Insulin Promotes Rat Retinal Neuronal Cell Survival in a p70S6K-dependent Manner*

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The purpose of this study was to examine the role of the ribosomal protein S6 kinase (p70S6K), a protein synthesis regulator, in promoting retinal neuronal cell survival. Differentiated R28 rat retinal neuronal cells were used as an experimental model. Cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% newborn calf serum, and during the period of experimentation were exposed either to the absence or presence of 10 nM insulin. Insulin treatment induced p70S6K, mTOR, and Akt phosphorylation, effects that were completely prevented by the PI3K inhibitor, LY294002. Insulin-induced phosphorylation of p70S6K and mTOR was prevented by the mTOR inhibitor, rapamycin. Apoptosis, induced by serum deprivation and evaluated by Hoechst staining, was inhibited by insulin treatment in R28 cells, but not in L6 muscle cells. This effect of insulin was also largely prevented by rapamycin. Inhibition of p70S6K activity by exogenous expression of a dominant negative mutant of p70S6K prevented insulin-induced cell survival, whereas, overexpression of wild type p70S6K or expression of a rapamycin resistant form of the kinase enhanced the effect of insulin on survival. Enhanced cell survival under the latter condition was accompanied by increased p70S6K activity and phosphorylation. Rapamycin did not inhibit insulin induced p70S6K phosphorylation and activity in cells transfected with the rapamycin-resistant mutant. Together, these results suggest that p70S6K plays a key role in insulin stimulated retinal neuronal cell survival.

Apoptotic cell death of retinal neuronal and vascular cells contributes to the pathogenesis of diabetic retinopathy (1, 2) but the mechanism of this process is uncertain. Several studies have identified activation of phosphoinositide 3-kinase (PI3K) as a necessary step in the cell survival pathway that is stimulated by a number of growth factors and insulin (3–5). Akt/ PKB, a protein kinase that functions downstream of PI3K in the insulin signal transduction pathway, is also involved in the regulation of cell survival (6). Evidence for a role of Akt in IGF-1-mediated cell survival was provided by Dudek et al. (7) showing that overexpression of Akt prevents apoptosis in primary cultures of cerebellar neurons induced by survival factor withdrawal or chemical inhibition of PI3K. The expression of dominant-negative forms of Akt interferes with growth factor-mediated survival in these cells, indicating that Akt is necessary and sufficient for neuronal survival. We have also shown that both IGF-I and insulin can rescue retinal neuronal cells from apoptosis through a PI3-kinase/Akt mediated mechanism (3), and that systemically administered insulin activates the retinal insulin receptor, PI3K, and Akt1 in normal rats (8).

The 70 kDa ribosomal protein S6 kinase, p70S6K, is one of the downstream effectors of PI3K (9–12), (13). It is now believed that there are five domains in the primary structure of S6K1. Beginning with the N terminus, they are: acidic, catalytic, linker, autoinhibitory, and the C-terminal domain (14–18). The mechanism through which S6K1 is activated is complicated (19–22), and involves interactions among four of the five domains and phosphorylation of at least seven specific regulatory sites (17). Of those 7 sites, phosphorylation of Thr389 and Thr229 are critical for p70S6K activation. To date, PI3K (23, 24), PDK1, Akt/PKB (25–28), protein kinase C (PKC) (29, 30), the Rho family of small G proteins and the mammalian target of rapamycin protein kinase (mTOR) (31–36) are thought to be the upstream effectors of S6K1 phosphorylation. Many growth factors, including insulin, activate p70S6K in a PI3K-dependent manner. In addition, amino acids can activate S6K1 in a PI3K-independent manner. Compared with the upstream effectors, the only known downstream effector of S6K1 is the 40S ribosomal S6 protein. Phosphorylation of S6 allows translational up-regulation of mRNAs containing 5’-tracts of pyrimidines (TOP), which encode for components of the translational apparatus (37), cell cycle-related (G1 to S transition) transcription factor E2F and insulin transcription (38–40).

Most studies of S6K1 including ours have to date focused on its role in protein synthesis and cell cycle regulation. In those studies, we reported that insulin controls protein synthesis in skeletal muscle through activation of p70S6K (41, 42). However, a number of recent studies have suggested that S6K1 may be intimately involved in mediating cell survival. Wan and Helman (43) found that inhibition of the p70S6K pathway may enhance chemotherapy-induced apoptosis in the treatment of IGF-II-overexpressing tumors. Agents that cause apoptosis inactivate mTOR signaling as a common early response prior to caspase activation (44). Rapamycin, a macrolide immunosuppressant that is a specific inhibitor of mTOR, represses p70S6K, prevents phosphorylation of Ser136 on BAD, and
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blocks cell survival induced by IGFI. IGFl-induced phosphorylation of BAD Ser398 is abolished in p70S6K-deficient cells (45). In addition, rapamycin induces apoptosis in different cell types (46–49). Overall, the results of these studies strongly support a role for p70S6K in promoting cell survival.

Based on the results of the studies described above, we hypothesize that insulin promotes retinal neuronal cell survival in a p70S6K-dependent manner. In the present study, the rat retinal neuronal cell line R28 was used as an in vitro model to examine this hypothesis. We found that insulin promotes phosphorylation and activation of p70S6K and exogenous expression of wild type S6K1 decreased pyknotic cell numbers in serum-starved cells and insulin further decreased apoptosis in such cells. Rapamycin did not block the insulin cell survival effect in R28 cells expressing a rapamycin-resistant p70S6K, indicating that insulin promotes R28 cell survival in a p70S6K-dependent manner. These data suggest that p70S6K phosphorylation and activity may contribute to retinal cell survival in vivo.

EXPERIMENTAL PROCEDURES

Plasmids—S6K1 constructs were a kind gift from Dr. John Blenis (Department of Cell Biology, Harvard Medical School, Boston) (50, 51). The constructs include control vector pRK7, wild type construct HA-p70S6KI-pRK7, kinase inactive construct HA-p70S6KI-FL5A, and C-terminal truncated construct HA-p70S6KI-E389-ΔCT. Detailed primary sequence alignment has been previously reported (52). Plasmids were prepared using a Qiagen EndoFree Plasmid Kit.

Cell Culture—R28 cells were a generous gift from Dr. Gail M. Seigel, State University of New York, Buffalo (53). They were grown in Dulbecco’s modified Eagle’s medium (DMEM) containing 5% newborn calf serum (Hyclone). They were also cultured in DMEM supplemented with 10% newborn bovine serum.

Transfection—R28 cells were seeded 24 h before transfection at 3 x 10^4/g/dish (60-mm dishes) of serum-starved cells and insulin further decreased apoptosis in such cells. Rapamycin did not block the insulin cell survival effect in R28 cells expressing a rapamycin-resistant p70S6K, indicating that insulin promotes R28 cell survival in a p70S6K-dependent manner. These data suggest that p70S6K phosphorylation and activity may contribute to retinal cell survival in vivo.

Insulin Induces Phosphorylation/Activation of p70S6K Kinase in a Time-Dependent Manner in R28 Rat Retinal Neuronal Cells—Insulin induction of P70S6K phosphorylation has been demonstrated in several types of cells and tissues, but the response has not been previously studied in retinal cells. Previous studies from this laboratory (3) showed that insulin induces phosphorylation of Akt in R28 cells through PI3K-mediated signaling, and that 10 nM insulin activates the insulin receptor but not the IGF-I receptor. Because P70S6K acts downstream of PI3K, we asked if insulin activates the insulin receptor in R28 cells. To this end, we first sought to demonstrate an effect of insulin on p70S6K. Serum-deprived cells were incubated in medium containing 10 nM insulin for time periods ranging from 15 min to 24 h. The results show that insulin induced phosphorylation of p70S6K within 15 min, and the effect was maintained for at least 4 h (Fig. 1A). A similar time course for changes in phosphorylation was also observed for Akt (Fig. 1A). After 24 h, both Akt and P70S6K phosphorylation returned to basal values. Insulin also stimu-
lated S6 kinase activity by ~3-fold (p < 0.01) within 15 min as measured in extracts of insulin-treated compared with control cells (Fig. 1D). These data indicate that insulin can induce phosphorylation and activation of p70S6K in rat retinal neuronal cells, as has been observed for other types of cells.

Insulin Induces Phosphorylation of p70S6K through the PI3K/Akt pathway in R28 Cells—We next asked if the activation of p70S6K by insulin occurs through the PI3K/Akt pathway, so the PI3K inhibitor LY294002 and the mTOR inhibitor rapamycin were employed. As shown in Fig. 2, both LY294002 and rapamycin completely inhibited insulin-induced p70S6K phosphorylation. In contrast, LY294002 inhibited insulin-induced phosphorylation of Akt, but rapamycin did not (Fig. 2). To determine if insulin induces mTOR phosphorylation in R28 cells, cells were treated with either the PI3K inhibitor LY294002 or the mTOR inhibitor rapamycin prior to analysis.
of mTOR phosphorylation on Ser2448. As shown in Fig. 3, insulin increased mTOR phosphorylation by 2.5-fold (p < 0.01) in R28 cells, and both LY294002 and rapamycin completely inhibited insulin-induced mTOR phosphorylation. Together, these data suggest that insulin induces phosphorylation of mTOR through the PI3K pathway, and that insulin activates p70S6K via PI3K/Akt/mTOR in retinal neurons.

**Rapamycin Blocks the Survival Effect of Insulin in R28 Cells**—Previous studies have suggested that insulin protects R28 cells from apoptosis through a PI3K/Akt mediated mechanism (3). As a downstream effector of Akt, p70S6K may be involved in the cell survival effect of insulin. This idea is supported by the results of several studies showing that rapamycin is a proapoptotic agent (39, 47, 49), but its role in retinal cells is unknown. Here we investigated whether or not rapamycin affects the ability of insulin to promote cell survival in R28 cells. Differentiated R28 cells were incubated in serum free medium in the presence or absence of insulin (10 nM) or rapamycin for 1 h, followed by 15 min insulin treatment. Control, INS, insulin; LY, LY294002; RAP, rapamycin. Data shown are mean ± S.E. of three independent experiments (*, p < 0.01).

To address this question, variants of p70S6K were introduced into R28 cells by cDNA transfection. The cDNA used encoded the wild type enzyme HA-p70S6K1, a kinase-inactive variant HA-p70S6K1-F5/A, a C-terminally truncated variant HA-p70S6K1-E389-ΔCT, which is rapamycin-resistant, and the empty vector, pRK7. For each construct three different conditions were examined: control, insulin, and rapamycin + insulin. 24 h after transfection, cell culture medium was replaced with medium lacking serum with or without insulin or rapamycin for additional 24 h. As expected, the percentage of pyknotic cell numbers of control vector transfection in different conditions is similar to those in Fig. 4B. Counting of pyknotic cells that were also HA-positive showed that wild type S6K1 transfection reduced the percentage of pyknotic cell number as compared with control vector pRK7 transfection (Fig. 5, A and D). Insulin treatment further significantly reduced the pyknotic cell number to 25% of control (p < 0.001), and the effect of the hormone was completely blocked by rapamycin. In contrast, in cells transfected with the kinase inactive HA-p70S6K1-F5/A variant, insulin did not affect HA-positive pyknotic cell number (Fig. 5, B and D). Rapamycin failed to block the cell survival effect of insulin in HA-p70S6K1-E389-ΔCT-transfected cells, strongly indicating that insulin promotes R28 cell survival in a rapamycin-sensitive and p70S6K-dependent manner. The percentage of pyknotic cells was equivalent among different transfections (Fig. 5D). We conclude therefore that insulin specifically promotes retinal cell survival in a rapamycin-sensitive and p70S6K-dependent manner.

**Insulin Induces Phosphorylation/Activation of both Endogenous and Transfected p70S6K in R28 Cells**—Among the 8 phosphorylation sites in p70S6K, Thr346 and Thr389 are vital for rapamycin-sensitive and p70S6K-dependent manner. The percentage of pyknotic cells was equivalent among different transfections (Fig. 5D). We conclude therefore that insulin specifically promotes retinal cell survival in a rapamycin-sensitive and p70S6K-dependent manner.

**Insulin Promotes R28 Cell Survival in a p70S6K-dependent Manner**—Recent evidence suggests p70S6K is involved in suppressing apoptosis in various cell types (39, 45, 55, 56). We then asked if the cell survival effect of insulin is p70S6K-dependent.
Fig. 4. Rapamycin blocks survival effect of insulin in R28 cells but not L6 cells. Cultures of R28 and L6 cells were supplemented with DMEM containing 10% new born calf serum for 24 h followed by 24 h of serum starvation in medium supplemented with insulin (10 nM) or rapamycin (10 nM). Serum-free and serum-containing cultures supplemented with or without rapamycin served as controls. R28 and L6 cells were then fixed in 1% paraformaldehyde and stained with Hoechst dye. Pyknotic cell number is expressed as percentage of total cells. A, R28 Hoechst staining results. B, R28 pyknotic cell number. C, L6 Hoechst staining results. D, L6 pyknotic cell number. Pyknotic cells are denoted by arrows (↑). Data shown are mean ± S.E. of three independent experiments (*, p < 0.01; **, p < 0.001; NS, p > 0.05).
FIG. 5. Insulin promotes R28 rat retinalneuronal cellsurvival in a p70S6K-dependent manner. R28 cells were transiently transfected with pRK7, HA-p70S6K1, HA-p70S6K1-F5A, and HA-p70S6K1-E389-ΔCT for 24 h. Cells were deprived of serum for 24 h in medium supplemented with or without insulin (10 nM) or rapamycin (10 nM). Cells were then fixed in 1% paraformaldehyde, stained with anti-HA antibody (1:200) and Hoechst dye (1:2000), and percentage of pyknotic cells in HA-positive cells was counted. A, HA immunocytochemistry and Hoechst staining for pRK7-transfected R28 cells. B, HA immunocytochemistry and Hoechst staining for HA-p70S6K1-transfected R28 cells. C, HA immunocytochemistry and Hoechst staining for HA-p70S6K1-E389-ΔCT-transfected R28 cells. D, pyknotic cell counting for HA-positive cells (*, p < 0.05; **, p < 0.001; NS, p > 0.05). HA-positive cells are denoted by yellow arrow. Pyknotic cells are denoted by orange arrow. Data shown are mean ± S.E. of three independent experiments.
Mammalian retinas exhibit high level of expression of cell surface receptors for a variety of growth-promoting hormones including insulin (57–60) and IGF-I (61–63). The purpose of this study was to determine the effects of insulin on p70S6K activity and its role in retinal cell survival. We show here for the first time that p70S6K in rat retinal neuronal cells is phosphorylated and activated in response to insulin via the PI3K/Akt/mTOR signal transduction pathway, and that p70S6K mediates nearly all of the insulin-mediated survival effects, similar to reports on skeletal muscle (64), liver (65), kidney (66), and myocardium (67). These studies are important because they provide further evidence that the retina is sensitive to the anabolic effects of insulin (8).

P70S6K is a protein synthesis regulator that mediates the effects of hormones on global and selective patterns of mRNA translation (37). The results of the present study demonstrate that p70S6K is also involved in rat retinal neuronal cell survival. When R28 cells were pretreated with rapamycin, insulin did not rescue serum-starved R28 cells from apoptosis. In contrast, rapamycin had no effect on serum-mediated cell survival, suggesting a specific role of p70S6K in the insulin-mediated cell survival effect. p70S6K also appears to exert a cell-specific survival effect in response to insulin because rapamycin did not block the survival effect of insulin in L6 myocytes. The cause for this difference is not known currently. Wild type S6K1 transfection resulted in a high basal level of phosphorylation and activity in the absence of insulin. With insulin stimulation, wild type p70S6K phosphorylation and activity were further increased. These results are consistent with those of other studies in 293 cells (68). Counting of pyknotic cells in HA-positive cells showed a reduced number of pyknotic cells in both control and insulin stimulated conditions, but rapamycin blocked the anti-apoptotic effect of insulin. The other evidence that also strongly supports the hypothesis comes from cell transfected with a rapamycin-resistant construct, HA-F5A- E389-CT. The p70S6K variant encoded by this construct contains a Thr to Glu mutation at residue 389 and a truncation of the C terminus. Among the many phosphorylation sites in S6K1, Thr389 contributes in large part to S6K1 activation and rapamycin sensitivity. When this site is mutated, the kinase becomes rapamycin-resistant (51).

Phosphorylation of the C-terminal autoinhibitory domain of p70S6K resulting in an increased activity. This is achieved through phosphorylation of Thr389 and Thr229, which is a critical step in the activation of p70S6K. Insulin induces both endogenous and exogenous p70S6K phosphorylation and activity in R28 cells. R28 cells were transiently transfected with pRK7, HA-S6K1, HA-F5A-KR, or HA-F5A-E389-CT. 24 h after transfection, cells were deprived of serum for 2 h and pretreated with rapamycin for 30 min. Cells were then stimulated with or without insulin for 15 min (Western blot) or 30 min (S6 kinase assay), and harvested with IP buffer. A, cell lysates were separated on 10% SDS-polyacrylamide gels, transferred to nitrocellulose membranes, and blotted with phospho-p70S6K (Thr389 or Thr229) or total p70S6K antibodies. HA immunoblotting was also applied to analyze p70S6K protein expression. Both endogenous and transfected p70S6K phosphorylation can be seen in HA-S6K1 transfection and HA-F5A-E389-CT transfection. Only endogenous p70S6K phosphorylation is seen in control vector pRK7 and kinase-inactive construct HA-F5A-KR transfections. C, control; I, insulin; R, rapamycin (10 nM). B, cell lysates were immunoprecipitated with anti-HA polyclonal antibody bound to protein A-Sepharose beads followed by p70S6K kinase assay. The results are mean ± S.E. of three independent experiments (*, p < 0.05; **, p < 0.01; NS, p > 0.05).
but not sufficient for maximum activation of p70S6K. Transfection of this construct into R28 cells resulted in increased basal phosphorylation and activation of p70S6K, and this was further increased by insulin stimulation. As predicted, rapamycin did not block insulin activation of the HA-F5A-E389-ΔCT variant. We noticed that both the phosphorylation and the activity of the HA-F5A-E389-ΔCT variant were less than that of exogenously expressed wild type p70S6K. A possible explanation for the reduced activity may be that C-terminal truncation removes several phosphorylation sites that may play a role in activation of p70S6K. This result differs from that reported by Schalm and Blenis (51), and the discrepancy may be because of the different cell types used in the two studies. Accordingly, counting of pyknotic cells among those that are also HA-positive showed that in all three conditions, including cells transfected with the rapamycin resistant construct and pretreated with rapamycin, cell death was reduced in HA-F5A-E389-ΔCT expressing R28 cells. This finding and results obtained in cells exogenously expressing wild type p70S6K are of particular importance and are strong evidence that support S6K1 as an important cell survival factor in retinal neurons. Previous studies have showed indirect evidence for a role for S6K1 in cell survival (43–49), but, to the best of our knowledge, this is the first study to demonstrate directly that activation of S6K1 modulates retinal neuronal cell survival.

The specific mechanism by which p70S6K supports insulin-stimulated retinal cell survival remains uncertain. Recent work by Holcik et al. (69) suggests that internal ribosomal initiation of mRNA translation, a step that is affected by rapamycin and p70S6K, is critical for survival of cells under transient apoptotic stress. Postmitotic retinal neurons require protein synthesis for survival (70), so it is possible that insulin stimulates protein synthesis via a rapamycin-dependent mechanism. Indeed, the same concentration of rapamycin used in this study completely blocks insulin stimulated protein synthesis in R28 cells. In addition, rapamycin partially blocks insulin-induced Foxo1 phosphorylation and translocation from nucleus to the cytosol, and co-transfection of wild type S6K1 and Foxo1 causes Foxo1 translocation to the cytosol3 suggesting a potential mechanism for S6K1-mediated cell survival in retinal neuronal cells.

The physiologic significance of these in vitro studies remains to be determined but p70S6K in whole retinas also responds to insulin stimulation, so it is likely that p70S6K activity also supports retina cell survival and protein synthesis in vivo. Studies are in progress to examine this question and the role of p70S6K activity in diabetic retinopathy.

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