IGF-I inhibits apoptosis through the activation of the phosphatidylinositol 3-kinase/Akt pathway in pituitary cells

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Abstract

In previous studies we demonstrated that IGF-I induces proliferation of pituitary lactotrophs. In addition to its mitotrophic actions, IGF-I is known to prevent apoptosis induced by diverse stimuli in several cell types. In this study, we investigated the action of IGF-I on pituitary cell survival and the intracellular signaling transduction pathway implicated in this effect. Treatment of cultured male rat pituitary cells with IGF-I (10⁻⁷ M) for 24 h prevented pituitary cell death induced by serum deprivation. The protective effect of IGF-I was blocked by phosphoinositide 3-kinase (PI3-kinase) inhibitor, LY294002, but was unaffected by PD98059, which inhibits MAP/ERK kinase (MEK1). IGF-I activation of PI3-kinase induced the phosphorylation and activation of the serine/threonine kinase Akt. Moreover, IGF-I increased the phosphorylation of the pro-apoptotic factor Bad and the levels of the anti-apoptotic protein Bcl-2 through the PI3-kinase pathway in primary pituitary cells.

Journal of Molecular Endocrinology (2004) 33, 155–163

Introduction

Insulin-like growth factor-I (IGF-I) is known to support growth and to prevent apoptosis in many cell types (LeRoith et al. 1995). The positive effect of IGF-I on cell survival has been described in several systems, using various apoptotic stimuli (Butt et al. 1999).

The actions of IGF-I are mediated through a tyrosine kinase receptor that leads to the activation of phosphoinositide 3-kinase (PI3-kinase) and the mitogen-activated protein kinase (MAPK) (LeRoith et al. 1995). Several studies have demonstrated that IGF-I induces cell survival via activation of the PI3-kinase pathway (Párrizas et al. 1997, Gagnon et al. 2001). Akt, a target of PI3-kinase, partially inhibits apoptosis (Kulik et al. 1997) by phosphorylating and inactivating the pro-apoptotic Bcl-2 member, Bad, at Ser136, blocking the Bad-induced death (Datta et al. 1997, 1999). Phosphorylated Bad is sequestered by 14–3–3 protein, leading to its down-regulation. In addition, the PI3-kinase/Akt pathway also increases the levels of anti-apoptotic proteins including Bcl-2 and Bcl-xL (Chrysis et al. 2001). The protective effect of IGF-I in PC12 cells has been associated both with an increase of Bcl-xL mRNA and protein levels (Párrizas & LeRoith 1997), and with the activation of Bcl-2 expression at the transcriptional level (Pugazhenthi et al. 2000). Alternate IGF-I-dependent survival signaling pathways, including the Ras/MAPK cascade and p38 MAPK, have also been proposed (Párrizas et al. 1997, Kulik & Weber 1998). However, current data suggest that IGF-I-mediated MAPK-dependent survival may predominate only when the PI3-kinase pathway is damaged (Peruzzi et al. 1999).

IGF-I has been implicated in the regulation of pituitary hormones synthesis and release (Yamashita & Melmed 1986, Lara et al. 1994). In mice with disrupted IGF-I gene, structural and functional alterations occur in somatotrophs and lactotrophs (Stefaneanu et al. 1999). Pituitary secretory cells, which play a key role in hormone
homeostasis, require trophic support for survival in culture. In previous studies we demonstrated that IGF-I stimulates proliferation of cultured rat pituitary lactotroph cells via activation of the MAPK pathway (Fernández et al. 2003). While the effectiveness of IGF-I on inhibition of apoptosis in many cell types (LeRoith et al. 1995) is well established, the effect of IGF-I on primary pituitary cell survival, the signaling pathways leading to apoptosis, and the mechanisms of action by which IGF-I and other agents prevent apoptosis are largely unknown.

Therefore, the aim of this study was to determine whether IGF-I is capable of protecting primary pituitary cells from apoptosis induced by serum deprivation, which is an established model for analyzing apoptotic pathways (Kennedy et al. 1997), and to investigate the mechanisms activated by IGF-I and their role in pituitary cell survival. We have studied the implication of the PI3-kinase/Akt pathway in the protective effect of IGF-I and whether IGF-I may induce Bad phosphorylation. The contribution of the IGF-I-induced PI3-kinase/Akt pathway in increasing the levels of the anti-apoptotic protein Bcl-2 was also examined.

Materials and methods

Reagents

Recombinant human IGF-I (rhIGF-I) was purchased from PreproTech EC (London, UK). All antibodies were purchased from Dako (Glostrup, Denmark) unless otherwise specified. Poly-L-ornithine was purchased from Sigma (St Louis, MO, USA). The chemical inhibitors PD98059 and LY294002 were obtained from Alexis Corp. (San Diego, CA, USA).

Buffers and media

Dulbecco’s modified Eagle’s medium (DMEM), fetal calf serum (FCS), Hank’s balanced salt solution (HBSS), phosphate-buffered saline (PBS), penicillin–streptomycin, and L-glutamine were purchased from Bio Whittaker (Walkersville, MD, USA).

Defined medium consisted of DMEM (glucose 1 g/l) supplemented with: BSA 1%, Hepes (15 mM), hydrocortisone (0·1 µM), triiodothyronine (T3; 0·5 nM), transferrin (10 µM), glucagon (10 nM) and parathyroid hormone (PTH; 0·2 nM) from Sigma, L-glutamine (4 mM) and penicillin–streptomycin (100 U/ml).

Cell culture

Preparation of primary rat anterior pituitary cell cultures was carried out as previously described (Cacicedo & Sánchez-Franco 1986). Two-month-old male Sprague–Dawley rats were supplied by Charles River (Barcelona, Spain). Animal handling was conducted in accordance with the guidelines on protection of animals used in scientific research set by Real Decreto 223/1988 14 March and Orden 13 October 1989. The animals were killed by decapitation and their pituitary glands were removed under sterile conditions. The neurohypophyses were discarded and the anterior pituitaries collected and mechanico-enzymatically dispersed with 0·1% papain, 0·1% neutral protease and 0·1% DNase for 1 h at 37 °C. The dispersed pituitary cells were re-suspended in defined medium containing FCS (3%) and were plated on poly-L-ornithine-coated 35 mm tissue culture dishes and seeded at a density of 5 × 10^3 cells per dish. Cultures were kept in a humidified atmosphere of 5% CO_2:95% air at 37 °C.

For cell survival studies, cultures were kept for 72 h in serum-supplemented defined medium. Then a group of plates (t=0) were removed and cells counted in duplicate using a Neubauer chamber. Another group of plates was cultured in serum-free medium (control) or IGF-I (10^-7 M) alone or in the presence of the PI3-kinase inhibitor, LY294002 (20 µM), or the MAP/ERK kinase inhibitor, PD98059 (10 µM). After 24 h cells were counted and cell viability was assessed by the trypan blue dye exclusion. Results are expressed as the percentage of viable adherent cells relative to serum-fed controls (t=0).

Cell death assessment

Cell death detection assay was undertaken according to the manufacturer’s instructions (Roche Applied Science, Barcelona, Spain). Fluorescein-16-deoxy-UTP was used and TUNEL signal was visualized with a fluorescent microscope (Nikon, Eclipse 400, Madrid, Spain). Anterior pituitary cells were plated on poly-L-ornithine-coated glass coverslips in 24-well culture plates and
incubated as described above. Cultures were kept for 72 h in serum supplemented medium. Thereafter a group of coverslips ($n=4$) ($t=0$) was removed and cells were fixed. Another group of cells was cultured in serum-free medium ($n=20$) or IGF-I ($10^{-7}$ M) ($n=20$). After 3, 6, 12 ($t=12$), 18 and 24 h cells (eight coverslips/experimental time period) were fixed, cell death was assessed by TUNEL. Nuclei were counterstained with 4-6-Diamidino-2-phenyindole, dilactate (DAPI). The number of TUNEL-positive cells per coverslip was scored by counting all TUNEL-positive cells in every coverslip. Results express the percentage of TUNEL over DAPI-positive nuclei relative to serum-fed controls ($t=0$).

**Experimental design**

Signaling pathway studies were determined in anterior pituitary cells by quantitation of phosphorylated forms of Akt (Ser473) and Bad (Ser136). Pituitary cells were plated on poly-l-ornithine-coated 35 mm tissue culture dishes as described above. After 72 h of incubation in serum-supplemented medium, cells were cultured in defined medium (serum free) for 24 h. Then, the medium was replaced by defined medium containing IGF-I ($10^{-7}$ M) for the times indicated in the figures. The levels of phosphorylated Akt and Bad were quantitated by Western immunoblotting as described below.

The anti-apoptotic action of IGF-I was determined by quantitation of Bcl-2. Pituitary cells were incubated as described above, and cells were then exposed to defined medium containing IGF-I ($10^{-7}$ M) for 24 or 48 h. Bcl-2 protein levels were determined by Western immunoblotting as described below.

To determine the signaling pathways involved in the action of IGF-I on Akt activation and the levels of the anti-apoptotic protein Bcl-2, cells were pretreated with the PI3-kinase inhibitor, LY294002 (20 µM) for 45 min prior to the addition of IGF-I. Cells were then incubated for the times indicated in the figures. LY294002 was dissolved with methanol at a concentration of 3·2 mM and was diluted immediately before use.

**Western immunoblots**

Anterior pituitary cells, growing in 35 mm dishes, were lysed in a buffer containing 0·1 M NaCl, 0·01 M Tris–HCl pH 7·6, 0·001 M EDTA, 0·1% NP-40, 1 µg/ml aprotinin and 100 µg/ml phenyl-methylsulfonyl fluoride (PMSF). Equal amounts of protein extracts (40 µg) from pools of three samples were resolved by SDS-PAGE and transferred to a PVDF membrane. After blocking the membranes, immunodetection was performed using antibodies that recognize phosphorylated forms of Akt (Ser473) (1:350 dilution, New England Biolabs, Ozyme, France) and Bad (Ser136) (1:500 dilution, Cell Signaling Technology, Beverly, MA, USA), and Bcl-2 antibody (1:10 000 dilution, St Cruz Biotechnology, CA, USA), followed by incubation with a goat peroxidase-conjugated anti-mouse or anti-rabbit secondary antibodies (Dako). Membranes were re-probed with phosphorylation state-independent antibodies, Akt (New England Biolabs) and Bad (Cell Signaling Technology). For Bcl-2, the membranes were systematically treated with Coomassie brilliant blue R-250 solution (0·125%), as a loading control.

**Statistical analysis**

Statistical analysis was performed using pooled data from three independent experiments. Triplicate wells were tested within each experiment. All data are expressed as the means ± s.e. Tests for significance between sample groups were performed with a two-tailed $t$-test. For multiple comparisons, ANOVA was used with the Fisher test for post hoc comparisons. Differences were considered statistically significant if $P<0·05$.

**Results**

**IGF-I reverses apoptosis in pituitary cells through activation of the PI3-kinase pathway**

The ability of IGF-I to protect pituitary cells from apoptosis was studied by incubating the cells in starvation medium with IGF-I. Pituitary cells placed in starvation medium for 24 h reduced by 41% the number of viable cells ($t=0$, 100%; $t=24$ h, 59 ± 7·8%) ($P<0·05$). Addition of IGF-I ($10^{-7}$ M) to the starvation medium for 24 h prevented cell death ($t=24$ h, 101 ± 3%) (Fig. 1). TUNEL labeling was increased in rat pituitary cells placed in starvation medium for 12, 18 and 24 h. A maximum effect was observed at 12 h ($t=0$, 100%; $t=12$, 281·05 ± 3·75%, $P<0·01$). The addition of
IGF-I (10^{-7} \text{ M}) to the starvation medium rescued rat pituitary cells from apoptosis (t=0, 100%; IGF-I 12 h, 69.66 ± 14.91%, NS). Pituitary cells cultured in starvation medium showed a high proportion of nuclei of small size, round in shape and stained brightly with DAPI (data not shown). This dose of IGF-I was shown to be optimal for the proliferation of pituitary cells (Fernández et al. 2003). These data indicate that IGF-I is a survival factor for primary pituitary cells.

PI3-kinase and MAPK have been proven to be involved in the IGF-I anti-apoptotic effect in PC12 (Butt et al. 1999). To identify whether these signals are important for the protective action of IGF-I in pituitary cells, cells were incubated in starvation medium for 24 h, in the presence or absence of IGF-I in combination with a variety of specific inhibitors. The anti-apoptotic properties of IGF-I were completely blocked after addition of 20 \mu M LY294002, an inhibitor of PI3-kinase (t=24 h, 55 ± 1.2% \text{ (P}<0.05\text{ compared with IGF-I treatment alone)}. However, 10 \mu M PD98059, an inhibitor of MEK1, had no significant effect on IGF-I-dependent survival in pituitary cells (t=24 h, 88 ± 2.1%). These inhibitors alone, in the absence of IGF-I, had no significant effect on the survival of pituitary cells.

IGF-I stimulates Akt activation in pituitary cells

Akt has been shown to participate in growth factor-induced cell survival against several apoptotic stimuli (Kulik et al. 1997). To investigate the role of IGF-I on Akt activation, dissociated rat anterior pituitary cells were incubated with IGF-I (10^{-7} \text{ M}) for varying times. The levels of phosphorylated Akt were examined using an antibody that recognizes phospho-Ser473 Akt. Treatment of pituitary cells with IGF-I resulted in the rapid and sustained phosphorylation of Akt, which is indicative of its activity. Maximal phosphorylation (tenfold) was observed at 2 min and maintained for 60 min after IGF-I treatment. Phosphorylation remained elevated for 180 min (P<0.01) (Fig. 2). To determine whether the effect of IGF-I on Akt phosphorylation involved the
PI3-kinase, cells were pretreated with the PI3-kinase inhibitor, LY294002 (20 µM). As shown in Fig. 3, the effect of IGF-I on Akt activation was completely abolished \( (P<0.01) \) by LY294002. These results indicate that IGF-I induces Akt phosphorylation via activation of the PI3-kinase signaling pathway.

**IGF-I phosphorylates Bad in pituitary cells**

Extracellular factors may promote cell survival by stimulating the phosphorylation of Bad, which reduces Bad–Bcl-2 heterodimer formation and thereby potentiates the effects of anti-apoptotic mediators such as Bcl-2. To investigate the role of IGF-I on Bad phosphorylation, rat anterior pituitary cells were incubated with IGF-I \((10^{-7} \text{ M})\) for varying times. The levels of phosphorylated Bad were examined using an antibody that recognizes phospho-Ser136 Bad. As shown in Fig. 4, treatment with IGF-I resulted in the rapid and transient phosphorylation of Bad. Maximum levels (fourfold) were observed at 5 and 10 min after IGF-I treatment, declining to basal levels at 60 min. These results suggest that IGF-I induces Ser136 phosphorylation of Bad in primary pituitary cells.

**IGF-I increases the anti-apoptotic protein Bcl-2 via the PI3-kinase pathway in primary pituitary cells**

Up-regulation of Bcl-2 expression has been identified as a critical mechanism by which growth factors promote cell survival (Singleton et al. 1996, Pugazhenti et al. 2000). The ability of IGF-I to induce Bcl-2 protein levels was studied by incubating the pituitary cells with varying concentrations of IGF-I. IGF-I was effective in inducing Bcl-2 protein in both a time- and a dose-dependent fashion. The effect of IGF-I was detected at 24 h and at a concentration of \(10^{-7} \text{ M}\), with maximal effects seen at 48 h. Lower concentrations of IGF-I \((10^{-8} \text{ M})\) were not effective in inducing Bcl-2 protein (Fig. 5). To determine whether the effect of IGF-I on the induction of Bcl-2 in pituitary cells
involved the PI3-kinase pathway, cells were pretreated for 45 min with LY294002 (20 µM). As shown in Fig. 6, the effect of IGF-I (10^{-7} M) on Bcl-2 expression was completely abolished by the PI3-kinase inhibitor (P<0.01). These results support the conclusion that IGF-I induces Bcl-2 protein levels and that the PI3-kinase/Akt pathway plays a role in mediating this effect.

**Discussion**

We have identified IGF-I as a survival factor for primary pituitary cells and characterized its intracellular signaling targets. IGF-I protects pituitary cells from apoptosis, induced by serum deprivation, by a mechanism dependent on the activation of PI3-kinase. Furthermore, we show that IGF-I induces the phosphorylation of the pro-apoptotic factor Bad, and increases the levels of the anti-apoptotic protein Bcl-2 via the PI3-kinase/Akt signaling pathway.

We found that IGF-I prevented the loss of pituitary cells induced by serum withdrawal. This could reflect actions of IGF-I on cell proliferation as we have previously demonstrated that IGF-I induces proliferation of pituitary cells (Fernández et al. 2003). However the proliferative action of IGF-I was effective as from 48 h, but was not evident at 24 h, the time at which the survival effects of IGF-I were observed. Furthermore, the TUNEL assay confirmed that the addition of IGF-I to the starvation medium rescued rat pituitary cells from apoptosis. Thus the present results support the conclusion that IGF-I promotes survival of primary pituitary cells and confirm recent evidence which has demonstrated that the signaling through the IGF-I system plays a powerful role in cell survival and prevention of programmed cell death in various cell types (LeRoith et al. 1995).

The identity of the cell types within the pituitary that IGF-I protects from apoptosis, induced by serum deprivation, is not entirely clear. IGF-I receptor mRNA is abundantly and homogeneously distributed throughout the anterior pituitary and intermediate lobes (Bach & Bondy 1992). Immunocytochemically, IGF-I receptors have been shown to be present in gonadotrophs (Unger & Lange 1997), corticotrophs and somatotrophs (Takahashi et al. 1997). The anti-apoptotic actions of IGF-I on pituitary cells have been previously reported using primary tilapia pituitary cell cultures (Melamed et al. 1999). A very recent report (Arroba et al. 2003) demonstrates that lactotrophs were TUNEL positive.
labeled in pituitaries of diabetic rats which have low insulin and peripheral IGF-I. Our data suggest the possibility that survival of various cell types, including lactotrophs, may be regulated by IGF-I.

Survival signaling through the IGF-I receptor is dependent on the kinase activity of the receptor. PI3-kinase is a major effector for signaling by IGF-I receptor. We have demonstrated that the activation of the PI3-kinase/Akt pathway seems to be required for IGF-I-dependent pituitary cell survival. We found that the ability of IGF-I to protect cells from apoptosis was fully blocked by LY294002. Thus activation of PI3-kinase is necessary for protecting primary pituitary cells from apoptosis. The involvement of the MAPK pathway in the IGF-I dependent pituitary cell survival was tested using PD98059 and we found that MAPK signaling was not required for IGF-I-dependent pituitary cell survival. This pathway of IGF-I signaling has been closely related with cell differentiation, migration and proliferation (Fernández et al. 2003), but in some cases it can also regulate the machinery of apoptosis (Párrizas et al. 1997).

We considered the possibility that the effects of IGF-I on cell survival were mediated by the protein kinase Akt, a downstream effector for PI3-kinase, that is activated by a number of growth factors, including insulin, through a PI3-kinase-dependent mechanism (Cross et al. 1995). Our results indicate that IGF-I induces a rapid and sustained stimulation of the Akt phosphorylation, suggesting that Akt may play a critical role in promoting IGF-I-dependent survival in primary pituitary cells. As previously shown (Yao & Cooper 1995), we confirmed that activation of Akt is blocked by the PI3-kinase inhibitor LY294002, which indicates that this activation is dependent on PI3-kinase.

The mechanism by which PI3-kinase/Akt offers cytoprotection remains unclear. Several factors involved in apoptosis and cellular survival have been shown to be regulated by Akt (Kennedy et al. 1997, Datta et al. 1999). Thus Akt may use a variety of pathways to promote cell survival (Datta et al. 1997, Khwaja 1999). One mechanism by which Akt may promote survival is through the inhibition of components of the cell death pathway. Our results indicate for the first time that IGF-I induces a transient phosphorylation and inactivation of the pro-apoptotic Bcl-2 family member, Bad, in primary pituitary cells. Previous studies have demonstrated that IGF-I leads to Bad phosphorylation at Ser136 and blocks the Bad-induced death through the PI3-kinase/Akt signaling pathway in several cell types (Vincent & Feldman 2002). It has been proposed that phosphorylation of Bad may lead to the prevention of cell death via a mechanism that involves the selective association of the phosphorylated forms of Bad with 14–3–3 proteins (Zha et al. 1996). The induced association of Bad with 14–3–3 appears to prevent Bad association with Bcl-xL or Bcl-2 (Datta et al. 1997). The transient rise in Bad has been described by other authors using human SH-SY5Y cells exposed to methylpyridinium (MPP) (Halvorsen et al. 2002). The significant reduction in Bad phosphorylation by 30 min observed in the present study cannot be accounted for by reduction in activating phosphorylation of Akt. This paradox suggested the possibility of the appearance of phosphatases capable of dephosphorylating and inactivating Bad (Keyse 2000).

However, these results do not rule out the possibility that Akt promotes cell survival by other mechanisms in addition to that mediated by phosphorylation of Bad. Recent studies also implicate Akt upstream of caspase activation in the apoptosis cascade, regulating mitochondrial membrane integrity and cytochrome c release, independently of Bad phosphorylation (Kennedy et al. 1999). In addition, the activity of Akt also increases the levels of anti-apoptotic proteins including Bcl-2 (Chrysis et al. 2001). Our results indicate that IGF-I increased the levels of Bcl-2 in primary pituitary cells. This finding is in agreement with previous studies, which have demonstrated that IGF-I was able to elevate Bcl-xL mRNA and protein levels in PC12 cells (Párrizas & LeRoith 1997). Bcl-2 is well known as an important factor promoting cell survival in several cell preparations (Reed 1997). Bcl-2 and other anti-apoptotic members of this family such as Bcl-xL partially suppress apoptosis by blocking the release of apoptogenic molecules from mitochondria such as cytochrome c.

Up-regulation of Bcl-2 expression has been identified as a critical mechanism by which growth factors promote cell survival (Singleton et al. 1996, Pugazhenthi et al. 1999). Our results do not show a direct link between the protective effect of IGF-I and its up-regulation of Bcl-2. However previous data showing that Bcl-2 overexpression can protect serum-deprived PC12 cells (Batistatou et al. 1993)
argues for a role of Bcl-2 in the IGF-I-induced survival of primary pituitary cells. The present study also examined whether IGF-I-induced Bcl-2 protein levels occur through the PI3-kinase/Akt pathway. The data shown indicate that IGF-I increased Bcl-2 protein levels via the PI3-kinase/Akt signaling pathway. Previous studies reported that IGF-I-induced Bcl-2 expression involves a signaling cascade mediated by PI3-kinase/PDK1/Akt in PC12 cells (Pugazhenthii et al. 2000).

In summary, we have demonstrated that the PI3-kinase/Akt pathway is required for IGF-I-regulated pituitary cell survival. The inactivation of Bad and the increase of Bcl-2 protein levels suggests that these proteins may be involved in IGF-I-induced pituitary cell survival in a PI3-kinase-dependent manner.

Acknowledgements

We thanks Constanza Navarro for technical assistance, Mary Harper for the preparation of the manuscript and Dr J. Jordan for a critical reading of the manuscript. This work was supported by grants SAF-2001–0016 from the Ministerio de Ciencia y Tecnología, and FIS 01/0127 and P1020720 from the Fondo de Investigaciones Sanitarias.

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In summary, we have demonstrated that the PI3-kinase/Akt pathway is required for IGF-I-regulated pituitary cell survival. The inactivation of Bad and the increase of Bcl-2 protein levels suggests that these proteins may be involved in IGF-I-induced pituitary cell survival in a PI3-kinase-dependent manner.

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Received in final form 14 February 2004
Accepted 23 February 2004
Made available online as an Accepted Preprint