Overexpression of Akt inhibits NGF-induced growth arrest and neuronal differentiation of PC12 cells

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SUMMARY

To investigate the role of Akt in nerve growth factor (NGF)-induced neuronal differentiation, PC12 cells ectopically expressing wild-type or dominant-inhibitory forms of Akt were analyzed. NGF-induced neurite outgrowth was greatly accelerated in cells expressing dominant-inhibitory Akt, compared to parental PC12 cells, but was almost completely blocked in cells expressing wild-type Akt. Since neuronal differentiation requires an arrest of cell growth, several aspects of cell growth of the different cell lines were compared. Cells expressing wild-type Akt were not susceptible to the growth-arresting effect of NGF, whereas parental PC12 cells and notably cells expressing mutant Akt were so affected. Accompanying this, the expressions of CDKs and p21WAF1 were down- and up-regulated, respectively, in both parental PC12 cells and cells expressing mutant Akt. When treated with some growth arrest-inducing agents such as sodium nitroprusside, forskolin and butyrolactone I, cells expressing wild-type Akt regained their responsiveness to the effects of NGF on differentiation. In summary, our results indicate that Akt overrides the growth-arresting effect of NGF and thereby, negatively regulates neuronal differentiation.

Key words: Akt, PC12 cell, Neuronal differentiation, NGF, Cell cycle, CDK, Growth arrest

INTRODUCTION

The differentiation of precursor cells into neuronal cells requires a series of distinct biological processes. For example, PC12 cells undergo proliferation followed by growth arrest and subsequent differentiation in response to nerve growth factor (NGF) (Greene and Tischler, 1976; Kaplan and Stephens, 1994; Penuova and Enikolopov, 1995). These NGF-induced processes are complex and well coordinated, involving the participation of a number of signaling proteins. Phosphatidylinositol 3-kinase (PI3-kinase) is one of the proteins activated by the receptor tyrosine kinase after NGF stimulation (Carter and Downes, 1992; Soltoff et al., 1992). However, the role of PI3-kinase in neuronal differentiation has not been unambiguously resolved. A study with cells expressing mutant growth factor receptors, which lack the PI3-kinase binding site has suggested that PI3-kinase is not crucial in the process of neuronal differentiation (Obermeier et al., 1994). Other studies using PI3-kinase inhibitors and constitutively active forms of PI3-kinase in PC12 cells showed conflicting results that PI3-kinase is involved in neuronal differentiation (Kimura et al., 1994; Jackson et al., 1996; Kobayashi et al., 1997). More confusingly, PI3-kinase appears to function as a negative regulator of cellular differentiation in other cell lines such as pancreatic and aortic smooth muscle cells (Ptasznik et al., 1997; Bacqueville et al., 1998). It is, however, widely accepted that PI3-kinase is implicated in the survival of neurons and other cell types (Vemuri and McMorris, 1996; Dudek et al., 1997; Crowder and Freeman, 1998).

Downstream molecules of PI3-kinase include Akt, p70S6 kinase, certain isoforms of protein kinase C, and the Rho family of small GTPases (Cheatham et al., 1994; Burgering and Coffer, 1995; Chou and Blenis, 1996; Moriya et al., 1996). Like many other intracellular signaling proteins, Akt is activated by a variety of mitogenic factors and cytokines as well as NGF (Franke et al., 1995; Alessi et al., 1996; Park et al., 1996; Ahmed et al., 1997; Andjelkovic et al., 1998). While intracellular proteins that Akt interacts with are largely unknown, glycogen synthase kinase-3 and BAD, the Bcl-2 homologue have been identified as downstream effectors of Akt (Cross et al., 1995; del Peso et al., 1997).

Akt has been shown to mediate the growth factor-dependent inhibition of apoptosis in various types of cells including neurons (Ahmed et al., 1997; Dudley et al., 1997; Kulik et al., 1997; Philpott et al., 1997; Songyang et al., 1997; Crowder and Freeman, 1998). For instance, Akt plays a critical role in NGF- or insulin-like growth factor 1-dependent survival of cerebellar, superior cervical, and sympathetic neurons (Dudek et al., 1997; Philpott et al., 1997; Crowder and Freeman, 1998). Another important effect of NGF on neuronal cells is to induce their differentiation. For these reasons, we investigated whether Akt is also involved in this neuronal differentiating effect of NGF. We demonstrated that the reduction of Akt activity by
transfection with the dominant-inhibitory mutant form of Akt accelerates neurite outgrowth. Overexpression of wild-type Akt, however, causes the inhibition of cell growth arrest and neurite formation in PC12 cells. These results suggest that Akt is likely to play a role as a negative regulator of the NGF-induced neuronal differentiation of PC12 cells.

**MATERIALS AND METHODS**

**Cell culture and treatments**

PC12 cells obtained from Dr David Kaplan (Montreal Neurological Institute, Canada) were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% heat-inactivated horse serum, 5% heat-inactivated fetal calf serum, and antibiotics (complete medium) at 37°C under 5% CO2. Chemicals used were 100 nM wortmannin (Sigma, St Louis, MO, USA), 50 µM LY294002 (Biolom, Plymouth Meeting, PA, USA), 0.5 mM sodium nitroprusside (Sigma), 25 µM forskolin (Calbiochem, La Jolla, CA, USA), 200 nM rapamycin, 30 µM oloymoucine, and 30 µM butyrolactone I (Calbiochem).

**Transfection**

Hemagglutinin (HA) epitope-tagged vectors (pCMV6-HA-Akt and pCMV6-HA-K179M) were generated from the pre-existing constructs (Franke et al., 1995). For stable transfections, PC12 cells (3×10⁶ cells per 60-mm culture dish) were transfected with 2 µg DNA using Lipofectamine Plus Reagent (Gibco BRL, Gaithersberg, MD, USA), according to the manufacturer’s instruction. Forty-eight hours after transfection, 500 µg/ml of G418 (Gibco BRL) was added to the medium. Stable clones expressing wild-type Akt and kinase-inactive Akt mutant (K179M) were maintained in complete medium containing 200 µg/ml of G418.

**Cell differentiation and proliferation assays**

Cells (3×10⁵ cells per 35-mm culture dish) were treated with 100 ng/ml of NGF (Promega, WI, USA) in complete medium. Cell processes with lengths equivalent to two diameters of a cell body were counted as neurites. A minimum of two hundred cells was examined in three fields for each datum point. For the analysis of proliferation, cells were trypsinized, and the number of viable cells was counted by the exclusion cells from trypan blue staining in triplicate using a hemocytometer.

**Western blot analysis**

Cells were washed three times with ice-cold Tris-buffered saline (TBS; 20 mM Tris-HCl, pH 8.0, 137 mM NaCl), and lysed with 0.2 ml of lysis buffer (TBS, 1% Nonidet P-40, 1 mM sodium orthovanadate, 10 µg/ml aprotinin, 10 µg/ml leupeptin, and 1 mM PMSF). Lysates were centrifuged for 10 minutes at 12,000 g and supernatants were analyzed for protein concentration using a BCA Protein Assay kit (Pierce, Rockford, IL). Equal amounts of proteins were separated on 7.5 or 10% polyacrylamide gels containing 1% SDS, transferred to nitrocellulose paper, and probed with antibodies against HA (Babco, Richmond, CA, USA), Akt (Park et al., 1996), phospho (Ser473)-Akt, phospho (Ser9)-GSK-3β (New England Biolabs, Beverly, MA, USA), GAP43 (Sigma), CDK1, CDK2, and p21(WAF1) (Santa Cruz Biotech., Santa Cruz, CA, USA).

**Immunoprecipitations and in vitro kinase assays**

Cell lysates were incubated with 5 µl of anti-HA or -Akt antibody for 3 hours at 4°C. Immunocomplexes were collected with 15 µl of Protein A-Sepharose slurry (50% v/v), washed three times with lysis buffer, and twice with kinase buffer (25 mM Hepes, pH 7.4, 1 mM dithiothreitol, 10 mM MnCl2, and 10 mM MgCl2). The immunoprecipitates were then incubated with 30 µl of kinase buffer containing 10 µCi [γ-32P]ATP (Amersham, Buckinghamshire, UK), 0.5 µg histone H2B as a substrate, and 50 µM cold ATP for 20 minutes at 30°C. The reaction was stopped by adding 4× SDS sample buffer followed by boiling for 5 minutes. For the CDK1 activity, immunoprecipitates of CDK1 were reacted with histone H1 as a substrate. Samples were then subjected to SDS-polyacrylamide gel electrophoresis on a 13% separating gel followed by autoradiography.

**RESULTS**

**Akt blocks NGF-induced neurite formation of PC12 cells**

To examine the role of Akt in NGF-induced neuronal differentiation, PC12 cells were stably transfected with cDNAs encoding the HA epitope-tagged wild-type enzyme (HA-Akt) or dominant-inhibitory mutant form (HA-Akt(K179M); Franke et al., 1995). Multiple clones (Akt-8, -116, -125 and K179M-0, -8, -10) were selected and analyzed for Akt expression. The expression level of HA-Akt and HA-Akt(K179M) in these clones was higher than that of the endogenous Akt in parental PC12 cells (Fig. 1A). When the activity of Akt was analyzed after immunoprecipitation with anti-HA or anti-Akt antibody (Fig. 1B), much higher enzymatic activity was observed in cells transfected with the wild-type HA-Akt (clone Akt-116) than in parental PC12 cells, confirming that ectopically expressed wild-type Akt is active. Furthermore, this NGF-induced activation of Akt was suppressed by pretreatment of cells with wortmannin, a PI3-kinase inhibitor, consistent with previous observations that Akt is activated via a PI3-kinase-dependent pathway. In contrast, Akt activity was barely detectable in cells expressing dominant-inhibitory mutant form of HA-Akt(K179M) (clone K179M-10). The activity of Akt in cells transfected with the wild-type HA-Akt and dominant-inhibitory mutant form of Akt(K179M) was assessed by the phosphorylation level of GSK-3β, a target molecule of Akt, as previously reported (Pap and Cooper, 1998). Overexpressed wild-type HA-Akt(clone Akt-116) remarkably increased the phosphorylation of GSK-3β in response to NGF treatment in the parental PC12 cells, which is inhibited by pretreatment of cells with wortmannin. Whereas, in cells expressing dominant-inhibitory mutant form of HA-Akt(K179M) (clone K179M-10) the phosphorylation of GSK-3β was not detected (Fig. 1C). This result indicates that HA-Akt(K179M) is really acting as a dominant negative mutant Akt.

Nerve growth factor induces differentiation as well as survival of PC12 cells by stimulating numerous signal transducing molecules. Akt, a downstream molecule of PI3-kinase, was known to be activated by NGF in PC12 cells (Park et al., 1996; Andjelkovic et al., 1998). Moreover, activity of
Akt was increased relatively high level at early time in response to NGF and decreased thereafter (data not shown), which might be inversely correlated with neurite outgrowth of PC12 cells. Therefore, to confirm whether Akt affects neuronal differentiation, parental PC12 cells and the above several transfected clones were treated with NGF, and the extent of neurite formation was measured. Parental PC12 cells started to extend neurites following three days’ treatment with NGF (Fig. 2). In comparison, cells expressing the dominant-inhibitory mutant form of Akt (K179M-0, -8, and -10) bore a greater number and a larger size of neurites during this time (Fig. 2A). These neurites are structurally normal, because they were positively stained with an antibody against GAP43, a growth cone marker protein (data not shown). Quantitative analysis (Fig. 2B) reveals that, throughout the 8 days period of treatment, the neurite formation in cells from the K179M clones appeared to have accelerated by 2 to 4 days. Thus, at 4 days after NGF treatment, the extent of neurite formation in all of the K179M clones ranged between 68-85%, being 3- to 4-fold higher than that (approximately 20%) in parental cells. Surprisingly, however, cells of clones overexpressing wild-type Akt (Akt-8, -116, and -125) failed to show any significant number of neurites (Fig. 2A). None of these cells was found to make neurites at 4 days after NGF treatment, and the extent of neurite formation did not reach even 10% by 8 days of NGF treatment (Fig. 2B). But yet, when the activity of Akt in Akt-116 cells was decreased to the untreated basal level as analyzed using anti-phospho Akt antibody by pretreatment of cells with LY294002 another PI3-kinase inhibitor (Fig. 2C), the cells became able to extend neurites of significant number and size (Fig. 2A), and highly expressed GAP43 protein (Fig. 2C).

These results indicate that, depending upon whether the Akt activity in the cells was increased by transfection with the wild-type Akt cDNA or decreased by its dominant-inhibitory mutant form, cells exhibited the extremes to each other in neurite formation, while parental cells were in-between. These results also imply that the opposing phenotype observed in Akt and K179M clones is not due to the clonal variations but incurred by the differential Akt activity between these cell lines.

**NGF’s effects on the growth arrest are inhibited by Akt**

We have shown above that NGF-induced neurite formation is affected by the total activity of Akt in the cells. For PC12 cells to differentiate, a preceding growth arrest is required (Greene and Tischler 1976; Kaplan and Stephens, 1994; Peunova and Enikolopov, 1995). We thus examined whether the opposite phenotype in neuronal differentiation between Akt and K179M clones has something to do with the growth arrest and cell proliferation. Cells were incubated in the complete medium without or with NGF, and cell numbers were counted each day. Under the routine culture conditions without NGF (Fig. 3A), all of the clones were able to proliferate well, but there were some differences in the proliferation rate among the clones. Cells overexpressing wild-type Akt proliferated faster than the parental cells, and cells transfected with mutant Akt (K179M) did less. This observation is consistent with the fact that Akt promotes cell proliferation (Kandel and Hay, 1999; Zimmermann and Moelling, 1999).

When cultured in the presence of NGF, however, proliferation of all of these cells was slowed down, but in different extents (Fig. 3B). Despite the decrease in the proliferation rate by NGF, Akt-116 cells still continued proliferating at 8 days after NGF treatment. In contrast, parental PC12 cells started to reach a plateau in their cell number at 5 to 6 days after NGF treatment, and even strikingly K179M-10 cells stopped proliferating almost from the beginning of NGF treatment. These results indicate that depending upon the total cellular activity of Akt, cells exhibit the differential susceptibility to the growth-arresting effect of NGF; K179M-10 cells expressing dominant-inhibitory mutant Akt were the most susceptible to the growth-arresting effect of NGF, while Akt-116 cells overexpressing wild-type Akt were the least.

Since PC12 cells accumulate in the G1 phase of the cell cycle in response to NGF (Rudkin et al., 1989), we next determined the relative distribution of cells in different phases of the cell cycle by FACs analysis. Clear distinctions were observed among the different cell lines of PC12 (Fig. 3C); in response to NGF, parental PC12 cells were arrested in the G1 phase of the cell cycle. In case of Akt-116 cells, the cell cycle distribution was not much changed between the cells cultured with and without NGF (Fig. 3C) in the complete medium, indicating that Akt-116 cells were in the proliferative state under the NGF treatment. K179M-10 cells, however, exhibited a strikingly different cell cycle distribution. Compared to PC12 and Akt-116 cells, a much higher percentage of K179M cells was in the G2/M phase (45% in K179M-10 vs 15-25% in PC12 and Akt-116 cells).

**Akt blocks NGF-induced neurite formation in PC12 cells**

Growth arrest is regulated at least in part by proteins such as CDK1, CDK2, and p21^WAF1_. As a cyclin-dependent kinase (CDK) which promotes cell cycle progression, CDK2 is required for G1/S transition and CDK1 (also termed as CDC2) for G2/M transition (van den Heuvel and Harlow, 1993; Sherr, 1994). p21^WAF1_ inhibits these CDKs’ activity and induces the cell cycle arrest at G1/S or G2/M (Parker et al., 1995; Billon et al., 1996; Poluha et al., 1996). The expression and the activity of these CDKs have been shown to be down-regulated by NGF in PC12 cells, while expression of p21^WAF1_ is increased (Buchkovich and Ziff, 1994; Decker, 1995; Yan and Ziff, 1995; van Grunsven et al., 1996). We thus compared the expression and the activity of these proteins in between the parental PC12 and the transfected cells. Consistent with the above-mentioned findings, parental PC12 cells underwent the gradual reduction in the expression of CDK1 and CDK2, and the activity of CDK1 in response to NGF treatment (Fig. 4A). As contrasted with this in parental cells, the reduction in the expression and the activity of CDK1 was more obvious and rapid in K179M-10 cells. In case of Akt-116 cells, however, the reduction in the expression of CDK2 was only slight, and the expression and the activity of CDK1 were not affected. The expression of p21^WAF1_ protein showed the opposite response. p21^WAF1_ expression was markedly increased in K179M-10 cells after NGF treatment compared to that in parental PC12 cells, while barely detectable levels of protein were expressed in Akt-116 cells. These results indicate that Akt inhibits the NGF’s effects on the expression and the activity of CDK1 and the expression of p21^WAF1_.
Sodium nitroprusside, forskolin and butyrolactone I restore the NGF-induced growth arrest and neurite formation in Akt-116 cells

The above results suggest that insignificantly minimal differentiation of the Akt-116 cells is due to their failure to undergo the NGF-induced growth arrest, a step necessary for differentiation to occur. This led us to suspect that these Akt-116 cells might gain the ability to differentiate in response to NGF, but become able to differentiate in response to NGF because of their escape from the growth arrest-inducing action of NGF. Thus, it seems that Akt-116 cells treated with SNP, forskolin, or butyrolactone I but not with rapamycin or olomoucine were found to respond to the differentiating action of NGF by making neurites. Therefore, the activity of Akt assessed by the phosphorylations at serine 473 was not changed (Fig. 5C). These agents by themselves were not able to provoke the neurite formation (data not shown). The extent of neurite formation was increased approximately 5- to 8-fold as compared with that in control culture with NGF alone (Fig. 5E).

These results indicate that SNP, forskolin, and butyrolactone I have the property to prime the Akt-116 cells to differentiate in response to NGF, suggesting that this priming effect probably come from the ability of these agents to induce the G2/M arrest in these otherwise resistant Akt-overexpressing cells. It can be also said that cells overexpressing Akt hardly differentiate in response to NGF because of their escape from the growth arrest-inducing action of NGF, but become able to differentiate once induced to enter into the growth arrest by using SNP, forskolin, or butyrolactone I.

DISCUSSION

In this study, we have examined the role of Akt in NGF-induced neuronal differentiation by characterizing PC12 cells that were managed to stably overexpress the wild-type HA-Akt or the dominant-inhibitory mutant form of Akt, HA-Akt(K179M). We show that, compared to in parental PC12 cells, NGF-induced neurite outgrowth was greatly enhanced in cells from several independent Akt(K179M) clones, while it was significantly blocked in cells overexpressing wild-type Akt. These results in all indicate that Akt inhibits the NGF-induced neurite formation of PC12 cells.

There have been several other papers reporting the role of PI3-kinase, an upstream molecule of Akt, on the neuronal differentiation of PC12 cells (Obermeier et al., 1994; Kimura et al., 1994; Jackson et al., 1996; Kobayashi et al., 1997). In most cases, though, these papers have suggested that PI3-kinase have a positive role in NGF-induced neuronal differentiation of PC12 cells (Kimura et al., 1994; Jackson et al., 1996; Kobayashi et al., 1997), thus seeming contradictory to our present result. However, PI3-kinase exerts diverse effects...
Akt blocks NGF-induced neurite formation in PC12 cells

on PC12 cells including survival, proliferation, and differentiation by utilizing its various downstream effectors, each of which probably mediates the distinct function (Cheatham et al., 1994; Burgering and Coffer, 1995; Chou and Blenis, 1996; Moriya et al., 1996). Thus, it might be that depending upon the magnitude of the PI3-kinase activity and the ratio of activities among PI3-kinase effectors as well as their temporal regulation, PC12 cells might undergo different cell fate decisions between proliferation and differentiation. Certainly, one function of Akt at the normal level has been shown to mediate the survival signal of PI3-kinase in neuronal cells (Dudek et al., 1997; Philpott et al., 1997; Crowder and Freeman, 1998).

At any rate, our results suggest that Akt, when overexpressed, can virtually cause the blockage to the neuronal differentiation of PC12 cells by sustaining the proliferation of PC12 cells as discussed below.

NGF initially causes a transient promotion of proliferation, but it subsequently leads to the cessation of proliferation and the induction of neuronal differentiation (Rudkin et al., 1989; Buchkovich and Ziff, 1994; Yan and Ziff, 1995; Billon et al., 1996). Differentiation of PC12 cells is blocked by some oncogenes such as E1A and c-Myc that stimulate cell cycle progression (Kalman et al., 1993; Owada et al., 1997).

Fig. 2. Neurite outgrowth in PC12 cell lines. (A) Phase-contrast photomicrographs. Parental PC12 cells, Akt-8, -116, -125 cells, and K179M-0, -8, -10 cells were treated with 100 ng/ml NGF in complete medium for 4 days, and random fields were photographed. For LY294002 treatment in Akt-116 cells (Akt-116/LY), cells were preincubated with 50 μM LY294002 for 16 hours.

(B) Quantification of neurite formation. Cells with processes longer than two diameters of cell body were counted as positive on the indicated day. The data represent averages of three independent experiments with standard deviation.

(C) Expression level of GAP43 and phosphorylation level of Akt protein in Akt-116 cells treated with LY294002. Cells were preincubated with 0.1% DMSO (–) or 50 μM LY294002 (+) for 16 hours and then cultured with NGF in complete medium for 4 days. Untreated Akt-116 cell was used as a negative control. Lysates were subjected to the immunoblot analysis using anti-phospho Akt (Ser 473) and -GAP43 antibodies.

Fig. 3. Cell proliferation and cell cycle analysis in transfected PC12 cell lines. Cells were grown in complete medium without NGF for 3 days (A) or with NGF for 8 days (B). After trypsinization, viable cells were counted on the indicated day. (C) Cells were cultured without NGF (–NGF) or with NGF (+NGF) for 6 days and the relative distribution of cells in the different phases of the cell cycle was assessed by FACS analysis as described in Materials and Methods. The data represent averages of four independent experiments with standard deviation.
Therefore, inhibition of cell proliferation/cell cycle progression is essential for NGF-induced neuronal differentiation. Here, we present several lines of evidence that cell cycle progression is positively regulated by the total cellular activity of Akt. Firstly, cell proliferation was affected by Akt; the ascending order of cell proliferation rate was Akt(K179M)-expressing cells, parental PC12 cells, wild-type Akt-overexpressing cells. Secondly, a high percentage of cells expressing Akt(K179M) was arrested in the G2/M phase of the cell cycle. Finally, the expression level and the activity of CDK1 were decreased more abruptly in cells expressing Akt(K179M) than those in parental PC12 cells, while CDK1 level in cells overexpressing wild-type Akt was rather increased, although slightly. Conversely, cells expressing Akt(K179M) had the highest expression level of p21WAF1. Taken together, these results indicate that NGF-induced growth-arrest is inhibited in cells having high activity of Akt but is enhanced in cells with low activity of Akt as in K179M clones.

We have shown that cells overexpressing wild-type Akt became primed for NGF-induced neuronal differentiation after treatment with some agents such as SNP, forskolin, and butyrolactone I. Although there is a caveat using these chemical agents that can often exert diverse effects on cells, the results that they possessed the property to induce the growth arrest with the characteristic modulation of CDK1 argue that the priming action of these agents originates from their attribution to induce growth arrest of the cell cycle. This implies that the reason why, if not aided by these agents, cells overexpressing Akt are hardly susceptible to the differentiating action of NGF is because these cells escape from the prerequisite growth arrest-inducing action of NGF.

It is not known how an increase in total cellular activity of Akt results in the inhibition of NGF-induced growth arrest. CDK1 or p21WAF1 could be a Akt’s target but it remains to be determined whether this is the case. It is noteworthy that not all of these...
growth arrest-inducing agents but only those that led to the arrest specifically at G2/M were able to endow the Akt-116 cells to differentiate in response to NGF. Thus, it might be that the three agents, SNAP, forskolin, and butyrolactone I act on the regulation of cellular proliferation under the control of PI3-kinase/Akt pathway (Ahmed et al., 1997; Brennan et al., 1997).

In summary, we have demonstrated that Akt negatively regulates the NGF-induced neuronal differentiation of PC12 cells by inhibiting NGF-induced growth arrest. Based on this, it can also be inferred that the activity of Akt must be fine regulated during neurogeneration and that if erratic high activation of Akt occurs somehow, for instance by mutation, before the neurons fully develop, it will probably cause a hyponeurogenesis. Interestingly, it has recently been shown that the expression of Akt in neuronal cell types is high in embryos but decreases gradually to adult levels during postnatal development (Owada et al., 1997).

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