Phosphatidylinositol 3-Kinase (PI-3K)/Akt but Not PI-3K/p70 S6 Kinase Signaling Mediates IGF-1-Promoted Lens Epithelial Cell Survival

Gudiseva Chandrasekher and Dasetty Sailaja

PURPOSE. To investigate the ability of insulin-like growth factor (IGF)-1 to prevent apoptosis in lens epithelial cells and the involvement of phosphatidylinositol 3-kinase (PI-3K)/Akt and PI-3K/p70 S6 kinase (p70 S6K) signaling in the cell-survival process.

METHODS. Apoptosis in rabbit lens epithelial cell cultures was induced by staurosporine (10 ng/mL). Cellular apoptosis was detected by identifying the characteristic ladder-like fragmentation of genomic DNA in agarose gels and the intense blue fluorescence exhibited by apoptotic nuclei of cells in live cultures in the presence of Hoechst 33,258 dye. Proliferation of lens epithelial cells grown in culture was measured with a DNA-binding fluorescent dye. Overexpression of the constitutively active Akt (CA-Akt) in epithelial cells was achieved by the transfection of cells using Fugene 6 reagent with a plasmid carrying Akt cDNA. Western immunoblotting was performed to identify various proteins of interest.

RESULTS. IGF-1 (5 to 50 nM) and insulin (100 to 400 nM) suppressed lens epithelial cell apoptosis in a dose-dependent manner, as determined by a significant inhibition of genomic DNA fragmentation and the decreased number of intense blue fluorescent Hoechst stain-positive apoptotic nuclei in live cultures. DNA degradation was almost completely inhibited in the presence of 50 nM IGF-1 or 400 nM insulin. PI-3K inhibitors wortmannin and LY294002 blocked the IGF-1 effect on cell survival. Stimulation of lens epithelial cells with IGF-1 for 10 minutes to 24 hours resulted in the sustained activation of both Akt and p70 S6K. IGF-1 also induced the phosphorylation of Bad (a pro-apoptotic protein of the Bcl-2 family), which was inhibited by PI-3K inhibitors, but not by the p70 S6K inhibitor rapamycin. Furthermore, activation of Akt but not p70 S6K signaling by IGF-1 resulted in the inhibition of caspase-3 endogenous substrate poly (ADP-ribose) polymerase (PARP) degradation and apoptosis. The overexpression of CA-Akt in lens epithelial cells inhibited PARP breakdown and suppressed apoptosis. Inhibition of p70 S6K activation by rapamycin blocked IGF-1-promoted lens epithelial cell proliferation but not the cell-survival effect.

CONCLUSIONS. These studies demonstrated a role for IGF-1 in the prevention of the lens epithelial cell apoptosis process. Furthermore, these studies indicated that anti-apoptotic and proliferative signals from IGF-1 bifurcate downstream of PI-3K. Whereas IGF-1-mediated PI-3K/Akt signaling plays a pivotal role in cell survival by inactivating proapoptotic Bad protein and suppressing caspase activation, its stimulation of the PI-3K/p70 S6K cascade promotes proliferation. (Invest Ophthalmol Vis Sci. 2004;45:3577–3588) DOI:10.1167/iovs.04-0279

Apoptosis is a cell-death program that plays an important role in normal development and homeostasis. If this process is not regulated, it leads to irreparable damage to tissues of multicellular organisms and contributes to various diseases such as neuronal degeneration, autoimmune disorders, and even cancer. An apoptosis-like process occurs in lens epithelial cells during their differentiation into fiber cells. Cellular events such as loss of organelles and degradation of nuclei that take place during terminal differentiation resemble apoptosis. Apoptosis has also been implicated in lens cataract development. The monolayer of epithelial cells covering the entire core of the lens body filled with fiber cells is susceptible to apoptosis, because several external cataractogenic factors, such as UV radiation, chemicals, and oxidative stress, induce cell apoptosis. Maintaining the integrity of the lens epithelium and survival capability of epithelial cells during adverse conditions is the utmost priority for the lens, because the rest of the tissue depends on these cells for the maintenance of transparency. Therefore, it is important to identify the factors that play a role in lens epithelial cell survival. Many growth factors such as nerve growth factor (NGF), fibroblast growth factor-2 (FGF-2), and insulin-like growth factor (IGF)-1, in addition to their roles in growth and differentiation, participate as crucial cell-survival promoters. A variety of intracellular signaling molecules, such as mitogen-activated kinases (MAP kinases), protein kinase C isoenzymes, and phosphatidylinositol 3-kinase (PI-3K), mediate the signals from growth factors in lens epithelium and survival capability of epithelial cells during adverse conditions is the utmost priority for the lens, because the rest of the tissue depends on these cells for the maintenance of transparency. Therefore, it is important to identify the factors that play a role in lens epithelial cell survival. 3K/p70 S6K cascade promotes proliferation. PI-3K is one of the key signal-transduction enzymes that has been implicated in the control of apoptosis and promotion of cell growth. A group of lipid kinases that phosphorylate the 3'-OH group of the inositol ring in phosphoinositides belong to the PI-3K family. Classical PI-3K is a heterodimer and represents class I kinases that contain a p110 catalytic subunit and a p85 regulatory subunit. The lipid products of PI-3K, phosphatidylinositol-3,4,5-triphosphate and phosphatidylinositol-3,4-bisphosphate, recruit a diverse set of signaling proteins with pleckstrin homology (PH) domains to the membrane where they are activated. Phosphoinositide-dependent kinase (PDK)-1 is a PH domain-containing enzyme that modulates the phosphorylation of Akt (also called protein kinase B) and p70 S6 kinase (p70 S6K), the two downstream serine/threonine protein kinases. Both Akt and p70S6K are involved in cell survival as well as growth. Whereas IGF-1 and IGF-1-like molecules which are present in lens and its surrounding fluids play a role in the development and growth of the lens, their involvement in the prevention of lens epithelial cell apoptosis has not been elucidated.
Although changes in the regulation of lens epithelial cell receptor activity and signaling cascades appear to be taking place in cataract lenses,\(^{28-34}\) it is not clearly known whether such disturbances in the signaling pathways cause apoptosis and contribute to the development of cataract. Nevertheless, impairment of survival-signaling pathways could compromise the ability of lens to protect its cells from apoptosis. Therefore, it is worthwhile to identify the specific intracellular signaling pathways responsible for mediating lens epithelial cell survival by suppressing apoptosis, because this could identify potential sites for therapeutic intervention in pathologic situations in lens. Previous studies from our laboratory\(^ {35}\) show that PI-3K signaling is altered in a selenite cataract model. IGF-1 and insulin also stimulate the PI-3K signaling pathway in lens epithelial cells,\(^ {18}\) and inhibition of PI-3K signaling blocks lens epithelial cell proliferation.\(^ {40}\) Cell culture and transgenic animal models are being widely used to analyze the molecular mechanisms of many vital cellular processes. The present study investigated the ability of IGF-1 to suppress apoptosis in rabbit lens epithelial cell culture and assessed the role of PI-3K/Akt and PI-3K/p70 S6K signaling during the cell-survival process. The lens epithelial cell culture system is an in vitro model without epithelial-fiber cell interactions and differs from the in vivo model, where lens is surrounded by ocular humors and other tissues. Because lens epithelium is continuously exposed to growth factors of the ocular humor in vivo, a lens epithelial cell culture system is an ideal model that provides greater flexibility for the manipulations required to understand the role of growth factors and signaling cascades in different cellular processes. Studies using growth factors such as FGF, IGF-1, and insulin on cell proliferation, differentiation or activation of signaling molecules with in vivo lens models, lens epithelial explants, and lens epithelial primary cultures and organ cultures have yielded similar or comparable results.\(^ {18,20,36-59}\) Our current studies showed that IGF-1 exhibited a potent anti-apoptotic property and that PI-3K transduced survival signals of IGF-1 through a downstream signaling cascade involving the activation of Akt and subsequent phosphorylation and inactivation of Bad, the pro-apoptotic member of the Bcl2 family. Furthermore, activation of PI-3K/p70 S6K signaling by IGF-1 resulted in the promotion of proliferation but did not affect the cell-survival process.

**MATERIALS AND METHODS**

Insulin, wortmannin, and staurosporine were obtained from Sigma Chemical Company (St. Louis, MO). Polyclonal and monoclonal antibodies for anti-phospho Akt (ser473), anti-Myc tag (clone 9E10), transfection-grade eukaryotic empty expression vector (pUSEamp), and Akt cDNA (activated) in pUSEamp expression vector were obtained from Upstate Biotechnology (Lake Placid, NY). The transfection reagent Fugene 6 was purchased from Roche Diagnostics (Indianapolis, IN). Polyclonal antibodies for p70 S6K, PARP, and Bad (ser136) were from Santa Cruz Biotechnology (Santa Cruz, CA). Recombinant human IGF-1 was a product from R&D Systems (Minneapolis, MN). Anti-mouse and anti-rabbit horseradish peroxidase secondary antibodies were procured from Transduction Laboratories (Lexington, KY). All SDS-PAGE reagents were from Bio-Rad (Hercules, CA). Nitrocellulose membrane and enhanced chemiluminescence (ECL) reagents kits were from Amersham (Arlington Heights, IL). Dulbecco’s modified Eagle’s medium (DMEM) and fetal calf serum (FCS) were from Gibco (Grand Island, NY). The biotinylated protein markers kit was from New England Bio (Beverly, MA). LY294002 and rapamycin were from Calbiochem (La Jolla, CA). CyQUANT cell-proliferation assay kit (C-7026) and Hoechst 33,258 reagent were purchased from Molecular Probes (Eugene, OR). DNA-purification kit (A1120) was from Promega (Madison, WI).

**Epithelial Cell Culture and Preparation of Cell Extracts**

Lens epithelial cells were cultured as described previously.\(^ {36}\) Rats were anesthetized by intraperitoneal injection of pentobarbital (60 mg/kg), and the posterior lens epithelium was dissected from the lens capsule with adhering epithelial cells. The isolated epithelial cells were cultured in DMEM supplemented with 10% fetal calf serum and 1% antibiotic/antimycotic solution at 37°C in 5% CO\(_2\)/95% O\(_2\). Second-passage cells were used in all experiments. Serum-starved rabbit lens epithelial cell cultures (80% to 90% confluent) were treated with insulin (0–400 nM), or IGF-1 (0–50 nM) at 37°C for different periods of time as per the requirements of the experiments described in the results. Cell extracts were prepared in lysis buffer (20 mM HEPES, 2 mM Mg\(^{2+}\), 2 mM EGTA, 2 mM orthovanadate, 2 mM DTT, 1 mM phenyl methyl sulfonyl fluoride [PMSF], 0.1 mM leupeptin, 150 mM NaCl, 1% Triton X-100, and 0.5% Nonidet P 40 [NP-40]). Epithelial cells were homogenized in lysis buffer using a glass homogenizer, centrifuged at 14,000 rpm for 30 minutes, and the supernatant used as the source for different proteins. All the operations were conducted at 4°C. Protein content of the extracts was determined by the Bio-Rad dye-binding method.

**Transfection of Lens Epithelial Cells with Akt Expression Vector**

Rabbit lens epithelial cells were transfected with an A tensed for fusion protein (Myc-Has tagged Akt) under the control of a CMV promoter. The constitutively active Akt (CA-Akt) construct has the c-src myristoylation sequence (required for the activation) fused in-frame to the n terminus of the Myc-Akt. The construct was amplified in competent HB101 cells and purified by ultracentrifugation through a CsCl gradient.\(^ {40}\) The eukaryotic empty expression plasmid vector pUSE was used for vector-alone mock transfection. The rabbit lens epithelial cells at 50% to 55% confluence were transiently transfected with either pUSE-myc-Akt plasmid or pUSE using Fugene 6 (Roche) and 1 µg cDNA/3 µL transfection reagent/tissue culture dish (35 mm diameter) in the presence of DMEM containing 10% FCS, according to the manufacturer’s specifications. After 20–24 hours, medium containing Fugene 6 transfection reagent was removed and cells were grown further in DMEM containing 10% FCS as per the requirements of the experiments.

**Induction of Apoptosis**

Apoptosis was induced in lens epithelial cells with staurosporine (10 nM/mL). Staurosporine activates the apoptotic death pathway by affecting mitochondrial metabolism\(^ {41}\) and is widely used to induce apoptosis in lens epithelial cells.\(^ {42-45}\) Epithelial cells (90% confluent) were incubated with staurosporine dissolved in DMEM containing 0.25% FCS for 2–3 hours. The medium was then removed and cells were washed with the same medium without staurosporine and incubated for another 6–8 hours (in some experiments incubations were continued up to 30 hours). Next, these cultures were stained for Hoechst-positive cells or subjected to analysis for DNA fragmentation as described below.

**Hoechst Staining**

Hoechst stain was used to detect apoptotic cells by fluorescence imaging. Hoechst reagent is readily taken up by cells during the initial stages of apoptosis.\(^ {46}\) Doubly and selectively stains nuclei of apoptotic cells with intense fluorescent blue. Cell cultures were washed twice with PBS and incubated with Hoechst reagent (20 µM) for 30–45 minutes at 37°C in the dark. The stained cells were then visualized by a fluorescence microscope (Nikon, Tokyo, Japan) using UV filters. The images were captured by a Nikon (DXM1200) digital camera attached to the microscope and processed by MetaVue software program (Nikon). Apoptotic cells in the cultures were counted in at least three to four
random fields and expressed as percentage of total cells. The statistical significance of the data was evaluated using Student’s t-test.

**Isolation of DNA from Epithelial Cells and Agarose Gel Electrophoresis**

The DNA from epithelial cells was purified using a Promega Wizard genomic DNA-purification kit according to the protocol provided by the manufacturer. Briefly, epithelial cell cultures from experiments of interest were trypsinized and the cell nuclei were lysed with nuclear lysis solution. The lysates were treated with DNase-free RNase (3 μg) at 37°C for 15 minutes. The cellular proteins were then removed by salt precipitation. The DNA was finally precipitated with isopropanol and washed once with 70% ethanol. The DNA was dissolved in 10 mM Tris-HCl buffer (pH 7.4) containing 1 mM EDTA and its concentration was determined at 260 nm by spectrophotometry. The purified DNA was subjected to agarose (1.8%) gel electrophoresis and the characteristic breakdown/fragmentation of the chromatin during apoptosis was visualized with a UV light after staining the gel with ethidium bromide.
Proliferation Assay

Cell proliferation was assessed as reported previously. Rabbit lens epithelial cells were seeded in 96-well plates in DMEM containing 10% FCS at a density of \(5 \times 10^3\) cells/well and incubated overnight at \(37^\circ C\) in 5% CO\(_2\)/95%O\(_2\). Nonadherent cells were removed and the attached cells were incubated in DMEM containing 0.2% BSA with or without insulin (200 nM), IGF-1 (50 nM), or 50 nM rapamycin (eight wells per each experimental condition) for 48 hours. At the end of the experiment, wells were washed in PBS and cells were frozen at \(-80^\circ C\). After 2 hours, the cells were thawed and the DNA content of the cells in each well was determined as before by measuring the fluorescent dye CyQUANT GR, which exhibits strong fluorescence enhancement when bound to DNA (excitation wavelength at 485 nm and emission at 536 nm) using the CyQUANT cell-proliferation assay kit according to the manufacturer's instructions. The statistical significance of the data was evaluated using Student's t-test and values were considered significant when \(P < 0.05\).

Western Immunoblot Analysis

The lens epithelial cell extracts (25–35 μg protein) were subjected to SDS-PAGE in 9% gels. The proteins were transferred to nitrocellulose membranes. The membranes were blocked with 5% nonfat dry milk. The membranes were then probed with specific antibodies as indicated in the results. The blots were incubated at room temperature with primary antibodies for 2 hours, washed six times with TBS (20 mM Tris-HCl, 150 mM NaCl [pH 7.4]/0.05% Tween 20) and further incubated for 2 hours with horseradish peroxidase-conjugated secondary antibodies. The protein bands of interest were identified by an ECL kit. The chemiluminescent protein bands on x-ray film were quantified by densitometric scanning. Molecular sizes were determined by comparing with the mobilities of biotinylated protein markers that were run simultaneously during electrophoresis.

RESULTS

Influence of IGF-1 and Insulin on Lens Epithelial Cell Death by Apoptosis

To investigate the ability of IGF-1 and insulin to suppress the programmed cell-death, apoptosis was induced in rabbit lens epithelial cells by treatment with staurosporine (10 ng/mL), a common inducer of cell apoptosis. Incubation of rabbit lens epithelial cells with staurosporine for 2–3 hours produced significant apoptosis within 8–10 hours. Apoptosis was assessed by several criteria: the characteristic ladder-like DNA fragmentation, nuclear staining with Hoechst fluorescent reagent, and cell morphology. As shown in Figure 1A, staurosporine induced significant breakdown in cellular DNA. The presence of various concentrations of IGF-1 (5–50 nM) suppressed the staurosporine-induced apoptosis in a dose-dependent manner. A marked inhibition in DNA fragmentation was observed with a 5–10 nM concentration range of IGF-1. DNA degradation was almost completely blocked in the presence of 50 nM IGF-1. Insulin also exhibited a similar anti-apoptotic effect (Fig. 1B). There was a gradual decrease in apoptosis induced by staurosporine when cells were treated with increasing concentrations of insulin (100–400 nM). The percentage of apoptosis-positive cells detected by Hoechst staining was significantly reduced in the presence of IGF-1 and insulin (Figs. 1C and 1D). Approximately 40% to 50% of the cells in cultures treated with staurosporine showed the characteristic intense blue nuclear stain due to DNA condensation. Whereas the presence of 10 nM IGF-1 or 400 nM insulin decreased the number of apoptotic cells to approximately 16%, only approximately 5% of cells were found to be apoptotic with 50 nM IGF-1. The ability of IGF-1 to suppress apoptosis was also examined over a prolonged time period. While staurosporine induced significant change in cuboidal shape of cells, cell detachment, and death due to apoptosis (Fig. 2), IGF-1 was able to rescue the lens epithelial cells from apoptotic cell death even after 30 hours (Figs. 2A and 2B, right panel). A diffuse appearance of cellular contents with no clear boundaries be-
between the cells and pyknosis of nuclei without distinct shape were the morphologic changes observed when staurosporine was present in the cultures (Fig. 2B, middle panel). The presence of IGF-1 blocked the staurosporine effect on cell morphology, which remained similar to that of control cells not treated with staurosporine. Very few cells were detached in these samples after 30 hours. In staurosporine-treated cultures there was an increase in DNA breakdown with time (Fig. 2A, left and middle panels), and after 30 hours, low-molecular-weight DNA fragments disappeared due to increased breakdown (Fig. 2A, right panel). In the presence of IGF-1 (50 nM), very little DNA fragmentation was detected after a 30-hour period (Fig. 2A, right panel).

**Effect of PI-3K Inhibitors on IGF-1–Promoted Lens Epithelial Cell Survival**

Previously, IGF-1 was shown to stimulate PI-3K activity in lens epithelial cells. To determine whether the PI-3K pathway is involved in the suppression of lens epithelial cell apoptosis by IGF-1, specific inhibitors of this enzyme, wortmannin and LY294002, were used. The cells were pretreated with wortmannin (200 nM) or LY294002 (10 μM) for 30 minutes before stimulation with IGF-1. Then apoptosis was induced with staurosporine. The presence of wortmannin and LY294002 blocked the ability of IGF-1 to protect the lens epithelial cells from apoptosis, as evidenced by an increase in DNA laddering (Fig. 3A, compare lanes 4 and 5 with lane 3). The numbers of Hoechst stain-positive apoptotic nuclei seen in the presence of wortmannin or LY294002, plus IGF-1 and staurosporine, were similar to those observed with staurosporine alone (35%–40% vs. 45%, Figs. 3B and 3C). Wortmannin and LY294002 by themselves did not induce significant DNA fragmentation or produce increased numbers of Hoechst stain-positive cells in the cultures under the experimental conditions of this study (data not shown).

**Sustained Activation of Akt and p70 S6 Kinase by IGF-1**

Activation of Akt and p70 S6K is considered to be an important event in the cell-survival mechanism. The effect of IGF-1 on the activation of Akt and p70 S6K and the impact of PI-3K inhibitors on their activation was examined. Consistent with our previously published results, IGF-1 induced approximately a 10-fold increase in Akt activation as measured by identifying the phosphorylated form of Akt (phospho-Akt) in 10 minutes (Fig. 4A, top panel). Activation of the enzyme by IGF-1 is not transient. A significant activation of Akt in the presence of IGF-1 was observed even after 4–8 hours. Although there was a decrease in Akt activation, an approximately 100%–200% increase in the phospho-Akt level was still observed after 24 hours. Total Akt levels remained same at all time points (Figs. 4A and 4C, bottom panels). IGF-1 also pro-
duced the activation of p70 S6K. Activation of p70 S6K was analyzed by identifying the characteristic upward shift in the mobility of the p70 S6K band in gels due to phosphorylation. Interestingly, IGF-1 caused a gradual increase in p70 S6K activation as a function of time. The 70-kDa protein band shifted upwards slowly from 0 to 60 minutes and remained shifted even after 24 hours, demonstrating a sustained activation of the enzyme (Fig. 4B). These results indicate the differential effect of IGF-1 on the kinetics of Akt and p70 S6K activation. As presented in Figures 4C (top panel) and 4D, PI-3K inhibitors LY294002 and wortmannin (data not shown) inhibited the phosphorylation of both Akt and p70 S6K, induced by IGF-1. These results show that Akt and p70 S6K are downstream of PI-3K and that their activation is differentially regulated through PI-3K. Sustained activation of these two kinases by IGF-1 could be important for the survival-promoting property of this growth factor. The presence of rapamycin, an inhibitor of p70 S6K activation, suppressed the IGF-1-induced phosphorylation of p70 S6K but had no effect on Akt activation (Fig. 4C, top panel, and 4D).

**Involvement of Akt but Not p70 S6K in the Phosphorylation of Bad**

Bad is a pro-apoptotic member of the Bcl2 family proteins. Phosphorylation of Bad is a key step in the signaling events that come into play in the rescue of cells from apoptosis. To investigate the mechanism by which IGF-1 inhibits apoptotic cell death, the effect of IGF-1 and PI-3K inhibitors on the phosphorylation status of Bad was examined. As shown in Figure 5A, stimulation of lens epithelial cells with IGF-1 resulted in the phosphorylation of Bad (at SEM136). Activation of Bad was dependent on the PI-3K pathway, because wortmannin and LY294002 inhibited the IGF-1-mediated phosphorylation of Bad. However, rapamycin, the specific inhibitor of p70 S6K, showed no significant effect in blocking Bad phosphorylation. These results showed that PI-3K/Akt, rather than the PI-3K/p70 S6K signaling cascade, is involved in Bad activation.

**Suppression of Apoptosis in Lens Epithelial Cells Overexpressing Constitutively Active Akt (CA-Akt)**

To assess the potential involvement of Akt in the regulation of lens epithelial cell survival, rabbit lens epithelial cells were transfected with an expression vector containing Myc-tagged Akt. Transfected cells expressed significant levels of Myc-Akt (Fig. 6A). A fast-migrating band shown in the blot (just below the Myc-Akt band) probed with anti-pAkt antibody represents endogenous p-Akt and empty vector did not cause any changes in the endogenous levels of Akt (Fig. 6A, right panel). Apoptosis was induced in transfected epithelial cells with staurosporine as described above. Cells overexpressing active Akt protein were significantly less susceptible to apoptosis compared with control, untransfected (Fig. 6B, left panel) or empty vector-transfected cells (Fig. 6B, right panel) after staurosporine treatment, as determined by a decrease in DNA fragmentation and DNA content (Fig. 6B, right panel).
p70 S6K plays a key role in the growth of cells. It is involved in the regulation of the G1/S check-point of the cell cycle.49,50 The present study examined the ability of IGF-1 to protect lens epithelial cells from apoptosis and the involvement of the PI-3K signaling cascade in the rescue process. IGF-1 was shown to serve as an anti-apoptotic agent for lens epithelial cells, and the functional significance of IGF-1-activated PI-3K/Akt cascades in the prevention of apoptosis of lens epithelial cells was documented. IGF-1 at concentrations as low as 5–25 nM was very potent in suppressing staurosporine-induced apoptosis, whereas a much higher concentration of insulin (400 nM) was required for a comparable effect. Earlier studies by Rampalli et al.8 suggest that apoptosis could be a factor in cataract, whereas a much higher concentration of insulin (400 nM) was required for a comparable effect. Earlier studies by Rampalli et al.8 suggest that apoptosis could be a factor in cataract, whereas a much higher concentration of insulin (400 nM) was required for a comparable effect. Earlier studies by Rampalli et al.8 suggest that apoptosis could be a factor in cataract, whereas a much higher concentration of insulin (400 nM) was required for a comparable effect. Earlier studies by Rampalli et al.8 suggest that apoptosis could be a factor in cataract, whereas a much higher concentration of insulin (400 nM) was required for a comparable effect. Earlier studies by Rampalli et al.8 suggest that apoptosis could be a factor in cataract, whereas a much higher concentration of insulin (400 nM) was required for a comparable effect. Earlier studies by Rampalli et al.8 suggest that apoptosis could be a factor in cataract, whereas a much higher concentration of insulin (400 nM) was required for a comparable effect. Earlier studies by Rampalli et al.8 suggest that apoptosis could be a factor in cataract, whereas a much higher concentration of insulin (400 nM) was required for a comparable effect. Earlier studies by Rampalli et al.8 suggest that apoptosis could be a factor in cataract, whereas a much higher concentration of insulin (400 nM) was required for a comparable effect. Earlier studies by Rampalli et al.8 suggest that apoptosis could be a factor in cataract, whereas a much higher concentration of insulin (400 nM) was required for a comparable effect. Earlier studies by Rampalli et al.8 suggest that apoptosis could be a factor in cataract, whereas a much higher concentration of insulin (400 nM) was required for a comparable effect.
FIGURE 6. Expression of constitutively active Akt suppresses apoptosis. (A) Overexpression of CA-Akt in lens epithelial cell cultures. Rabbit lens epithelial cells were transfected with a vector expressing Myc-tagged Akt (active) or empty vector (mock transfection). Lysates from transfected and untransfected cell cultures, and untransfected cells stimulated with IGF-1 (50 nM) for 10 minutes, were prepared. Extracts containing approximately 15–20 μg protein were separated by SDS-PAGE and then blotted with anti-pAkt or anti-Myc antibodies. (B) and (C) Epithelial cells in the presence or absence of IGF-1 (50 nM) and cells expressing CA-Akt were treated with staurosporine (10 ng/mL) in DMEM containing 0.2% FCS for 2 to 3 hours to induce apoptosis. Culture medium was then removed and replaced with fresh medium with or without IGF-1 and incubated for another 6 to 8 hours. Cell cultures were washed twice with PBS, and subjected to either DNA extraction followed by agarose gel electrophoresis (B), or stained with Hoechst fluorescence reagent to identify apoptotic cells (C). All experiments were repeated at least two to four times. The ratio of Hoechst stain-positive apoptotic cells to total cells was determined in three to five different fields. The values shown (C) represent averages of three independent experiments.

Members of the Bcl2 family that consist of both pro-apoptotic and anti-apoptotic proteins play a central role in regulating apoptosis.59,60 The pro-apoptotic protein Bad is inactive on phosphorylation as it dissociates from the Bad-Bcl-xl complex. Phosphorylated Bad sequesters in the cytoplasm with 14-3-3 protein, and blocks damage to mitochondria that would result in the release of cytochrome c, and is thus unable to promote apoptosis.51 In lens epithelial cells, PI-3K inhibitors blocked the phosphorylation of Bad at ser 136, demonstrating the importance of PI-3K signaling in cell survival. Although IGF-1-mediated PI-3K stimulation led to the activation of both Akt and p70 S6K in lens epithelial cells, inhibition of p70 S6K activation by rapamycin did not block the phosphorylation of Bad, suggesting that PI-3K/Akt rather than PI-3K/p70 S6K activation is essential for Bad phosphorylation at ser 136. Furthermore, inhibition of p70 S6K activation with rapamycin did not affect the ability of IGF-1 to suppress apoptosis in the presence of staurosporine. These studies indicate that p70 S6K activation is not a requirement during the lens epithelial cell survival process. Further studies conducted with lens epithelial cells overexpressing CA-Akt (Figs. 6 and 8) allowed us to confirm the role of Akt in suppressing apoptosis. Valverde et al.42 showed that Akt is also activated during Gas6/Axl-mediated anti-apoptotic signaling in human lens epithelial cells. We have demonstrated that cells expressing CA-Akt were able to resist apoptosis. However, we found that overexpression of Akt did not result in a complete inhibition of apoptosis, as observed with 50 nM IGF-1. This could be due in part to the fact that the level of CA-Akt expression in these cells was insufficient to block apoptosis totally. It is also possible that IGF-1 suppresses apoptosis through other signal-transduction cascades.53 The ability of CA-Akt to inhibit apoptosis, considered together with the increase in Akt activation after IGF-1 treatment, suggests that Akt may function to transduce the IGF-1-initiated anti-apoptotic signals through the phosphorylation and inactivation of pro-apoptotic Bad.

Caspases are closely linked to the pro-apoptotic signals generated by Bcl2 proteins.55 Damage to mitochondria causes the release of cytochrome c, resulting in the stimulation of apoptosis-activating factor (Apaf-1) and leading to an increase demonstrated that the protective effect of IGF-1R is more pronounced in vivo than in vitro and the number of expressed receptors is a critical determinant for cell survival. The domain of IGF-1 receptor required for anti-apoptotic function has also been identified.55 As the studies presented here suggest, it is possible that IGF-1 also protects lens cells in vivo against apoptosis. However, in addition to IGF-1, other growth factors such as FGF may also share a cell-survival function in lens epithelial cells.57,58 Wortmannin, an inhibitor of PI-3K, binds irreversibly to the catalytic subunit (p110), thereby inhibiting the signaling pathway after PI-3K activation. In this study, wortmannin inhibited the effect of IGF-1 in suppressing apoptosis, indicating that PI-3K is involved in the regulation of this process. Another PI-3K inhibitor, LY294002, also gave essentially the same result. In both cases, characteristic DNA ladder-forming, which coincided with the appearance of Hoechst stain-positive apoptotic cell nuclei, was observed even in the presence of 50 nM IGF-1. PI-3K stimulation by IGF-1 produced a sustained but differential activation of two downstream kinases, Akt and p70 S6K (Fig. 4), which are considered important for the survival of cells. The specific roles of these two protein kinases in the regulation of apoptotic cascades were further investigated.
in the activity of caspases (such as caspases-3 and -9) which execute the degradation of nuclear and cytoskeletal proteins to cause cell death. \textsuperscript{51-53} Staurosporine also produces apoptosis by similar mechanism. \textsuperscript{31,64} We have found that in lens epithelial cells staurosporine treatment caused the degradation of PARP, the endogenous substrate of caspase-3. Many of the stresses (such as UV radiation or oxidative stress due to \textsubscript{2}H\textsubscript{2}O\textsubscript{2}) that lens cells are likely to experience in vivo, also activate caspases in several cell types. \textsuperscript{65-66} In lens epithelial cells, IGF-1 inhibited caspase-3 activation and blocked the cleavage of its intracellular substrate protein PARP after treatment with staurosporine. Thus, IGF-1 could play a role in protecting lens in vivo against apoptosis induced by UV and oxidative stress. PI-3K but not p70 S6K inhibitors also opposed the IGF-1 effect in maintaining PARP levels. Furthermore, expression of CA-Akt in lens epithelial cells resulted in the suppression of caspase-3 activation and prevented PARP degradation in the presence of staurosporine. These results are consistent with a role for PI-3K/Akt signaling in the IGF-1-mediated survival of lens epithelial cells.

Proliferation and apoptosis are opposing cellular processes that are very closely interconnected. A balance in the regulation of apoptosis and survival/proliferation signals is necessary for cellular homeostasis. Growth factors often produce simultaneous yet distinct cell-survival and growth-related signals. When the cell receives apoptotic signals, while it responds by activating anti-apoptotic pathways, it also stimulates mitogenic cascades to compensate for eventual cell death due to apoptosis. Similarly, growth responses are counterbalanced by activation of apoptotic cascades. Thus, dual signaling by growth factors helps in regulating homeostasis. The essential role of IGF-1/IGF-1R system in promoting growth was demonstrated with transgenic mice carrying mutations in IGF-1 and IGF-1R genes. \textsuperscript{67} Newborn mice lacking IGF-1 (IGF\textsuperscript{-/-}) and IGF-1R (IGF-1R\textsuperscript{-/-}) have birthweights 40% lower than normals and most die immediately after birth; a small number that survive show severe growth retardation. Decreased cell proliferation coupled with apoptosis in tissues could contribute to growth deficiency and death of IGF-1/IGF-1R knockout mice. Increased epithelial cell proliferation in lens of newborn transgenic mice overexpressing IGF-1 has also been reported. \textsuperscript{68} We have previously shown that IGF-1 promotes mitogenesis in lens, and the inhibition of PI-3K signaling activated by IGF-1 blocks lens epithelial cell proliferation. \textsuperscript{36} The present study shows that IGF-1 is capable of activating both Akt and p70 S6K.
cascades through PI-3K. Although PI-3K/p70 S6K signaling does not seem to have an essential role in IGF-1–mediated cell survival, this pathway appears to be important in cell proliferation. The p70 S6K inhibitor rapamycin significantly suppressed IGF-1–induced lens epithelial cell proliferation. p70 S6K contributes to cell growth by regulating translation of key messenger RNAs and coordinating cell-cycle progression.69 Whereas the studies presented here demonstrated the absolute requirement for PI-3K/Akt signaling for lens epithelial cell survival promoted by IGF-1, the involvement of this pathway in mitogenic reactions may also be necessary. Akt catalyzes the phosphorylation of glycogen synthase kinase 3 (GSK3) resulting in its inactivation. GSK3 promotes cyclin D degradation.70 Inhibition of GSK3 by Akt contributes to the accumulation of cellular cyclin D levels and cell-cycle entry.71 It is possible that during IGF-1-promoted lens epithelial cell proliferation, Akt and p70 S6K produce signals that modulate different phases of cell-cycle events.

In summary, our results demonstrated that IGF-1 serves as a potent anti-apoptotic factor for lens epithelial cells. As depicted in Figure 9, the protective effect of IGF-1 against staurosporine-induced apoptosis is dependent on the PI-3K/Akt signaling pathway, and Akt activation modulates the inactivation of the pro-apoptotic Bcl2 family protein, Bad, thereby suppressing caspase-3 activation and lens epithelial cell death. Furthermore, overexpression of active Akt in lens epithelial cells inhibited the degradation of caspase-3 substrate PARP and apoptotic cell death, indicating the necessity for Akt activation for cell survival. Interestingly, activation of the PI-3K/p70 S6K cascade by IGF-1 was not a requirement for lens epithelial cell survival, but it was necessary for cell proliferation. Thus dual signaling by IGF-1 through PI-3K mediated growth as well as survival reactions in lens epithelial cells. Targeting the PI-3K pathway with factors that promote its survival reactions and suppress mitogenic signaling could be a useful approach to overcome defects in diseases of the lens that involve apoptosis and proliferation.

Acknowledgments
The authors thank Haydee E. P. Bazan for helpful discussions, and Pranab K. Mukherjee for his help in purifying Akt-cDNA plasmids.

References


