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

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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 fluorescence 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. 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.

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Supported by National Eye Institute Grant NIH-NEI RO1 EY 12701 and Core Grant NEI EY 02577.

Submitted for publication March 11, 2004; revised May 16, 2004; accepted June 12, 2004.

Disclosure. G. Chandrasekher, None; D. Sailaja, None.

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Although changes in the regulation of lens epithelial cell receptor activity and signaling cascades appear to be taking place in cataract lenses,29-34 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 laboratory35 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.46 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 system 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 have yielded similar or comparable results.18-20,36-39 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 biotinylated protein markers kit was from New England BioLabs (Beverly, MA). The biotinylated protein markers kit was from New England BioLabs (Beverly, MA). Biotinylated protein markers kit was from New England BioLabs (Beverly, MA). Epitope dilution was performed in 0.25% BSA in PBS. Protein concentrations were determined using the Bio-Rad dye-binding method. The biotinylated protein markers kit was from New England BioLabs (Beverly, MA).

Epithelial Cell Culture and Preparation of Cell Extracts

Lens epithelial cells were cultured as described previously.36 Lenses from rabbit eyes (Pel-Freeze Biologicals, Rogers, AR) were separated, the capsule with adhering epithelial cells was dissected from the lens, and primary cultures of epithelial cells were grown in DMEM supplemented with 10% fetal calf serum and 1% antibiotic/antimycotic solution at 37°C in 5% CO2/95% O2. 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 MgCl2, 2 mM EDTA, 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 Akt eukaryotic expression plasmid (pUSE) in which mouse-activated Akt was expressed as fusion protein (Myc-His 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 metabolism41 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.46,47 It 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.
Approximately 3–5 μg DNA were loaded in each sample well. A 100-base-pair DNA ladder was used as standard.

**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 × 10³ cells/well and incubated overnight at 37°C in 5% CO₂/95%O₂. Next day, 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°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-
tween 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-

**FIGURE 3.** Effect of PI-3K inhibitors on IGF-1–mediated suppression of apoptosis. Rabbit lens epithelial cell cultures were pretreated for 30 minutes with or without wortmannin (200 nM) or LY294002 (10 μM) in DMEM containing 0.2% FCS before incubating with or without IGF-1 (50 nM). Thirty minutes after the addition of IGF-1, cultures were incubated in the presence of staurosporine (10 ng/mL) for 2 to 3 hours. Medium was then removed and replaced with fresh medium containing IGF-1 or IGF-1 and PI-3K inhibitors. Cultures were incubated under these conditions for another 6 to 8 hours. At the end of the experiment, cell cultures were washed twice with PBS and subjected to either DNA extraction followed by agarose gel electrophoresis (A), or stained with Hoechst fluorescence reagent to visualize cells containing apoptotic nuclei (B). The ratio of Hoechst stain-positive apoptotic cells to total cells was determined by counting cells in three to five different fields. The values shown (C) represent averages of three independent experiments. These experiments were repeated three to four times and similar results were obtained. Scale bar, 100 μm.
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 (200 nM) or p70 S6K inhibitor, rapamycin (25 nM) for 20 minutes before stimulating with IGF-1 (50 nM) for 10 minutes. At the end of the experiments, cells were washed three times with ice-cold PBS and cellular proteins were extracted in lysis buffer. Protein extracts (15–20 µg) were subjected to SDS-PAGE and Western blot analysis. Blotted membranes were probed with anti-phospho-Akt (A and C, top panels) or anti-Akt (A and C, bottom panels) or anti-p70 S6K antibodies (B) and (D). The data were from one experiment and similar results were obtained in two other independent experiments. Arrows in (B) show different levels of the p70 S6K bandshift due to phosphorylation.

**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...
the regulation of the G1/S check-point of the cell cycle.49,50 p70 S6K plays a key role in the growth of cells. It is involved in the number of apoptotic cells (data not shown) in the cultures. Figure 5: IGF-1-induced phosphorylation of Bad is blocked by wortmannin and LY294002 but not by rapamycin. Serum-starved rabbit lens epithelial cell cultures in DMEM supplemented with 0.2% FCS were preincubated with or without PI-3K inhibitors wortmannin (200 nM) or LY294002 (10 μM), or p70 S6K inhibitor, rapamycin (25 nM) for 20 minutes before stimulating with IGF-1 (50 nM) for 10 minutes. Cells were then washed in PBS and extracted in lysis buffer. Extracts containing approximately 25–30 μg protein were separated by SDS-PAGE and then blotted with anti-phospho Bad (top panel) or anti-Bad (bottom panel) antibodies. A typical experiment, which was repeated two more times, is presented.

presence of Hoechst stain-positive apoptotic cells (Fig. 6C). The level of apoptotic nuclei present in CA-Akt-transfected cells was significantly less compared to empty vector-transfected cells after staurosporine treatment (approximately 15% vs. 50%). These results demonstrate that Akt plays an essential role in PI-3K signaling-dependent rescue of lens epithelial cells from apoptosis.

Involvement of p70 S6K in Lens Epithelial Cell Proliferation but Not in Cell Survival

Inhibition of p70 S6K activity causes apoptosis in several cell lines.54,55 Since IGF-1 also activated p70 S6K signaling through PI-3K, we investigated whether suppression of p70 S6K activation by rapamycin could inhibit the IGF-1 ability to protect lens epithelial cells during staurosporine-induced apoptosis. Selective inhibition of p70 S6K activation by rapamycin did not result in any significant DNA breakdown (Fig. 7A) or increase in number of apoptotic cells (data not shown) in the cultures. p70 S6K plays a key role in the growth of cells. It is involved in the regulation of protein translation through the phosphorylation of the 40S ribosomal protein subunit S6. It participates in the regulation of the G1/S check-point of the cell cycle.59,50 Because IGF-1 promotes lens epithelial cell proliferation,56 we investigated whether inhibition of IGF-1-induced p70 S6K activation by rapamycin affects lens epithelial cell proliferation. When lens epithelial cells were grown in the presence of IGF-1 (50 nM) or insulin (200 nM), there was an approximately 40% increase in cell proliferation compared to control cultures (Fig. 7B). These data are consistent with our previous findings.56 The presence of rapamycin resulted in a significant decrease in IGF-1- and insulin-promoted cell proliferation. Rapamycin alone had no appreciable effect on cell proliferation under the experimental conditions of this study. These results suggest that IGF-1-induced activation of p70 S6K is not essential for lens epithelial cell survival but is necessary for the growth of the cells.

Influence of IGF-1-Induced PI-3K/Akt Signaling on PARP (Caspase-3 Substrate) Degradation

Caspase activation is a characteristic feature of apoptosis. Activation of these proteases results in the degradation of cellular proteins leading to apoptotic cell death.51,52 To further understand the mechanism by which IGF-1–dependent PI-3K/Akt activation prevents cell death, the effect of the PI-3K/Akt signaling cascade on the activation status of caspase-3 during staurosporine-induced apoptosis was studied. For this, the degradation of PARP protein, the endogenous substrate of caspase-3, was analyzed by Western immunoblot analysis. In cells treated with staurosporine, there was a significant increase in the degradation of PARP in 8–10 hours (Fig. 8A, top panel), correlating with cell death. In cultures where cell death was suppressed due to the presence of IGF-1, breakdown of PARP was blocked. However, when PI-3K inhibitors wortmannin and LY294002 were present, the ability of IGF-1 to arrest the degradation of PARP protein was markedly decreased. Similarly, in lens epithelial cells expressing CA-Akt, staurosporine treatment did not result in significant degradation of PARP protein (Fig. 8B, top panel). Furthermore, inhibition of IGF-1–induced p70 S6K activation by rapamycin did not cause any noteworthy decrease in PARP level. These results show that activation of Akt (Figs. 8A and 8B, middle panels) is pivotal for inhibiting caspase-3 activation and therefore lens epithelial cell apoptosis. The level of total Akt remained unchanged during the experimental conditions (Figs. 8A and 8B, bottom panels).

DISCUSSION

Whereas the apoptosis-like process appears to be important for lens fiber cell differentiation,4,5 the significance of actual apoptosis that results in epithelial cell death in lens pathology is not clear. Although studies reported by Li et al.7 and others8,11,15 suggest that apoptosis could be a factor in cataract, studies by Harcopolos et al.53 indicate that human senile cataract formation is not associated with increased epithelial cell death due to apoptosis. Throughout its lifespan, lens experiences several stresses such as UV-radiation, chemicals, and oxidative and osmotic stresses which can cause apoptosis. These stresses are considered to be causative factors in the development of lens cataract over a period.10 Therefore it is reasonable to assume that apoptosis could have an adverse effect on lens metabolism. On the other hand, apoptosis that occurs after cataract surgery9 may be beneficial because it can result in the suppression of epithelial cell viability and limit the spread of secondary cataract. Understanding the factors which influence signaling pathways involved in the regulation of apoptosis could be helpful in managing lens-related diseases.

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 and Zelenka54 also demonstrated that insulin suppresses apoptosis in 6-day-old embryonic chicken lens epithelial explants. There is increasing evidence that suggests the IGF-1/IGF-1 receptor (IGF-1R) system is involved in the regulation of cell death in many mammalian tissues in vivo. Resnicoff et al.55
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-akt 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.
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{51-52} 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 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 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, 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{50} The present study shows that IGF-1 is capable of activating both Akt and p70 S6K

**FIGURE 7.** IGF-1-induced activation of the PI-3K/p70 S6K pathway is involved in cell proliferation but not in cell survival. (A) Differences in the effect of rapamycin and LY294002 on DNA degradation. Rabbit lens epithelial cell cultures were pretreated for 30 minutes with or without rapamycin (25 nM) or LY294002 (10 \textmu M) in DMEM containing 0.2% FCS before incubating with or without IGF-1 (50 nM) for 30 minutes. Cultures were further incubated in the presence of staurosporine (10 ng/mL) for 2–3 hours. Medium was then removed and replaced with a fresh medium containing IGF-1 or IGF-1 and inhibitors and incubated for another 6 to 8 hours. Extent of DNA breakdown was analyzed by agarose gel electrophoresis. (B) Effect of rapamycin on IGF-1-promoted cell proliferation. Rabbit lens epithelial cells were grown for 48 hours in the presence or absence of insulin (200 nM) or IGF-1 (50 nM) or rapamycin (50 nM) dissolved in DMEM containing 0.2% FCS. At the end of the experiment, medium was removed, cells were washed twice in PBS, and the cellular DNA levels were measured by fluorescence. The data were from three independent experiments. The values obtained for insulin (*P < 0.0009) and IGF-1 (**P < 0.0005) are significantly higher than controls. The values for insulin with rapamycin (**P < 0.001) are significantly lower than insulin alone. The values for IGF-1 with rapamycin (**P < 0.02) are significantly lower than IGF-1 alone.

**FIGURE 8.** Akt activation is necessary for the suppression of caspase-3 substrate PARP degradation. (A) Rabbit lens epithelial cell cultures were pretreated for 30 minutes with or without wortmannin (200 nM) or LY294002 (10 \textmu M) or rapamycin (25 nM) in DMEM containing 0.2% FCS before incubating with or without IGF-1 (50 nM). Thirty minutes after the addition of IGF-1, cultures were incubated in the presence of staurosporine (10 ng/mL) for 2 to 3 hr. Medium was then removed and replaced with a fresh medium containing IGF-1 or IGF-1 and inhibitors. Cultures were incubated under these conditions for another 8–10 hours. (B) Lens epithelial cells expressing CA-Akt were subjected to staurosporine treatment as described in the legend for Figure 6. At the end of the experiment cell cultures were washed with PBS and extracted in lysis buffer. Cell lysates (approximately 25 \mu g protein) were separated by SDS-PAGE and proteins were transferred to nitrocellulose membranes and probed with anti-PARP (A and B, top panels) or anti-pAkt (A and B, middle panels) or anti-Akt (A and B, bottom panels) antibodies. This experiment was repeated and similar results were obtained.
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.

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