Possible role for protein kinase B in the anti-apoptotic effect of prolactin in rat Nb2 lymphoma cells

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Abstract

Prolactin (PRL) is a mitogen for a number of cell types and its action as a survival factor has recently been demonstrated in Nb2 lymphoma cells. However, the intracellular signalling pathways by which PRL promotes the survival of Nb2 cells is unknown. In previous studies, we have shown that PRL caused the activation of phosphatidylinositol 3-kinase (PI3-kinase) and its association with tyrosine phosphorylated *fyn*. Protein kinase B (PKB), a serine/threonine kinase, is now known to be a downstream component of the PI3-kinase pathway. The aim of the present study was to examine the effect of PRL on the activation of PKB and to find out whether this has any role on the PRL-induced survival of Nb2 cells. Our studies have revealed the phosphorylation and activation of PKB in PRL-stimulated Nb2 cells. We have also observed, using confocal microscopy, translocation of PKB to the membrane of Nb2 cells in response to PRL. These effects

were blocked by the PI3-kinase inhibitor, LY294002 (10 µg/ml).

Apoptosis was induced by the general protein kinase inhibitor, staurosporine (STS; 0.1–1 µM), the synthetic glucocorticoid, dexamethasone (Dex; 100 nM) or ionising radiation by exposing Nb2 cells to X-irradiation (IR; 10 Gy). PRL had no effect on STS-induced apoptosis. On the other hand, PRL (100 ng/ml) inhibited apoptosis induced by Dex or IR; this effect of PRL was reversed by the addition of LY294002 (10 µg/ml). Furthermore, Western blot analysis using phosphospecific PKB antibody on lysates from PRL-treated Nb2 cells showed that phosphorylation of PKB in response to PRL was inhibited by STS (0.5 µM), but not by Dex (100 nM).

These results suggest that the PI3-kinase/PKB pathway may mediate the anti-apoptotic effect of PRL in Nb2 cells.

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Introduction

Prolactin (PRL) is a pleiotrophic hormone having more than 300 reported actions, but the mechanism of action of PRL is only just beginning to be understood (reviewed by Bole-Feyssot *et al.* 1998). Although PRL is a mitogen for several cell types, it is now becoming clear that it also acts as a survival factor in a number of cell types (Travers *et al.* 1996, Ahonen *et al.* 1999, Lee *et al.* 1999). PRL plays an important role in immune cells, increasing the proliferation of most haematopoietic cells and inhibiting apoptosis. The rat Nb2 lymphoma cell line has been a useful model for investigating the action of PRL (Gout *et al.* 1980). Indeed, in Nb2 cells, PRL has been shown to inhibit apoptosis induced by glucocorticoids (Witorsch *et al.* 1993) and to regulate the expression of proteins involved in the regulation of the apoptotic process such as members of the Bcl-2 family proteins (Leff *et al.* 1996, Clevenger *et al.* 1997, Krumenacker *et al.* 1998). A recent report suggested that PRL can block the loss in the mitochondrial

transmembrane potential caused by glucocorticoids (Weimann *et al.* 1999). However, the intracellular signalling pathway by which PRL promotes the survival of Nb2 lymphoma cells is unknown. We have previously shown that PRL activated phosphatidylinositol 3-kinase (PI3-kinase) and enhanced its association with tyrosine phosphorylated *fyn* (Al-Sakkaf *et al.* 1996, 1997) in Nb2 cells. However, the downstream events influenced by PRL-stimulated PI3-kinase remain to be fully elucidated.

Earlier work (Chung *et al.* 1992, Cheatham *et al.* 1994) has characterised at least some of the downstream enzymes of the PI3-kinase pathway. Protein kinase B (PKB), also referred to as RAC-PK and Akt, is a serine/threonine kinase (Bellacosa *et al.* 1991, Coffey & Woodgett 1991, Jones *et al.* 1991) which was also discovered to be downstream of PI3-kinase (Burgering & Coffey 1995, Franke *et al.* 1995). Subsequently, PKB was found to be activated by a serine/threonine kinase, 3-phosphoinositide-dependent kinase (PDK), both translocating to the cell membrane in response to PI3-kinase activation (Cohen

Figure 1 Western analysis of PKB phosphorylation. (a) Nb2 cells were incubated with varying doses of PRL (1–1000 ng/ml) for 15 min and lysed. The lysates were separated by SDS-PAGE. (b) Nb2 cells were incubated with PRL (100 ng/ml) for the times shown and lysed. Lysates were immunoprecipitated with anti-PKB antibody prior to SDS-PAGE and transfer to membranes. Membrane from both studies (a and b) were then immunoblotted (IB) with anti-phosphospecific PKB antibody (p-PKB). (c) The membrane in (b) was stripped and blotted with anti-PKB antibody to confirm efficient immunoprecipitation. The results are representative of two experiments with similar results.

et al. 1997). The PI3-kinase pathway has also been implicated in mediating a variety of growth factor-induced responses, such as inactivation of glycogen synthase kinase-3 (GSK-3) and the stimulation of cell growth. Furthermore, evidence suggests that PKB functions to promote cell survival by actively inhibiting apoptosis (Dudek *et al.* 1997). This effect of PKB has been suggested by the phosphorylation and inactivation of substrates involved in the regulation of apoptosis, such as Bad, a pro-apoptotic member of the Bcl-2 family (Datta *et al.* 1997, del Peso *et al.* 1997), and caspase-9, a cell death protease downstream of cytochrome C release (Cardone *et al.* 1998). It has also been shown to act through the regulation of the activity of transcription factors, such as members of the forkhead family (Paradis & Ruvkun 1998) and nuclear factor-kappa B (NF- κ B) (Ozes *et al.* 1999, Romashkova & Makarov 1999).

Here we report the phosphorylation and activation of PKB in response to PRL which is consequent upon PI3-kinase activation in Nb2 cells. We also demonstrate the anti-apoptotic effect of PRL in Nb2 cells, and the possible role of the PI3-kinase/PKB pathway in mediating this effect.

Materials and Methods

Materials

Ovine PRL (oPRL) was kindly provided by the National Hormone and Pituitary Program, NIDDK (Baltimore, MD, USA). Anti-rat PKB was purchased from UBI (distributed by TCS Biologicals Ltd, Botolph Claydon, Bucks, UK) and anti-phospho PKB antibody was

from New England BioLab Inc. (Beverly, MA, USA). Staurosporine (STS) was purchased from Alexis Biochemical (Nottingham, Notts, UK) and biotin-dUTP was from Boehringer (Mannheim, Germany). DNA polymerase I *E. coli* and all culture media and sera were obtained from Gibco (Paisley, Strathclyde, UK). LY294002 was purchased from BioMol (distributed by Affinity Research Product Ltd, Exeter, Devon, UK) and dexamethasone (Dex) was from Sigma (Poole, Dorset, UK).

Cell culture

Nb2 cells, the PRL-dependent thymocyte subline, were grown in suspension culture at 37 °C under 5% CO₂–95% air in RPMI 1640 supplemented with 10% fetal calf serum, 10% horse serum, 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 60 µM β -mercaptoethanol.

Immunoprecipitation and Western blotting

Nb2 cells (20×10^6), serum-starved for 16–18 h, were stimulated with PRL for the times indicated. The cells were then washed twice in ice-cold phosphate-buffered saline (PBS), and lysed in 1 ml ice-cold lysis buffer (10% PBS, 0.1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 0.2 mM activated sodium orthovanadate and 10 µg/ml of each of the anti-proteases, aprotinin, pepstatin-A and leupeptin). Cell lysates were immunoprecipitated overnight at 4 °C with anti-PKB antibody. Samples were subjected to 10% SDS-PAGE, transferred onto nitrocellulose membranes, probed with either anti-phosphospecific PKB antibody or anti-PKB

Figure 2 A typical autoradiograph showing the effect of PRL and/or LY294002 (LY) on PKB phosphorylation in Nb2 cells. Lysates from control and stimulated cells (for 15 min at 37 °C) were immunoprecipitated with anti-PKB antibody. The immunoprecipitates were incubated with [γ - 32 P]ATP in kinase buffer and the autophosphorylation assay was performed as described in Materials and Methods. Results are representative of three independent experiments.

antibody, and detected using the ECL Western blotting detection system (Amersham International plc, Aylesbury, Bucks, UK).

In vitro autophosphorylation assay

Cells were stimulated and lysed using a lysis buffer (50 mM Tris, pH 7.5, 0.1% Triton X-100, 10 mM β -glycerophosphate, 5 mM $\text{Na}_4\text{P}_2\text{O}_7$, 50 mM NaF, 1 mM Na_3VO_4 , 1 mM EDTA, 1 mM EGTA and 0.1% β -mercaptoethanol) containing microcystin (1 μM), and 10 $\mu\text{g}/\text{ml}$ of each of the anti-proteases, aprotinin, pepstatin-A and leupeptin. Cell lysates were immunoprecipitated with anti-PKB antibody as above.

The immunoprecipitates were incubated for 30 min at room temperature in 20 μl kinase buffer (10 mM HEPES, pH 8, 50 mM NaCl, 5 mM MnCl_2 , 5 mM MgCl_2 and 0.1 mM Na_3VO_4) containing 17 μM protein kinase A inhibitor peptide (PKI) and 0.25 mCi/ml [γ - 32 P]ATP. Samples were then washed four times in lysis buffer and resolved by SDS-PAGE on a 10% gel. Separated proteins were fixed using Coomassie blue and gels were then dried and visualised by autoradiography.

PKB kinase assay

Nb2 cells were stimulated, lysed and immunoprecipitated as above. Samples were incubated for 10 min at 30 °C in 10 μl kinase buffer (20 mM MOPS, pH 7.2, 25 mM β -glycerophosphate, pH 7, activated Na_3VO_4 (1 mM) and 1 mM dithiothreitol) containing 17 μM PKI (from UBI). Crosstide substrate peptide (10 μM ; from UBI) was added to the reaction mixture and the kinase assay was initiated by the addition of 10 μl [γ - 32 P]ATP (10 $\mu\text{Ci}/\text{assay}$) in kinase buffer. The phosphorylated substrate was separated by spotting aliquots of 20 μl of the reaction mixture onto 2.5 cm^2 squares of Whatman P81 chromatography

Figure 3 The effects of PRL and LY294002 (LY) on Crosstide phosphorylation. Nb2 cells were preincubated with or without LY294002 for 30 min at 37 °C before being stimulated with PRL (100 ng/ml) for 30 min. Lysates were immunoprecipitated with anti-PKB antibody and *in vitro* kinase assays were performed using Crosstide as substrate, as described in Materials and Methods. The results shown are the means $\pm$ S.E.M. of three independent experiments. ** $P < 0.005$ compared with basal activity; * $P < 0.05$, ** $P < 0.005$ compared with PRL stimulation (Student's *t*-test).

paper. The papers were washed with 0.75% phosphoric acid and then counted in a β -counter (LKB, Bromma, Sweden).

PKB translocation

Serum-starved Nb2 cells were stimulated with oPRL in the presence or absence of the PI3-kinase inhibitor, LY294002, for 15 min. The cells were then washed twice in ice-cold PBS, fixed in 1% paraformaldehyde and permeabilised with 75% ethanol overnight at -20 °C. Cells were then resuspended in PBS and cytospun. Slides were then incubated with PKB antibody, and detected by anti sheep-FITC-labelled antibody using confocal microscopy.

Determination of apoptosis: in situ nick translation (ISNT)

Treated cells ($1-2 \times 10^6$) were washed in PBS, fixed in 1% paraformaldehyde and permeabilised with 70% ethanol overnight at -20 °C. Cells were then washed in PBS, resuspended in 50 μl 'nick translation' buffer (2.5 mM MgCl_2 , 10 mM β -mercaptoethanol, 50 mM Tris, pH 7.8, 10 $\mu\text{g}/\text{ml}$ bovine serum albumin, 1 U *E. coli* DNA polymerase, 0.2 M biotin-dUTP and 0.2 nM each of unlabelled dATP, dGTP and dCTP) and incubated for 4-6 h at room temperature. Samples were washed and

Figure 4 Confocal microscopy images of cells treated with PRL (100 ng/ml) in the presence or absence of LY294002 (LY) for 15 min at 37 °C (samples were preincubated with LY294002 for 30 min before the addition of PRL). The cells (5×10^4) were harvested, fixed, cytopspun onto a slide and incubated with anti-PKB antibody for 10 min. Cells were then washed and labelled with anti-sheep-FITC conjugates and analysed.

resuspended in 100 µl staining buffer (600 mM NaCl, 60 mM sodium citrate, 2.5 µg/ml avidin-FITC, 0.1% Triton X-100 (w/v) and 5% (w/v) non-fat dry milk) for 30 min in the dark at room temperature. The samples were then washed and measured on a FACScan and analysed using the Cell Quest program (Beckton Dickinson, Oxford, Oxon, UK).

Results

Activation of PKB in PRL-stimulated Nb2 cells

Western blot analysis, using anti-phosphospecific PKB antibody, showed a dose-dependent increase in PKB

phosphorylation of Nb2 cells lysates (50 µg protein) treated with different concentrations of PRL (1–1000 ng/ml) for 15 min at 37 °C, with maximal phosphorylation being achieved with 100 ng PRL/ml (Fig. 1a). Figure 1b shows the time-dependent phosphorylation of PKB, in anti-PKB immunoprecipitates of Nb2 cells, in response to PRL (100 ng/ml). The reason why the anti-phosphospecific PKB antiserum detected two bands in immunoprecipitates but only one in lysates is unclear. This blot was stripped and reprobed with anti-PKB antibody to confirm efficient immunoprecipitation (Fig. 1c).

PKB activity was also measured directly in an *in vitro* kinase assay. Also, as PKB activation was shown to be consequent upon PI3-kinase activation (Burgering & Coffey 1995, Franke *et al.* 1995), PKB kinase activity was

Figure 5 Nb2 cells were treated with either 10 or 100 ng/ml PRL, alone or in combination with 0.1–1 µM STS for 24 h at 37 °C. Apoptosis was measured by the ISNT assay. Results shown are representative of three independent experiments (means $\pm$ S.E.M.).

analysed in the presence of LY294002, a specific PI3-kinase inhibitor (Vlahos *et al.* 1994). Nb2 cells were preincubated with or without LY294002 for 30 min at 37 °C before being stimulated with PRL (100 ng/ml) for 15 min. The results showed that, in anti-PKB immunoprecipitates, PRL stimulated PKB activity and that LY294002 (10 µg/ml) inhibited PKB autophosphorylation stimulated by 100 ng PRL/ml (Fig. 2). The inhibition of PKB by LY294002 confirms that PKB lies downstream of PI3-kinase in a signal transduction cascade initiated by the binding of PRL to its specific receptor on Nb2 cells. These cells also displayed some basal PKB activity which, interestingly, was also inhibited by LY294002.

The protein kinase, GSK-3, has been identified as a likely physiological target for PKB (Cross *et al.* 1995). PKB activity was quantified in anti-PKB immunoprecipitates by measuring the incorporation of 32 P from [γ - 32 P]ATP into Crosstide (GRPRTSSFAEG), a peptide corresponding to the sequence in GSK-3 surrounding the serine phosphorylated by PKB. Figure 3 shows that PRL caused an increase in the phosphorylation of Crosstide and also that LY294002 inhibited this activity in a dose-dependent manner.

As activation of PKB leads to its translocation to the cell membrane (Cohen *et al.* 1997), confocal microscopy was used to determine the effect of PRL on the intracellular location of PKB. The results in Fig. 4 demonstrate the translocation of PKB to the plasma membrane when cells were stimulated with PRL (100 ng/ml) for 15 min, and that this effect was inhibited by LY294002 (10 µg/ml).

Anti-apoptotic effect of PRL

Apoptosis induced in Nb2 cells was measured after 24-h incubation with STS (0.1–1 µM), Dex (100 nM) or

X-irradiation (IR; 10 Gy). The effects of these treatments and the influence of PRL are shown in Figs 5 and 6. STS induced both dose- (Fig. 5) and time- (data not shown) dependent increases in DNA fragmentation in Nb2 cells as measured by the ISNT assay; however, we failed to detect a protective effect with PRL. Nb2 cells had previously been shown to undergo apoptosis induced by Dex and measured by trypan blue exclusion and DNA laddering (LaVoie & Witorch 1995). Using the ISNT assay, it is clear that PRL abrogated the apoptotic effect of Dex, but only partially inhibited the effects of ionising radiation (Fig. 6). To determine whether the PI3-kinase/PKB

Figure 6 Nb2 cells were treated with Dex (100 nM) or exposed to 10 Gy of IR in the presence or absence of PRL (100 ng/ml) with or without LY294002 (10 µg/ml) for 24 h at 37 °C. The cells were harvested, fixed and labelled with biotinylated-dUTP. The labelled cells were then stained with avidin-FITC conjugates and analysed by flow cytometry. Shaded bars, no PRL; solid bars, 100 ng/ml PRL; open bars, 100 ng/ml PRL and 10 µg/ml LY294002. Results shown are representative of three independent experiments (means $\pm$ S.E.M.). ** $P < 0.005$ compared with Dex alone and ++ $P < 0.005$ compared with Dex+PRL. * $P < 0.05$ compared with IR alone and + $P < 0.005$ compared with IR+PRL (Student's *t*-test).

Figure 7 Nb2 cells were treated with PRL (100 ng/ml) in the presence or absence of Dex (100 nM), LY294002 (LY; 10 µg/ml), STS (0.5 µM) or the JAK2 inhibitor AG490 (5 µM) for 15 min at 37 °C. Cell lysates were subjected to 10% SDS-PAGE and probed with anti-phosphospecific PKB antibody. Results shown are representative of two independent experiments.

pathway mediates PRL-induced inhibition of apoptosis, Nb2 cells were incubated with the PI3-kinase inhibitor, LY294002 (10 µg/ml). It is clear from the data presented in Fig. 6 that, in these cells, LY294002 overcame the inhibitory effect of PRL to apoptosis-induced by Dex (100 nM). These findings were also confirmed using DNA fragmentation on agarose gel electrophoresis (data not shown). The effects of PRL and LY294002 on IR cells were also tested using the ISNT assay to detect apoptosis. PRL induced around 23–35% increase in cell viability of IR Nb2 cells as measured by this technique and this effect of PRL on survival was abolished by the addition of LY294002 (10 µg/ml) (Fig. 6). The effects of STS, Dex, LY294002 and the JAK2 inhibitor, AG490, on PKB phosphorylation was also tested. STS, but not Dex, inhibited PRL-stimulated PKB phosphorylation (Fig. 7); Dex alone had no effect on PKB phosphorylation (data not shown) and, as expected, LY294002 blocked PRL-stimulated PKB phosphorylation. Furthermore, AG490 (Meydan *et al.* 1996) had no effect on PRL-stimulated PKB phosphorylation, confirming the data reported previously by this laboratory of the lack of association between JAK2 and PI3-kinase in the response to PRL (Al-Sakkaf *et al.* 1996).

Discussion

Our previous studies have shown a relationship between the relatively rapid stimulation of PI3-kinase activity and more distal PRL-mediated effects, such as mitogenesis (Al-Sakkaf *et al.* 1996). However, although activation of PI3-kinase may be an important mediator of the effects of PRL on Nb2 cells, it may not be sufficient by itself to stimulate Nb2 cellular growth, as other pathways, such as those involving JAK2 receptor-associated tyrosine kinase, appear to be important for PRL receptor-mediated growth (Lebrun *et al.* 1994). The possible role of PRL-stimulated

PI3-kinase and downstream events is therefore important. Our current interest in PKB arose from the reports that PKB lies downstream of PI3-kinase (Burgering & Coffey 1995, Franke *et al.* 1995) and functions to promote cell survival in a number of cell types (Dudek *et al.* 1997).

We now report that PRL stimulated the activation of PKB and that LY294002 inhibited PRL-induced PKB activation (as measured by four separate techniques: by immunoblotting with phosphospecific PKB antisera, auto-phosphorylation, phosphorylation of Crosstide and translocation using confocal microscopy), confirming that activation of PKB is consequent to PI3-kinase activation by PRL. LY294002 is a competitive inhibitor for the ATP-binding site, specific for PI3-kinase and has no inhibitory effect against phosphatidylinositol 4-kinase nor a number of serine/threonine or tyrosine kinases (Vlahos *et al.* 1994). LY294002 has also been shown to block PI3-kinase activity in different systems including Nb2 cells mitogenesis (Al-Sakkaf *et al.* 1996).

PRL has been shown to inhibit apoptosis induced by glucocorticoids in Nb2 cells and here we confirm that PRL can significantly inhibit apoptosis induced by Dex or IR in Nb2 cells. To evaluate the functional significance of PKB activation by PRL, the effect of LY294002 on the anti-apoptotic action of PRL was examined. LY294002 completely abrogated the inhibitory effect of PRL on apoptosis stimulated by Dex or IR in Nb2 cells, suggesting that the PI3-kinase/PKB pathway may mediate the anti-apoptotic effect of PRL in Nb2 cells. These results are consistent with the induction of apoptosis following inhibition of the PI3-kinase pathway in other cell types stimulated by survival factors such as cytokines (Scheid *et al.* 1995, Ahmed *et al.* 1997). Interestingly, STS, a general protein kinase inhibitor which has also been shown to induce apoptosis in many cell types including Chang liver cells (Swe & Sit 1997) and fibroblasts (Jacobson *et al.* 1996), stimulated apoptosis in Nb2 cells, but this effect was not inhibited by PRL. Since we have

shown that STS inhibited PKB phosphorylation, this suggests that STS exerts its apoptotic action via effects on this survival pathway. Dex-induced apoptosis was not coincident with changes in PKB phosphorylation. Also, the JAK2 inhibitor had no effect on PKB phosphorylation.

In summary, these and previous observations may suggest that PRL can act as an anti-apoptotic and mitogenic factor via the PI3-kinase pathway. The role of PKB in promoting cellular survival has been suggested to involve phosphorylation of Bad (Datta *et al.* 1997, del Peso *et al.* 1997). However, this has been shown to be the case in response only to some cytokines (Scheid & Duronio 1998). Several other substrates involved in the regulation of apoptosis have also been suggested to be regulated by PKB to promote survival. Further studies are underway to characterise the role of the PI3-kinase/PKB pathway in the anti-apoptotic effect of PRL and to elucidate the downstream events by which PKB may promote Nb2 cell survival in response to PRL.

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