the vicinity of the E50K mutation. Therefore, it is likely that a conformational change induced by E50K mutation may alter its interaction with Rab8. It has been reported that in response to a high level of oxidative stress (25 mM H_2O_2), optineurin translocates to the nucleus in a Rab8-dependent manner, whereas the E50K mutant is unable to do so.[13] Whether the loss of interaction with Rab8 or a gain of interaction with some other protein is responsible for the ability of E50K mutant to induce cell death in RGC-5 cells will require further investigation. A cytoprotective role of optineurin has been suggested in the retina, though this remains to be demonstrated. In NIH 3T3 cells, optineurin (but not the E50K mutant) protects against cell death induced by a high level of oxidative stress.[13] Thus, it is likely that under certain conditions of stress, optineurin may have a cytoprotective function. However, our results suggest that

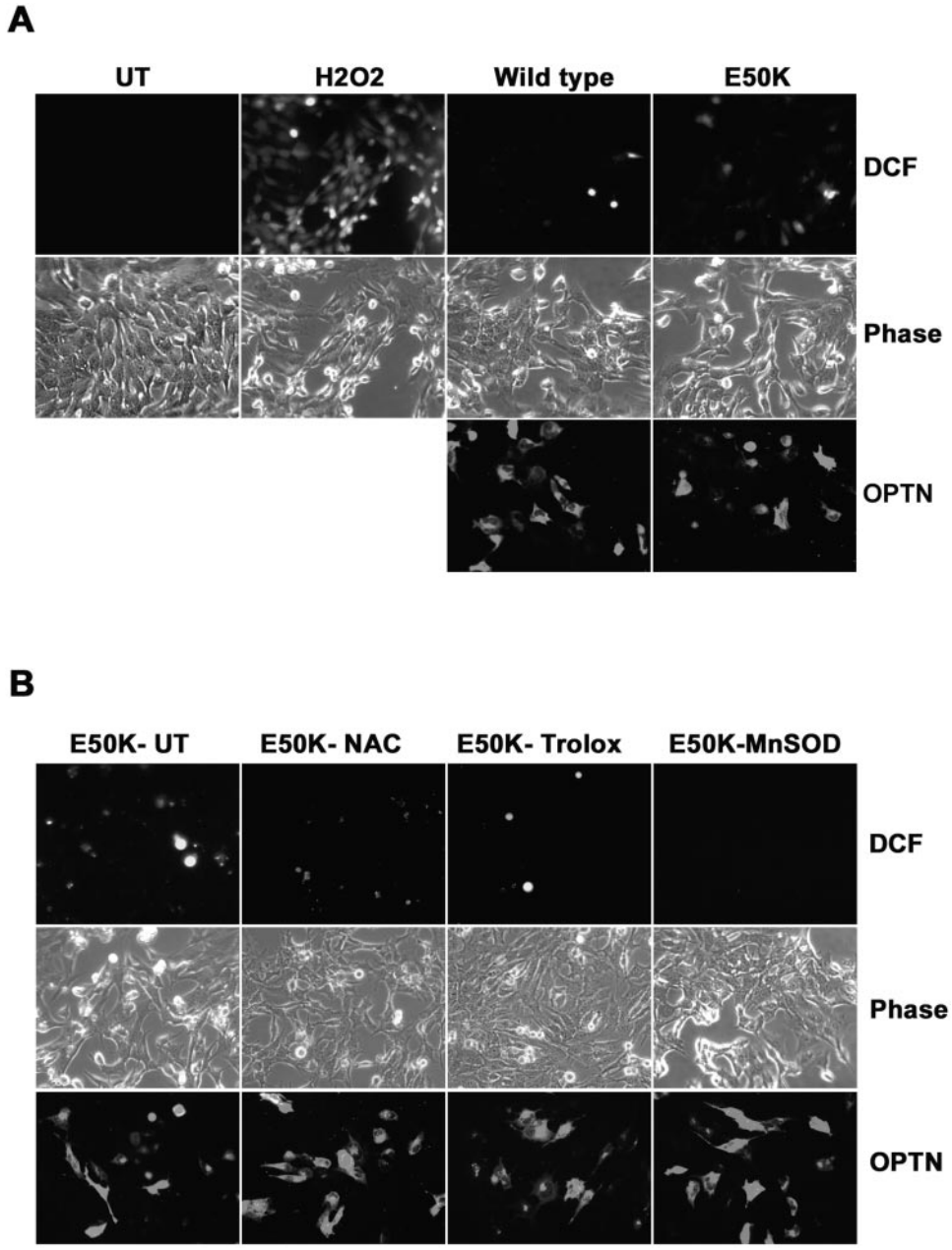

FIGURE 7. E50K mutant induces ROS production. (**A**) RGC-5 cells were transfected with WT optineurin or E50K mutant plasmids. After 30 hours of transfection, cells were washed and incubated with CM-H_2DCFDA. After 1 hour, cells were washed and visualized through an inverted microscope. Representative fields showing DCF fluorescence and corresponding phase-contrast images are shown. H_2O_2-treated cells were used as positive control. UT, untreated cells. Expression of E50K and WT optineurin was confirmed in each experiment by fixing these cells and staining with HA antibody. Representative fields from the same coverslips are shown in the *bottom panels*. (**B**) RGC-5 cells were transfected with E50K expression plasmid, alone or with MnSOD. E50K-transfected cells were treated with 5 mM NAC or 0.2 mM Trolox and were subjected to ROS detection assay. Representative fields showing DCF fluorescence are shown. MnSOD-transfected cells showed no DCF fluorescence.

a high level of WT optineurin can increase the TNF-α–induced death of RGC-5 cells, indicating that optineurin might not have a generalized cytoprotective function in these cells.

The mechanism of selectivity of E50K-induced cell death in RGC-5 cells (compared with other cells tested) is unclear. This selectivity is not the result of a higher level of E50K expression in RGC-5 cells. Earlier studies with neonatal rat retina have shown that, compared with other retinal cells, RGCs were more resistant to oxidative stress–induced cell death.[29] In another report,[27] it was observed that RGC-5 cells were less sensitive to oxidative stress-induced cell death than the hippocampal cell line HT22. Therefore, differential sensitivity of RGC-5 cells to oxidative stress is not likely to be the reason for the selectivity of E50K-induced cell death, which would require further investigation.

In conclusion, our results show that the E50K mutant of optineurin has acquired the ability to induce cell death selectively in RGCs. This cell death is inhibited by antioxidants, suggesting the potential of antioxidants to prevent or delay some forms of glaucoma. Optineurin affects TNF-α–induced cell death, suggesting that it may be a component of the TNF-α signaling pathway.

References

1. Resnikoff S, Pascolini D, Etya'ale D, et al. Global data on visual impairment in the year 2002. *Bull World Health Organ*. 2004;82:844–851.
2. Libby RT, Gould DB, Anderson MG, John SWM. Complex genetics of glaucoma susceptibility. *Ann Rev Genomics Hum Genet*. 2005;6:15–44.
3. Rezaie T, Child A, Hitchings R, et al. Adult-onset primary open-angle glaucoma caused by mutations in optineurin. *Science*. 2002;295:1077–1079.
4. Willoughby CE, Chan LL, Herd S, et al. Defining the pathogenicity of optineurin in juvenile open-angle glaucoma. *Invest Ophthalmol Vis Sci*. 2004;45:3122–3130.

5. Leung YF, Fan BJ, Lam DS, et al. Different optineurin mutation pattern in primary open-angle glaucoma. *Invest Ophthalmol Vis Sci*. 2003;44:3880–3884.
6. Fuse N, Takahashi K, Akiyama H, Nakazawa T, Seimiya M, Kuwahara S. Molecular genetic analysis of optineurin gene for primary open angle and normal tension glaucoma in the Japanese population. *J Glaucoma*. 2004;13:299–303.
7. Alward WL, Kwon YH, Kawase K, et al. Evaluation of optineurin sequence variation in 1048 patients with open angle glaucoma. *Am J Ophthalmol*. 2003;136:904–910.
8. Funayama T, Ishikawa K, Ohtake Y, et al. Variants in optineurin gene and their association with tumor necrosis factor-α polymorphisms in Japanese patients with glaucoma. *Invest Ophthalmol Vis Sci*. 2004;45:4359–4367.
9. Mukhopadhyay A, Komatireddy S, Acharya M, et al. Evaluation of optineurin as a candidate gene in Indian patients with primary open angle glaucoma. *Mol Vision*. 2005;11:792–797.
10. Aung T, Rezaie T, Okada K, et al. Clinical features and course of patients with glaucoma with the E50K mutation in the optineurin gene. *Invest Ophthalmol Vis Sci*. 2005;46:2816–2822.
11. Sarfarazi M, Rezaie T. Optineurin in primary open angle glaucoma. *Ophthalmol Clin North Am*. 2003;16:529–541.
12. Kroeber M, Ohlmann A, Russell P, Tamm ER. Transgenic studies on the role of optineurin in the mouse eye. *Exp Eye Res*. 2006; 82:1075–1085.
13. De Marco N, Buon M, Troise F, Diez-Roux G. Optineurin increases cell survival and translocates to the nucleus in a RAB8 dependent manner upon an apoptotic stimulus. *J Biol Chem*. 2006;281: 16147–16156.
14. Li Y, Kang J, Horwitz MS. Interaction of an adenovirus E3 14.7-kilodalton protein with a novel tumor necrosis factor alpha-inducible cellular protein containing leucine zipper domains. *Mol Cell Biol*. 1998;18:1601–1610.
15. Hattula K, Paranen J. FIP-2, a coiled-coil protein, links Huntingtin to Rab8 and modulates cellular morphogenesis. *Curr Biol*. 2000; 10:1603–1606.
16. Schwamborn K, Weil R, Courtois G, Whiteside ST, Israel A. Phorbol esters and cytokines regulate the expression of the NEMO-related protein, a molecule involved in a NF-kappa B-independent pathway. *J Biol Chem*. 2000;22780–22789.
17. Sahlender DA, Roberts RC, Arden SD, et al. Optineurin links myosin VI to the Golgi complex and is involved in Golgi organization and exocytosis. *J Cell Biol*. 2005;169:285–295.
18. Anborgh PH, Godin C, Pampillo M, et al. Inhibition of metabotropic glutamate receptor signaling by the Huntingtin-binding protein optineurin. *J Biol Chem*. 2005;280:34840–34848.
19. Shivakrupa R, Radha V, Sudhakar CH, Swarup G. Physical and functional interaction between Hck tyrosine kinase and guanine nucleotide exchange factor C3G results in apoptosis, which is independent of C3G catalytic domain. *J Biol Chem*. 2003;278: 52188–52194.
20. Ough M, Lewis A, Zhang Y, et al. Inhibition of cell growth by overexpression of manganese superoxide dismutase (MnSOD) in human pancreatic carcinoma. *Free Radical Res*. 2004;38:1223–1233.
21. Krishnamoorthy RR, Agarwal P, Prasanna G, et al. Characterization of a transformed rat retinal ganglion cell line. *Mol Brain Res*. 2001;86:1–12.
22. Gupta V, Swarup G. Evidence for a role of transmembrane protein p25 in localization of protein tyrosine phosphatase TC48 to the ER. *J Cell Sci*. 2006;119:1703–1714.
23. Yan X, Tezel G, Wax MB, Edward DP. Matrix metalloproteinases and tumor necrosis factor-α in glaucomatous optic nerve head. *Arch Ophthalmol*. 2000;118:666–673.
24. Tezel G, Li LY, Patil RV, Wax MB. TNF-α and TNF-α receptor-1 in the retina of normal and glaucomatous eyes. *Invest Ophthalmol Vis Sci*. 2001;42:1787–1794.
25. Yuan L, Neufeld AH. Tumor necrosis factor-α: a potentially neurodestructive cytokine produced by glia in the human glaucomatous optic nerve head. *Glia*. 2000;32:42–50.
26. Lin HJ, Tsai FJ, Chen WC, et al. Association of tumour necrosis factor alpha–308 gene polymorphism with primary open-angle glaucoma in Chinese. *Eye*. 2003;17:31–34.
27. Maher P, Henneken A. The molecular basis of oxidative stress-induced cell death in an immortalized retinal ganglion cell line. *Invest Ophthalmol Vis Sci*. 2005;46:749–757.
28. Maher P, Hanneken A. Flavonoids protect retinal ganglion cells from oxidative stress-induced death. *Invest Ophthalmol Vis Sci*. 2005;46:4796–4803.
29. Kortuem K, Geiger LK, Levin LA. Differential susceptibility of retinal ganglion cells to reactive oxygen species. *Invest Ophthalmol Vis Sci*. 2000;41:3176–3182.