Oxidant-mediated Akt Activation in Human RPE Cells

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PURPOSE. To determine whether a model oxidant, hydrogen peroxide (H$_2$O$_2$), influences Akt activation and, if so, whether Akt activation promotes retinal pigment epithelial (RPE) cell survival.

METHODS. Cultured human RPE cells were pretreated with medium alone, with LY294002 (LY), an inhibitor of phosphatidylinositol-3 kinase (PI3K) and its downstream effector Akt, or with Akt/protein kinase B signaling inhibitor (API)-2, a specific Akt inhibitor, and then were stimulated with H$_2$O$_2$ at different doses for various times. Akt phosphorylation was evaluated by Western blot using antibody against phosphorylated Akt (Ser473). The effect of Akt blockade on RPE cell viability was assessed by tetrazolium salt (WST-1) assay and a lactate dehydrogenase (LDH) release assay. Caspase-mediated cytokeratin cleavage, an early apoptosis marker, was assessed by M30 antibody staining. Caspase-independent apoptosis was determined by nuclear translocation of apoptosis-inducing factor (AIF). RPE cell morphology was evaluated by electron microscopy. The effect of H$_2$O$_2$ on downstream Akt targets was examined by Western blot using antibody against phosphorylated forkhead in rhabdomyosarcoma (FKHR) and phosphorylated glycogen synthase kinase (GSK)-3β.

RESULTS. H$_2$O$_2$ induced Akt phosphorylation in a dose-dependent manner and also induced the phosphorylation of downstream effectors FKHR and GSK-3β. LY markedly inhibited H$_2$O$_2$-mediated Akt phosphorylation and significantly enhanced caspase-associated and caspase-independent RPE cell death.

CONCLUSIONS. A model oxidant, H$_2$O$_2$, induces PI3K and thereby activates Akt. Akt activation enhances RPE cell survival and thus may protect RPE cells from oxidant-induced cell death under normal circumstances and in disease states such as age-related macular degeneration (AMD). (Invest Ophthalmol Vis Sci. 2006;47:4598–4606) DOI:10.1167/iovs.06-0140

Retinal pigment epithelial (RPE) cells are continually exposed to oxidants throughout life. These oxidants arise from endogenous sources that include photoreceptor outer segment phagocytosis, peroxidized lipid membranes, and photo-oxidative reactive-oxygen intermediates and from exogenous sources such as those found in cigarette smoke. Normally, RPE cells can survive this ongoing oxidant bombardment. However, prolonged oxidative exposure disrupts RPE cell junction function and barrier integrity and induces RPE cell blebbing, a sign of oxidant injury. Age-related macular degeneration (AMD) is the leading cause of irreversible vision loss in the Western world among persons older than 65 years of age. AMD progresses through two stages (early and advanced). Early AMD is characterized by the accumulation of lipid-rich deposits under the RPE, called drusen, basal linear deposits, and basal laminar deposits. Advanced AMD is characterized by two forms: geographic atrophy and choroidal neovascularization. Choroidal neovascularization is caused by the growth of pathologic new vessels under the retina. Geographic atrophy is caused by degenerative loss of the RPE, photoreceptors, and choriocapillaris and accounts for 25% of AMD-associated legal blindness. Growing evidence indicates that cumulative oxidative injury caused by reactive-oxygen intermediates (ROIs) contributes to AMD pathogenesis. Antioxidant vitamins prevent the formation of advanced neovascular AMD. However, even with antioxidant vitamin treatment, given as described in this study, it is not possible to prevent nonneovascular AMD progression to geographic atrophy. These data suggest that factors (for example, genetic) in addition to endogenous antioxidants protect RPE cells from oxidant injury in the earlier stages of the disease.

RPE cell survival is crucial for maintaining the normal function of the overlying neurosensory retina and the underlying choriocapillaris. Recently, we showed that RPE cells are naturally endowed with a variety of survival proteins that protect them from premature demise. However, the signaling pathways that help promote RPE cell survival have not been clearly defined.

Multiple signal transduction pathways that serve to coordinate the cellular response and ultimately determine cell fate are activated by oxidant injury. The phosphoinositide 3-kinase (PI3K)-Akt pathway is one such pathway that is regulated by ROIs. The serine/threonine kinase Akt (protein kinase B) is a key prosurvival molecule in a variety of cell types. Akt becomes phosphorylated in a PI3K-dependent manner not only in response to various growth factors but also in response to oxidants such as H$_2$O$_2$. Akt activation causes phosphorylation of proapoptotic molecules such as forkhead in rhabdomyosarcoma (FKHR), BAD, glycogen synthase kinase (GSK)-3β, and caspase-9, thereby inactivating them. In certain cells, Akt activation can protect cells from lethal oxidant injury. However, the effect of oxidants on RPE cell Akt activation, the effect of Akt activation on oxidant-induced necrotic and apoptotic cell death, and the mechanisms by which Akt exerts these effects have not been well described. To better understand the mechanisms by which oxidants influence RPE cell survival and death, we investigated whether a model oxidant, H$_2$O$_2$, modulated Akt and its downstream substrates and, if so, whether Akt blockade affected apoptotic and nonapoptotic RPE cell death.

MATERIALS AND METHODS

RPE Cell Culture and Treatments

Human donor eyes were obtained from the North Carolina Organ Donor and Eye Bank, Inc. (Winston-Salem, NC) in accordance with the provisions of the Declaration of Helsinki for research involving human tissue. RPE cells for culture studies were harvested from eyes, as previously described. Cells were grown in Eagle minimal essential media.
medium (MEM; Invitrogen, Carlsbad, CA) with 10% fetal bovine serum (FBS; HyClone Laboratories, Logan, UT) and 1 × antibiotic-antimycotic (Invitrogen) at 37°C in a humidified environment containing 5% CO₂. RPE cells (4 × 10⁵) were seeded in six-well plates (Corning-Costar Inc., Corning, NY), 1.1 to 2 × 10⁶ in 96-well plates (Corning-Costar Inc.), and 8 × 10⁴ in 8-well chamber slides (Nalge Nunc International, Naperville, IL). Twenty-four hours later, cells were incubated with fresh medium for an additional 24 hours and then were starved in serum-free MEM for 24 hours. The cells were then incubated for 1 hour with varying dosages of LY294002 (LY; Sigma, St. Louis, MO), an inhibitor of PI3K and its downstream effectors, or for 1 hour with 100 nM Akt/protein kinase B signaling inhibitor (API)-2 (Calbiochem, La Jolla, CA), a specific small-molecule Akt activation inhibitor. The cells were stimulated with varying H₂O₂ (Sigma) dosages in serum-free and phenol-free MEM for varying times.

Cell Extracts and Western Blot

Cell extracts were prepared, and Western blot analysis was performed as we have previously described. For Western blot analysis, membranes were incubated overnight at 4°C with the following antibodies (Cell Signaling Technology, Beverly, MA) diluted in 3% milk: rabbit polyclonal antibody directed against phospho-Akt (Ser473) (1:1000), Akt (1:1000), phospho-FKHR (Ser256) (1:1000), and phospho-GSK-3β (Ser9) (1:1000). The blots were then washed three times (20 minutes per wash) in TBST and incubated with anti-rabbit IgG conjugated with horseradish peroxidase (1:5000 in 2% milk; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) for 60 minutes at room temperature (RT). Immunoreactive bands were visualized using an enhanced chemiluminescence light (ECL) detection kit (Amersham, Piscataway, NJ). In all the figures with bar charts of densitometry data, optical density (OD) refers to the integrated density.

Cell Viability Assay

A colorimetric assay was performed based on the cleavage of the tetrazolium salt WST-1 (4-[3-(4-Iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate) by mitochondrial dehydrogenases in viable cells (Roche, Mannheim Germany). After pretreatment with vehicle alone or LY (10 μM) for 1 hour and stimulation with H₂O₂ at different doses for 5 hours, the WST-1 solution (10 μL/well) was added, and cells were further incubated for 2 hours at 37°C. The plate was read on a spectrophotometer at 440 nm with a reference wavelength at 690 nm.

Fluorescence Measurement of ROI

Formation of intracellular ROI was measured using 2′,7′-dichlorodihydrofluorescein diacetate (H₂DCFDA; Molecular Probes, Eugene, OR). Briefly, cells were rinsed twice with Hanks balanced salt solution (HBSS; plus calcium and magnesium; Invitrogen) and loaded with 10 μM H₂DCFDA diluted in HBSS for 30 minutes at 37°C followed by two washes with HBSS. After treatment with H₂O₂, cells were washed twice with HBSS, and the ability of intracellular ROI to oxidize the dye to its fluorescent product was measured using a fluorescence plate reader (492 nm excitation, 520 nm emission; Molecular Devices, Sunnyvale, CA).

Lactate Dehydrogenase Release Assay

During early necrotic cell death, cytoplasmic LDH is released through damaged cell membranes. A colorimetric assay kit (Roche) was used to quantify LDH released from cultured RPE cells into the surrounding culture medium according to the manufacturer’s recommendations. Briefly, after pretreatment with vehicle alone or LY (10 μM) for 1 hour and stimulation with H₂O₂ at different doses for 7 hours, the supernatant was carefully removed, centrifuged, and transferred to a separate 96-well plate. A reaction mixture consisting of catalyst/dye combination was prepared, and 100 μL was added directly to 100 μL of the cell supernatant. After incubation at 15°C to 25°C for 30 minutes, absorbance was read using a spectrophotometer at 490 nm with a reference wavelength at 690 nm.

Immunofluorescence Detection of M30

Cytokeratin cleavage is a specific early apoptotic event in epithelial cells. M30 is an antibody that specifically recognizes a neoepitope exposed by cytokeratin cleavage and has been widely used as a marker for early caspase-dependent apoptotic cell death. Akt medium was removed, and cells were rinsed twice with PBS, fixed with ice-cold 100% methanol for 30 minutes at −20°C, washed twice with PBS and then with washing buffer (PBS containing 0.1% Tween 20) for 15 minutes, and incubated with M30 mouse monoclonal antibody (1:50 in PBS containing 0.1% Tween 20 and 1% BSA; CytoDEATH [Roche]) for 1 hour at RT. Cells were washed with washing buffer three times and then incubated with fluorescein (FITC)–conjugated goat anti–mouse IgG antibody (1:100 in washing buffer; Jackson ImmunoResearch Laboratories, Inc.) for 30 minutes at RT. Cells were washed with washing buffer three times and then incubated with 4′,6-Diamidino-2-phenyldole dihydrochloride (DAPI; Sigma) in PBS for 5 minutes. Fluorescent stain was observed with a light microscope and epifluorescent attachment. A masked observer determined the percentage of cells with M30-positive stain out of the total number stained with DAPI. For this analysis, for each treatment group, three microscopic fields, each in triplicate wells, each containing approximately 200 cells, were examined.

Apoptosis-Inducing Factor Nuclear Translocation

Cell medium was removed, and cells were rinsed twice with PBS and fixed with 4% paraformaldehyde for 15 minutes at RT. Cells were blocked as described previously and then incubated with rabbit polyclonal apoptosis-inducing factor (AIF) antibody (1:50 in PBS containing 0.6% Triton X-100; Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4°C. After three washes with PBS, the cells were incubated with FITC-conjugated donkey anti–rabbit IgG antibody (1:50 in PBS containing 0.6% Triton X-100; Jackson ImmunoResearch Laboratories, Inc.) for 30 minutes at RT. Cells were washed with washing buffer three times and then incubated with DAPI (Sigma) in PBS for 5 minutes. Fluorescent stain was observed with a light microscope and epifluorescent attachment. AIF and DAPI images (two pairs per sample) were captured from three random fields from each of triplicate wells using appropriate filters and identical exposure conditions. Fluorescence intensities for AIF and DAPI-paired images were quantified (MetaMorph software). AIF nuclear signal for each cell in each captured field (pixels above background) was normalized to nuclear area (DAPI perimeter). Results for each condition are given as mean AIF signal intensity per total number of DAPI-stained nuclei.

Transmission Electron Microscopic Analysis

Cell medium was removed, and cells were trypsinized, rinsed with PBS, and fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer for 18 hours at 4°C. Cells were washed twice with 0.1 M sodium cacodylate buffer (15 minutes per wash), postfixed with 1% osmium tetroxide in 0.1 M sodium cacodylate buffer for 1 hour at 4°C, dehydrated, and embedded in Spurr embedding medium (Polysciences, Inc., Warrington, PA). Sections (70 nm) were cut on a microtome and stained with 2% uranyl acetate and 0.2% lead citrate and examined and photographed using a transmission electron microscope (1200Ex; JEOL, Tokyo, Japan).

Statistical Analysis

Data are expressed as the mean ± SD. Student t test was used to determine whether there were statistically significant differences between treatment groups determined by cell viability assay, intracellular ROI detection, LDH release assay, M30 assay, or AIF translocation assay. P < 0.05 was considered statistically significant. Western blot analysis performed in duplicate, cell viability assays in triplicate, LDH
release assay in triplicate, M30 assay in triplicate, and AIF translocation assay in triplicate were separately repeated three times in three individual experiments with similar results. Fluorescence measurement of intracellular ROI (seven replicate samples per treatment group) was separately repeated twice in two individual experiments with similar results. Data shown in Figures 1 to 15 are from representative experiments from the same donor.

RESULTS

H2O2-Induced Akt Phosphorylation

As an initial step to determine the effect of H2O2 on Akt-mediated RPE cell survival, we first determined whether H2O2 induced RPE cell Akt phosphorylation. When RPE cells were exposed to varying H2O2 doses for 15 minutes, Akt was phosphorylated in a dose-dependent manner (Fig. 1A). In contrast, total Akt levels were not greatly affected by any of the tested concentrations; this result also served to confirm equal protein loading on Western blot analysis (Fig. 1B).

To examine the kinetics of Akt activation, cells were exposed to 200 μM H2O2 for varying times. p-Akt levels increased to a maximum within 15 minutes and subsequently slowly declined (Fig. 2A). Total Akt levels were not greatly affected by this treatment (Fig. 2B). When cells were treated with 100 μM H2O2 for 1 hour, Akt phosphorylation was also observed, though expression levels were lower than they were with 200 μM H2O2 (not shown).

H2O2-Induced Intracellular ROI Accumulation

To quantify the intracellular oxidative stress that led to Akt activation, we measured intracellular ROI production after H2O2 addition. After treatment of RPE cells with varying H2O2 concentrations for 15 minutes, fluorescence was significantly increased in a dose-dependent manner and peaked at 200 μM (Fig. 3). Similar results were also observed at 30 minutes, 1 hour, 2 hours, and 3 hours (not shown).

Inhibition of H2O2-Induced Akt Phosphorylation

PI3K is known to be upstream of Akt because its product, PtdIns(3,4,5)P3, stimulates Akt activation.36 When PI3K is inhibited, Akt activation is blocked in a variety of cells.17,18,37,38 Pretreatment with different LY doses for 1 hour reduced 300 μM H2O2-induced RPE cell Akt phosphorylation to near basal values (Fig. 4A) without altering total Akt levels (Fig. 4B). To confirm the specificity of this effect, an experiment was performed that was nearly identical; the exception was that API-2, a specific Akt inhibitor, was substituted for LY. API-2 similarly, though less potently, inhibited H2O2-induced Akt activation (Fig. 5).

Effect of Akt Blockade on H2O2-Mediated RPE Cell Death

H2O2 is a strong oxidant, and high concentrations can be toxic to cells. Because the PI3K-Akt pathway inhibits the activity of

**Figure 1.** Dose-response of H2O2-induced Akt phosphorylation. RPE cells in duplicate wells were treated with medium alone or H2O2 at different doses for 15 minutes. Cytoplasmic proteins (30 μg) were separated by SDS-PAGE and transferred to a nitrocellulose membrane for Western blot analysis. (A) Western blot probed with antibody to p-Akt (Ser473). Relative quantities of p-Akt determined by densitometry are shown separately below each lane. (B) Blot in (A) was stripped and reprobed with antibody to Akt, a control for gel loading. Relative quantity of Akt protein is shown below each lane.

**Figure 2.** Time course of H2O2-induced Akt phosphorylation. RPE cells in duplicate wells were treated with medium alone or H2O2 at 200 μM for various times. Cytoplasmic proteins (30 μg) were separated by SDS-PAGE and transferred to a nitrocellulose membrane for Western blot analysis. (A) Western blot probed with antibody to p-Akt (Ser473). The relative quantities of p-Akt determined by densitometry are shown separately below each lane. (B) Blot in (A) was stripped and reprobed with antibody to Akt, a control for gel loading. Relative quantity of Akt protein is shown below each lane.

**Figure 3.** Fluorescence measurements of intracellular ROI. RPE cells in seven replicate wells for each treatment group were loaded with 10 μM H2DCFDA for 30 minutes at 37°C, washed with HBSS, and treated with medium alone or H2O2 at various doses for 15 minutes. Unstained cell autofluorescence values were subtracted from the raw fluorescence data. Results are expressed as mean ± SD (n = 7). *P = 0.00002; **P = 0.000002; ***P = 0.000001; ^P = 0.01.
proapoptotic molecules, we next investigated whether H$_2$O$_2$-treated cell viability is diminished when Akt activation is blocked. When RPE cells were pretreated with 10 nM LY followed by the addition of different H$_2$O$_2$ doses, RPE cell viability was significantly decreased (Fig. 6). These data suggest that PI3K-Akt activity is important for survival during the cellular response to H$_2$O$_2$ in RPE cells.

RPE cell death can occur by apoptosis or necrosis. We next determined whether Akt blockade enhanced H$_2$O$_2$-mediated RPE apoptotic cell death. A few M30-positive cells were observed when RPE cells were treated with H$_2$O$_2$ alone. However, when cells were pretreated with 10 nM LY followed by H$_2$O$_2$ exposure, the number of M30-positive cells was significantly increased (Fig. 7), consistent with caspase-dependent apoptotic RPE cell death.

AIF is a flavoprotein that is normally confined to mitochondria. AIF is translocated from the mitochondria to the nucleus after the induction of caspase-independent apoptotic cell death. More than 90% of RPE cells exposed to vehicle or LY alone had punctate AIF cytoplasmic staining. This staining pattern is typical for mitochondrial localization. A similar staining pattern was observed in H$_2$O$_2$-treated cells. However, the punctate AIF cytoplasmic staining pattern was lost, and AIF nuclear labeling intensity was significantly increased when cells were treated with LY plus H$_2$O$_2$ (Fig. 8).

To determine whether H$_2$O$_2$ treatment induced necrotic cell death and whether the effect depended on Akt, we measured RPE cell LDH release, an early marker of necrotic cell death, with or without LY pretreatment. As shown in Figure 9, when RPE cells were treated with higher H$_2$O$_2$ doses (200 μM and 300 μM), with or without LY pretreatment, LDH release was significantly increased compared with cells treated with vehicle alone, LY alone, or low H$_2$O$_2$ doses (50 μM and 100 μM). Interestingly, LDH release was greater in cells treated with higher doses of H$_2$O$_2$ alone than in cells that were first pretreated with LY and then exposed to these H$_2$O$_2$ dosages.

To further evaluate the effect of PI3K-Akt inhibition and oxidant injury on apoptotic and necrotic RPE cell death, cell morphology was examined by transmission electron microscopy. When RPE cells were pretreated with LY and then exposed to H$_2$O$_2$, cells that had features of apoptosis and cells that had features of necrosis were both observed (Fig. 10).
**Effect of H\textsubscript{2}O\textsubscript{2} on Downstream Akt Effectors**

To elucidate possible mechanisms for the effect of H\textsubscript{2}O\textsubscript{2} on RPE cell survival, we examined the effect of H\textsubscript{2}O\textsubscript{2} on downstream Akt effectors. First, we measured FKHR and a related family member, acute lymphocytic leukemia-1 fused gene from chromosome X (AFX). After stimulation with H\textsubscript{2}O\textsubscript{2} at various doses for 1 hour, FKHR and AFX were phosphorylated in a dose-dependent manner (Fig. 11A), whereas GAPDH was not affected by H\textsubscript{2}O\textsubscript{2} treatment (Fig. 11B).

To evaluate the time course of this effect, RPE cells were exposed to 200 \( \mu \text{M} \) H\textsubscript{2}O\textsubscript{2} for varying times. Both FKHR and AFX were rapidly and transiently phosphorylated from 30 minutes to 2 hours in a time-dependent manner (Fig. 12A), but GAPDH was not affected by these treatments (Fig. 12B). Pretreatment for 1 hour with LY (10 \( \mu \text{M} \)) or the specific Akt inhibitor, API-2 (100 nM), reduced H\textsubscript{2}O\textsubscript{2}-induced FKHR and AFX phosphorylation (Fig. 13A) without affecting GAPDH (Fig. 13B).

We next investigated the effect of oxidant exposure on GSK-3\( \beta \), an enzyme recently found to contribute to apoptosis.\textsuperscript{40,41} As shown in Figure 14, 30-minute treatment with H\textsubscript{2}O\textsubscript{2} (200 \( \mu \text{M} \)) caused GSK-3\( \beta \) phosphorylation in a dose-dependent manner. GSK-3\( \beta \) phosphorylation was detected after 15 minutes and remained elevated 3 hours after the addition of H\textsubscript{2}O\textsubscript{2} (Fig. 15).
FIGURE 9. Effect of Akt inhibition on H₂O₂-mediated RPE cell death. RPE cells in triplicate wells were pretreated with vehicle alone or LY at 10 μM for 1 hour and then were treated with H₂O₂ at different doses for 7 hours. Absorbance of samples against a background control was measured with a spectrophotometer at 490 nm with a reference wavelength of 690 nm. Results are expressed as mean ± SD (n = 3). *P = 0.01 compared with H₂O₂-treated cells; **P = 0.005 compared with H₂O₂-treated cells.

**DISCUSSION**

In the present study, we have shown that H₂O₂, used as a model oxidant, activates the survival-signaling pathway PI3K-Akt in human RPE cells. When RPE cell Akt is activated, downstream proapoptotic effector molecules are phosphorylated and thereby inactivated in a dose-dependent manner. Furthermore, when PI3K-Akt is inhibited, H₂O₂-induced apoptotic and nonapoptotic RPE cell death is significantly enhanced; these data support the hypothesis that the PI3K-Akt signaling pathway serves as an important protective mechanism to prevent oxidant-mediated RPE cell death.

H₂O₂ is a potent ROI generator that readily crosses the plasma membrane. There are a variety of types of ROI, including H₂O₂, and one of the major sources of intracellular ROI generation, especially for RPE cells, is lipid peroxidation from phagocytosed rod outer segments. Adding H₂O₂ to RPE cells significantly increased intracellular ROI production and, at the same time, caused detectable Akt activation. Similarly, H₂O₂ induces intracellular ROI production in ARPE-19 cells. Furthermore, our results suggest that caspase-activation in RPE cells through the EGF receptor.

FIGURE 10. Electron microscopic images of apoptotic and necrotic RPE cells. RPE cells were pretreated with vehicle alone (A) or 10 μM LY (B-D) and then were treated with 200 μM H₂O₂ (C, D) for 18 hours and thereafter were fixed for EM analysis. (A, B) Normal nucleus and cytoplasm. (C) Nuclear chromatin condensation characteristic of apoptosis. (D) Cytoplasmic vacuoles characteristic of necrosis. Bars, 1 μM.

FIGURE 11. Dose–response of H₂O₂-induced FKHR phosphorylation. RPE cells in duplicate wells were treated with medium alone or H₂O₂ at different doses for 1 hour. Cytoplasmic proteins (30 μg) were separated by SDS-PAGE and transferred to a nitrocellulose membrane for Western blot analysis. (A) Western blot probed with antibody to p-FKHR and p-AFX determined by densitometry are shown separately below each lane. (B) Blot in (A) was stripped and reprobed with antibody to GAPDH, a control for gel loading. Relative quantity of GAPDH protein is shown below each lane. These results are consistent with previous observations that activation of the PI3K-Akt signaling pathway protects cells from oxidant-mediated cell death. Protection by Akt from oxidant-induced RPE cell death has not been previously described. Our data indicate that Akt protects against oxidant-induced apoptotic RPE cell death. When Akt phosphorylation was inhibited, modest concentrations of H₂O₂ induced apoptotic morphology, as determined by immunofluorescence and electron microscopy. Similarly, Akt protects against oxidant-induced apoptosis in other cell types. Furthermore, our results suggest that caspase-
dependent and caspase-independent apoptotic cell death was prevented by activation of the PI3K-Akt signaling pathway. When oxidant-treated RPE cells were incubated with LY, the result was significantly enhanced staining of cleaved cytokeratins by M30 antibody, an early marker of epithelial cell caspase-dependent apoptosis.31,32 These data are consistent with a protective effect of Akt on caspase-dependent, oxidant-mediated cell death.

It has been previously demonstrated that RPE cells undergo caspase-independent cell death, as evidenced by AIF translocation, when they are exposed to menadione or high doses of hydroquinone (an important cigarette smoke oxidant).4,33 To our knowledge, a protective effect of Akt on caspase-independent, oxidant-induced apoptotic RPE cell death has not been previously described. In the present study, we have confirmed the finding that another model oxidant, H2O2, promotes AIF translocation and have gone beyond this observation to show that PI3K-Akt inhibition significantly increases AIF translocation. These data support the hypothesis that Akt protects against caspase-independent apoptotic RPE cell death. Together with the information that high concentrations of H2O2 induce RPE cell death, we hypothesize that Akt signaling can help to compensate for low to intermediate levels of oxidant injury and to prevent apoptotic cell death but that, at higher oxidant concentrations, this protective mechanism is insufficient, and cell death ensues.

In addition to apoptotic programmed cell death, depending on the specific cell type, Akt can also protect against nonapoptotic cell death.49 Consistent with these findings, we observed oxidant-injured RPE cells with necrotic and apoptotic morphologies, as determined by electron microscopy, after Akt was inhibited. Similarly, LDH release, a marker of early necrotic cell death, was significantly greater when Akt was inhibited in H2O2-treated cells than when Akt was blocked but cells were not treated with H2O2. However, less LDH was released when cells were treated with H2O2 after Akt inhibition than when cells were treated with H2O2 alone. This latter observation could be explained by Akt having a predominant protective...
RPE cells are exposed to H$_2$O$_2$, FKHR, another forkhead family member, and Akt in the present study. We have shown for the first time that when oxidant exposure activates Akt, which phosphorylates and inactivates proapoptotic GSK-3$\beta$, the free FHKR then binds to 14-3-3 proteins, which forms a complex that is transported out of the nucleus, thereby functionally inactivating the transcription factor. Activation of Akt also phosphorylates and inactivates several proapoptotic factors. Inactivation of these proapoptotic RPE cell death proteins then prevents oxidant-mediated apoptotic cell death. Inactivation of these proapoptotic factors from DNA. The effect on apoptotic RPE cell death compared with nonapoptotic cell death. Further investigations will be necessary to clarify the relative Akt protective effect on apoptotic and non-apoptotic RPE cell death.

The PI3K-Akt pathway mediates cell survival by phosphorylating and therefore inactivating several proapoptotic factors. For example, activated Akt phosphorylates and releases proapoptotic FHKR transcription factor proteins from DNA. The free FHKR then binds to 14-3-3 proteins, which forms a complex that is transported out of the nucleus, thereby functionally inactivating the transcription factor. Activated Akt also phosphorylates and inactivates proapoptotic GSK-3$\beta$ protein.

In the present study, we have shown for the first time that when RPE cells are exposed to H$_2$O$_2$, FKHR, another forkhead family member AFX, and GSK-3$\beta$ are phosphorylated. Similarly, in other cell types, these proteins are phosphorylated in response to oxidant injury. Furthermore, when Akt phosphorylation is inhibited, FHKR phosphorylation is blocked. Together, these data are consistent with a mechanism by which RPE cell oxidant exposure activates Akt, which phosphorylates and thereby inactivates proapoptotic proteins. Inactivation of these proteins then prevents oxidant-mediated apoptotic cell death.

We chose to examine the effect of H$_2$O$_2$ on RPE cell PI3K-Akt activation. The effect of other oxidants on this signaling pathway in RPE cells is unknown. It would be of interest to determine whether other relevant oxidants, such as hydroquinone and myoperoxidase, induce a similar protective response in RPE cells. These studies are beyond the scope of the present report but are under way in our laboratory.

In conclusion, our data suggest that oxidant-mediated Akt activation protects RPE cells from oxidant-induced cell death. We hypothesize that this signaling pathway may help to protect RPE cells from oxidant-induced cell death under normal conditions and in diseases such as AMD and that they may be a useful therapeutic target to improve RPE cellular viability against oxidant injury. Future studies to address these hypotheses are also under way in our laboratory.

**FIGURE 15.** Time course of H$_2$O$_2$-induced GSK-3$\beta$ phosphorylation. RPE cells in duplicate wells were treated with medium alone or H$_2$O$_2$ at 200 μM for various times. Cytoplasmic proteins (30 μg) were separated by SDS-PAGE and transferred to a nitrocellulose membrane for Western blot analysis. (A) Western blot probed with antibody to p-GSK-3β (Ser9). Relative quantities of p-GSK-3$\beta$ determined by densitometry are shown separately below each lane. (B) Blot in (A) was stripped and reprobed with antibody to GAPDH, a control for gel loading. Relative quantity of GAPDH protein is shown below each lane.

**References**


