Genetic and Pharmacologic Identification of Akt as a Mediator of Brain-Derived Neurotrophic Factor/TrkB Rescue of Neuroblastoma Cells from Chemotherapy-Induced Cell Death

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Abstract

Patients whose neuroblastoma tumors express high levels of brain-derived neurotrophic factor (BDNF) and TrkB have an unfavorable prognosis. Our previous studies indicated that BDNF activation of the TrkB signal transduction pathway blocked the cytotoxic effects of chemotherapeutic drugs via the phosphatidylinositol 3-kinase pathway. Akt is an important downstream target of phosphatidylinositol 3-kinase and functions to regulate cell survival, proliferation, and protein synthesis. In this study, we examined whether Akt is required and sufficient to mediate BDNF/TrkB protection of neuroblastoma cells from chemotherapy. Transient transfection of a constitutively active Akt (Akt-Myr) into TrkB-expressing SY5Y cells (TB8 cells) increases Akt activation and attenuates the cell death induced by chemotherapeutic reagents in the absence of BDNF. Furthermore, expression of a dominant-negative Akt (Akt-K179A) blocks the ability of BDNF to rescue TB8 cells from chemotherapy-induced cell death. Pharmacologic inhibition of Akt, with PIA6, a phosphatidylinositol ether lipid analogue (PIA), blocks BDNF-induced phosphorylation of Akt and the downstream target of Akt. PIA6 sensitizes neuroblastoma cells to chemotherapy and attenuates BDNF protection of neuroblastoma cells from chemotherapy. These results suggest that constitutively active Akt can substitute for BDNF and protect cells from chemotherapy. Furthermore, the BDNF-induced rescue of neuroblastoma cells from chemotherapy is blocked by both genetic and pharmacologic inhibition of Akt activity. These results suggest that drugs that inhibit Akt activity may enhance the effectiveness of chemotherapeutic reagents in the treatment of neuroblastoma tumors.

Materials and Methods

TrkB-Expressing Cell Line and Cell Culture. A TrkB-expressing SH-SY5Y neuroblastoma cell line (TB8) was used and cultured as described previously (5, 7).

DNA Vector Constructions. pCIS2, constitutively active Akt (Akt-Myr), and dominant-negative Akt (Akt-K179A) plasmids were provided by M.J. Quon (8).

Reagents. Recombinant human BDNF, P3K inhibitor LY294002, and Akt inhibitor PIA6 were obtained from PeproTech, Inc. (Rocky Hill, NJ), Cell Signaling Technology (Beverly, MA), and Calbiochem (Oakland, CA), respectively. Etoposide and cisplatin were obtained from Sigma (St. Louis, MO). Anti-Akt, anti-phospho-Akt (Ser473), anti-phospho-GSK-3β (Ser9), and anti-phospho-epha-1 (Ser1031/1033) were obtained from Cell Signaling Technology.

Transfections. TB8 cells (8 × 105) were transfected with green fluorescent protein (GFP) and either Akt-Myr, Akt-K179A, or pCIS2 by electroporation at 250 mF in 400 µL medium.

Treatments. Pharmacologic inhibitors were used as described previously (5). To study the effect of PIA6 (the Akt inhibitor) on cell survival, cells were first cultured in serum-free medium overnight and then treated with PIA6 for 1 hour before a 1-hour treatment with BDNF (100 ng/mL) or medium control and followed by a 24-hour treatment with chemotherapeutic reagents.

Western Blot. Western blot analyses were done as described previously (5). Briefly, protein lysates (30 µg) were analyzed by SDS-PAGE and the indicated primary antibodies were diluted in 1:1,000 and detected with horseradish peroxidase–conjugated goat anti-rabbit IgG (1:2,000).

Cell Survival Analysis. Cells were stained with trypan blue. The percentage of cell survival (survival rate) was calculated by dividing the number of survival cells by the total number of cells for each condition. The effects of BDNF are dependent on both its concentration and the level of TrkB receptor expression (5). Following binding of BDNF to its receptor TrkB, receptor dimerization leads to activation of the TrkB tyrosine kinase as well as activation of downstream signaling pathways, including the phosphatidylinositol 3-kinase (PI3K), Ras/mitogen-activated protein kinase, and phospholipase Cγ pathways (2). Recently, we have shown that activation of TrkB by BDNF protects neuroblastoma cells from chemotherapy-induced cell death via the PI3K pathway (5). The serine/threonine kinase Akt is a crucial mediator of this pathway (6). In this study, we tested the hypothesis that Akt is a key downstream target of PI3K by which the BDNF/TrkB pathway attenuates the effects of chemotherapy in neuroblastoma cells. We find that constitutively active Akt can substitute for BDNF and protect cells from chemotherapy. Furthermore, the BDNF-induced rescue of neuroblastoma cells from chemotherapy is blocked by both genetic and pharmacologic inhibition of Akt activity. These results suggest that drugs that inhibit Akt activity may enhance the effectiveness of chemotherapeutic reagents in the treatment of neuroblastoma tumors.

Introduction

Neuroblastoma, one of the most common solid pediatric tumors, is derived from the sympathoadrenal lineage of the neural crest (1). The expression of the neurotrophin brain-derived neurotrophic factor (BDNF) and its tyrosine kinase receptor TrkB are often detected in neuroblastoma tumors derived from patients with an unfavorable prognosis (2). BDNF is known to increase neuroblastoma cell survival, neurite extension, and cell invasion (3) as well as to protect cells from chemotherapy (4). Furthermore, the protective effects of BDNF are dependent on both its concentration and the level of TrkB receptor expression (5). Following binding of BDNF to its receptor TrkB, receptor dimerization leads to activation of the TrkB tyrosine kinase as well as activation of downstream signaling pathways, including the phosphatidylinositol 3-kinase (PI3K), Ras/mitogen-activated protein kinase, and phospholipase Cγ pathways (2). Recently, we have shown that activation of TrkB by BDNF protects neuroblastoma cells from chemotherapy-induced cell death via the PI3K pathway (5). The serine/threonine kinase Akt is a crucial mediator of this pathway (6). In this study, we tested the hypothesis that Akt is a key downstream target of PI3K by which the BDNF/TrkB pathway attenuates the effects of chemotherapy in neuroblastoma cells. We find that constitutively active Akt can substitute for BDNF and protect cells from chemotherapy. Furthermore, the BDNF-induced rescue of neuroblastoma cells from chemotherapy is blocked by both genetic and pharmacologic inhibition of Akt activity. These results suggest that drugs that inhibit Akt activity may enhance the effectiveness of chemotherapeutic reagents in the treatment of neuroblastoma tumors.

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Akt Mediates BDNF Protection of Neuroblastoma

Results

Akt Expression in SY5Y-TrkB-Expressing Cells. Our previous results showed that BDNF rescued TrkB-expressing neuroblastoma cells from chemotherapeutic-induced apoptosis via the PI3K pathway (5). Although Akt is predominantly activated in a PI3K-dependent manner, PI3K-independent mechanisms, such as those activated by agonists of protein kinase A or β-adrenergic receptors, have been reported (6, 9). To confirm that BDNF/TrkB-induced phosphorylation of Akt is mediated by the PI3K pathway, TB8 cells were treated with 100 ng/mL BDNF for indicated times after a 1-hour pretreatment with 20 μM LY294002, a PI3K inhibitor, or control solvent. Stimulation of BDNF induced a 12-fold increase of Akt phosphorylation (P-Akt [Ser473]) after 5 minutes, which declined by 1 hour, yet 3-fold increase was still detected after 6 hours. Pretreatment with LY294002 blocked BDNF-induced phosphorylation of Akt (Fig. 1A).

To directly evaluate the role of Akt in BDNF/TrkB rescue of cell death induced by chemotherapeutic reagents, different plasmids containing constitutively active Akt (Akt-Myr) or dominant-negative Akt (Akt-K179A) were transfected into TB8 cells. The respective P-Akt and phosphorylation of GSK-3β (P-GSK-3β [Ser9]), an Akt downstream target, and total Akt were evaluated. TB8 cells were also cultured in the absence or presence of BDNF. In Fig. 1B, extracts from cells transfected with Akt-Myr showed 10- or 18-fold higher P-Akt level (lane 1) compared with extracts from cells transfected with Akt-K179A (lane 2) or vector pCIS2 (lane 3), respectively. P-Akt increased after BDNF stimulation (lane 5) compared with unstimulated cells (lane 4). To assess the activity of Akt in cells transfected with recombinant Akt constructs, P-GSK-3β was evaluated. P-GSK-3β levels were 3- or 2.5-fold higher in Akt-Myr-transfected cells compared with Akt-K179A or vector pCIS2-transfected cells. BDNF stimulation increased P-GSK-3β level in untransfected cells. Total Akt level in Akt-Myr-transfected cells was 1.3- or 2.4-fold higher compared with Akt-K179A or vector pCIS2-transfected cells, respectively.

Transfection of Akt-Myr into TB8 cells and BDNF stimulation of TB8 cells increased the levels of P-Akt. To determine if transfection of dominant-negative Akt (Akt-K179A) into TB8 cells could block the BDNF-induced phosphorylation of Akt and its downstream targets, we transfected either Akt-K179A or the control vector (pCIS2) into TB8 cells followed by stimulation of BDNF. Densitometric analysis of appropriately scanned autoradiograms indicated that BDNF stimulated a 7.5-fold increase in P-Akt levels in pCIS2-transfected cells yet only a 3.1-fold increase in Akt-K179A-transfected cells (Fig. 1C and D, left). BDNF stimulated a 1.6-fold increase in P-GSK-3β levels in pCIS2-transfected cells but had little effect on P-GSK-3β level when Akt-K179A was transfected into the cells (Fig. 1C and D, right). BDNF stimulation did not markedly change the total Akt levels in either pCIS2-transfected or Akt-K179A-transfected cells (Fig. 1C). These data indicate that dominant-negative Akt blocks BDNF-induced phosphorylation of Akt and GSK-3β.

Figure 1. A. BDNF-induced phosphorylation of Akt was mediated by PI3K pathway. TB8 cells were cultured in RPMI 1640 containing 10% fetal bovine serum for 24 hours. Without or with pretreatment of 20 μM LY294002 for 1 hour, cells were stimulated with 100 ng/mL BDNF and harvested at indicated times (5, 60, 180, and 360 minutes). Whole cell lysates were extracted and total protein (30 μg) was analyzed for P-Akt (Ser473) and total Akt (lane 1) by Western blot. B. Expression of recombinant Akt constructs in transfected TB8 cells. GFP (8 μg) and either Akt-Myr (12 μg), Akt-K179A (12 μg), or pCIS2 (12 μg) were cotransfected into 8 million cells by electroporation. Cells were cultured in RPMI 1640 with 10% serum for 24 hours. Nontransfected cells were stimulated with 100 ng/mL in BDNF (+) samples. Protein lysate (30 μg) was analyzed by Western blot for P-Akt (Ser473) and total Akt (lane 4, left). C. Densitometric analysis of Western blot results: densities of P-Akt (Ser473) or P-GSK-3β (Ser9) bands were measured by densitometer and normalized by the densities of total Akt. RDU, relative density unit.
Constitutively Active Akt Substitutes for BDNF, whereas Dominant-Negative Akt Blocks BDNF Effect on Protecting TB8 Cells from Chemotherapy.

To determine whether BDNF rescues TrkB-expressing neuroblastoma cells from chemotherapy via the Akt pathway, we transfected Akt-Myr or pCIS2 into TB8 cells. After transfection (24 hours), the cells were treated with etoposide for 24 hours and cell survival was determined. In Fig. 2A, etoposide treatment induced 32% and 56% cell death in pCIS2-transfected cells at 0.25 and 0.5 μg/mL etoposide, respectively. However, in the Akt-Myr-transfected cells, only 13% and 23% of cells died at 0.25 and 0.5 μg/mL etoposide, respectively. Although the survival rate of Akt-Myr-transfected cells was slightly higher than that of pCIS2-transfected cells, these data indicate that expression of constitutively active Akt caused an ~30% reduction in the cell death induced by etoposide.

A comparison of the cell survival among pCIS2-transfected cells, Akt-Myr-transfected cells, Akt-Myr and Akt-K179A cotransfected cells is depicted in Fig. 2B. Etoposide induced obvious cell death in pCIS2-transfected cells, whereas transfection of Akt-Myr into TB8 cells blocked the etoposide-induced cell death. However, when Akt-K179A was cotransfected with Akt-Myr, the ability of Akt-Myr to protect TB8 cells from etoposide-induced cell death was attenuated. These data indicate that Akt kinase is important in mediating cell survival during chemotherapy, and transfection of a constitutively active Akt into cells can substitute for BDNF and protect TB8 cells from etoposide-induced cell death.

Figure 2. A, constitutively active Akt rescued TB8 cells from etoposide-induced cell death. GFP (8 μg) and either Akt-Myr (12 μg) or pCIS2 (12 μg) were cotransfected into 8 million cells by electroporation. Twenty-four hours after transfection, cells were treated with etoposide (0.25 and 0.5 μg/mL) for 24 hours. Cells were harvested and stained with trypan blue, and living cell number and total cell number were counted. Survival rate of pCIS2-transfected cells (with no-etoposide treatment) was set as 100%; other conditions were normalized to this value. Bars, SD. *, P < 0.05; **, P < 0.01, versus pCIS2-transfected cells.

B and D, dominant-negative Akt attenuated the cell protection of constitutively active Akt; GFP (10 μg) and either pCIS2 (10 μg) or Akt-Myr (10 μg), Akt-Myr (10 μg), and Akt-K179A (10 μg) were cotransfected into TB8 cells. Twenty-four hours after transfection, cells were treated with etoposide (B, 0.13, 0.25, and 0.5 μg/mL) or cisplatin (D, 0.5, 1, and 2 μg/mL) for 24 hours. 3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt assay was used to detect the cell survival. Survival rate in every condition was normalized to the no-drug control within every group. Bars, SD. *, P < 0.05; **, P < 0.01, versus pCIS2-transfected cells; #, P < 0.05; ##, P < 0.01, versus Akt-Myr-transfected cells.

C and E, dominant-negative Akt blocked BDNF effect on rescuing TB8 cells from chemotherapy. GFP (8 μg) and either pCIS2 (12 μg) or Akt-K179A (12 μg) were cotransfected into the cells by electroporation. Twenty-four hours after transfection, cells were stimulated with BDNF (1 hour, 100 ng/mL) followed by 24-hour treatment of etoposide (C, 0.25 and 0.5 μg/mL) or cisplatin (E, 1 and 2 μg/mL). Cell survival rate was calculated as described in A for C and in B for E. Bars, SD. *, P < 0.05; **, P < 0.01, versus no-BDNF-stimulated pCIS2-transfected cells.
The dominant-negative Akt (Akt-K179A) not only blocks the BDNF-induced phosphorylation of Akt and its downstream target GSK-3β (Ser9) but also blocks the ability of Akt-Myr to protect TB8 cells from etoposide-induced cell death (Fig. 2B). To test whether Akt-K179A can block BDNF rescue of cell death, we transfected Akt-K179A or pCIS2 into TB8 cells. After transfection (24 hours), the cells were treated with 2 μM PIA6 or medium control for 1 hour followed by 1-hour 100 ng/mL BDNF stimulation and treatment with etoposide (0.125, 0.25, and 0.5 μg/mL, respectively) or cisplatin (0.25, 0.5, and 1 μg/mL, respectively) or cisplatin (0.25, 0.5, and 1 μg/mL, respectively). 3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt assay was used to detect the cell survival. Survival rate in every condition was normalized to the no-etoposide or no-cisplatin control within every group. Bars, SD. *, P < 0.05; **, P < 0.01, versus no-PIA6-pretreated cells.

Besides etoposide, we evaluated the effect of cisplatin on TB8 cells. Cisplatin is another chemotherapeutic reagent used for neuroblastoma treatment. Similar results were observed. In Fig. 2D, transfection of Akt-Myr into TB8 cells caused up to 30% reduction of cisplatin-induced cell death. Cisplatin sensitized TB8 cells to chemotherapy. TB8 cells were first cultured in no-serum medium for 16 hours and then treated with 2 μM PIA6 or medium control for 1 hour followed by 1-hour 100 ng/mL BDNF stimulation and treatment with etoposide (D, 0.25, 0.5, and 1 μg/mL) or cisplatin (E, 0.5, 1, and 2 μg/mL) for 24 hours. 3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt assay was used to detect the cell survival. Survival rate in every condition was normalized to the no-etoposide or no-cisplatin control. Bars, SD. *, P < 0.05; **, P < 0.01, versus etoposide- or cisplatin-treated cells; #, P < 0.05; ##, P < 0.01, versus etoposide/BDNF- or cisplatin/BDNF-pretreated cells.

Pharmacologic Inhibition of Akt Blocks BDNF-Mediated Phosphorylation of Akt and BDNF-Mediated Protection of Neuroblastoma Cells from Chemotherapy-Induced Cell Death. Recently, small molecule inhibitors of Akt, phosphatidylinositol ether lipid analogues (PIAs), have been shown to block Akt kinase but not PI3K activity (10). To evaluate whether such an approach would
be effective in blocking the activation of Akt by BDNF stimulation, TB8 cells were treated with one of PIAs, PIA6, at indicated concentrations for 1 hour followed by a 1-hour stimulation of BDNF (100 ng/mL). Figure 3A showed that 10 μmol/L PIA6 partially blocked and 40 μmol/L completely blocked the BDNF-induced P-Akt and 40 μmol/L PIA6 partially blocked P-GSK-3β.

Because previous studies indicated that Akt inhibitors increased apoptosis in cancer cell lines with high levels of endogenous Akt activity (10), we sought to determine whether PIA6 could sensitize TB8 cells to chemotherapy. Concentrations ranging from 0.1 to 10 μmol/L (Fig. 3B) were tested, and 2 μmol/L PIA6 inhibited 50% of BDNF-induced P-Akt with only 50% decrease in cell viability.

To observe the effect of PIA6 on chemotherapy-induced cell death, TB8 cells were pretreated with 2 μmol/L PIA6 for 1 hour followed by a 24-hour treatment with etoposide or cisplatin. The cell survival rates of PIA6 treated groups were 22%, 42%, and 20% lower than those of control groups at 0.125, 0.25, and 0.5 μg/mL etoposide, respectively (Fig. 3C), and ~20% lower than control groups at 0.25, 0.5, and 1 μg/mL cisplatin (Fig. 3F). This result indicates that PIA6 increases the sensitivity of TB8 cells to chemotherapy. To determine whether PIA6 could block the effect of BDNF on chemotherapy-induced cell death, we stimulated the cells with 100 ng/mL BDNF or medium control after a 1-hour treatment with etoposide or cisplatin. Pretreatment of TB8 cells with PIA6 before BDNF stimulation completely (Fig. 3D) or partially (Fig. 3F) attenuated the protection of BDNF. These data indicate that pharmacologic inhibition of Akt blocked BDNF protection of TB8 cells from chemotherapy-induced cell death.

To determine whether PIA6 would sensitize other neuroblastoma cell lines to chemotherapy-induced cell death and whether PIA6 could block the protection of BDNF of neuroblastoma cell lines expressing endogenous TrkB from chemotherapy, we treated KCNR and NGP cells with all-trans retinoic acid (10 nmol/L, 3 days) to induce TrkB expression (3). PIA6 caused a 18% (Fig. 4A) and 20% (Fig. 4C) increase in etoposide-induced cell death and a 20% (Fig. 4B) and 16% (Fig. 4D) increase in cisplatin-induced cell death in KCNR and NGP cells, respectively. Furthermore, PIA6 pretreatment blocked the protection of BDNF of these cells from etoposide- or cisplatin-induced cell death (Fig. 4A–D).

**Discussion**

In the present study, we showed that Akt is a key regulator in BDNF protection of neuroblastoma cells from chemotherapy-induced cell death. Constitutively active Akt in neuroblastoma cells can substitute for BDNF and protect neuroblastoma cells from chemotherapy-induced cell death (Fig. 2A, B, and D). Several studies have shown increased Akt activity in a variety of cancers, including breast, glioblastomas, prostate, colon, and lung cancers, and cancers with high active Akt levels are resistant to different treatments (11–15). Castillo et al. reported that Akt inhibitors increase apoptosis 20- to 30-fold in lung cancer cells lines with high levels of endogenous Akt activity (10). In our model system, the endogenous P-Akt levels are low, but BDNF treatment markedly enhances the P-Akt (Fig. 1A). BDNF can protect neuroblastoma cells from chemotherapy (5). This is the first study showing that both genetic and pharmacologic inhibition of Akt activation can block BDNF protection of neuroblastoma cells from chemotherapy-induced cell death (Figs. 2C and E and 3D and F). In human leukemia cells and multiple myeloma cells, selective Akt pharmacologic inhibitors were shown to reduce the cell resistance to chemotherapeutic drugs (16, 17). Small molecule inhibitors, such as imatinib mesylate, have been shown to be a clinically effective strategy to block kinase activation in patients (18). The role of Akt in therapeutic resistance of cancers raises the possibility that by inhibiting Akt activity the sensitivity of tumors to chemotherapy will be improved. Such an approach may have less side effects compared with other PI3K inhibitors, such as LY294002. PIA6, a newly found Akt inhibitor, is one of a set of PIAs that have been designed by molecular modeling to mimic the interaction of PIP2 with the pleckstrin homology domain of Akt (19) and interferes with Akt activity. PIAs inhibit phosphorylation of Akt and its downstream targets, such as GSK-3β, FKHR, and 4EBP1, without influencing activity of upstream kinases, such as P38 or PDK-1 (10).

In our present study, PIA6 inhibited BDNF-induced phosphorylation of Akt and GSK-3β (Fig. 3A) and attenuated BDNF protection of neuroblastoma cells from chemotherapy-induced cell death (Figs. 3D and F and 4). Akt may be a potential target.
for neuroblastoma therapeutic approaches. Etoposide is a topoisomerase II inhibitor that is used for the treatment of neuroblastoma (20). Etoposide induces apoptosis via the intrinsic or mitochondrial pathway in which Bcl-2 family members, such as Bcl-2 and Bcl-XL, play a crucial role. Several Bcl-2 family members, such as Bad, are downstream targets of Akt. In the future, we will evaluate how Akt affects chemotherapy-induced apoptosis as this will enhance our understanding of how BDNF/TrkB path protects neuroblastoma cells from chemotherapy-induced cell death. Our future studies will be aimed at evaluating the in vivo effects of activation of the TrkB/Akt signal transduction pathway on chemotherapy, and once sufficient drug is available, testing the feasibility of Akt inhibitors alone or in combination with chemotherapy to treat neuroblastoma tumors.

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