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Mechanisms of Platelet-derived growth factor- Mediated Neuroprotection: Implications in HIV Dementia

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Abstract

Platelet-derived growth factor (PDGF) has been implicated in promoting survival and proliferation of immature neurons, and even protecting neurons from gp120-induced cytotoxicity. However, the mechanisms involved in neuroprotection are not well understood. In the present study we demonstrate the role of phosphatidylinositol 3-kinase (PI3K)/Akt signaling in PDGF-mediated neuroprotection. Pharmacological inhibition of PI3K greatly reduced the ability of PDGF-BB to block gp120 IIIB-mediated apoptosis and cell death in Human neuroblastoma cells. Role of Akt in PDGF-mediated protection was further corroborated using dominant-negative mutant of Akt, which was able to block the protective effect of PDGF. We next sequentially examined the signals downstream of Akt in PDGF-mediated protection in Human neuroblastoma cells. In cells pre-treated with PDGF prior to gp120, there was increased phosphorylation of both GSK-3 β and Bad, an effect that was inhibited by PI3-kinase inhibitor. Nuclear translocation of NF- κ B, which lies downstream of GSK-3 β , however, remained unaffected in cells treated with PDGF. In addition to inducing phosphorylation of Bad, PDGF-mediated protection also involved down-regulation of the pro-apoptotic protein Bax. Furthermore, PDGF-mediated protection also involved the inhibition of gp120-induced release of mitochondrial cytochrome C. Our findings thus underscore the roles of both PI3K/Akt and Bcl family pathways in PDGF-mediated neuroprotection.

Keywords

PDGF; Gp120; Neurons; HIV Dementia; Neuronal Signaling

Introduction

Human immunodeficiency virus type 1-associated dementia (HAD) is a severe form of neurological impairment found in almost 20–30% of HIV-1 infected patients in the late stages of AIDS (Kolson & Gonzalez-Scarano, 2000). Although HIV-1 does not directly infect the neurons in the CNS (Gartner, 2000; Kaul *et al.*, 2001), HAD is characterized by neuronal dysfunction and loss and is manifested clinically as cognitive, behavioral, and motor disorder (Bagasra *et al.*, 1996; Saha & Pahan, 2003). HIV-associated neuronal damage is thus thought to be due to an indirect mechanism whereby virus infected, as well as uninfected, activated glial cells can elaborate neurotoxins that can injure the neighboring neurons. Candidate toxins include cytokines, glutamate and virus encoded proteins such as the envelope glycoprotein gp120 and the HIV transactivator protein, Tat (Kaul *et al.*, 2001).

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Extensive studies have demonstrated the role of gp120 in inducing neuronal toxicity both *in vitro* and *in vivo* (Bagetta *et al.*, 1996; Garden *et al.*, 2002). Gp120 is an important member of the HIV coat proteins and is shed by HIV-infected mononuclear phagocytes in the CNS. The general consensus existing in the field is that caspase cascades contribute, in part, to the neurotoxicity induced by gp120 (Catani *et al.*, 2000; Nardacci *et al.*, 2005). Furthermore, Castedo *et al.* have also demonstrated that gp120 can induce neuronal apoptosis through mitochondria-dependent pathway which occurs early in gp120-mediated apoptosis (Castedo *et al.*, 2003). It has been suggested that gp120 induces the expression of pro-apoptotic protein such as Bax, which can contribute to mitochondrial membrane permeabilization by binding to and blocking anti-apoptotic Bcl-2, ultimately leading to the release of cytochrome c and subsequent caspase -9 and -3 activation (Castedo *et al.*, 2003).

Since neuronal apoptosis is often observed as a consequence of neuronal dysfunction in brains of patients with HAD, the need for identifying neuroprotective agent(s) is of paramount importance. Growth factors have been considered to play critical roles in promoting neurogenesis and survival of neurons (Pixley *et al.*, 1998; Jin *et al.*, 2002; Ohori *et al.*, 2006). Some of the factors, such as fibroblast growth factor and brain-derived neurotrophic factor, have been shown to protect neurons by down-modulating the expression of the coreceptor CXCR4, activating cell-survival signals, and inhibiting the internalization of HIV-1 coded proteins (Sanders *et al.*, 2000; Bachis *et al.*, 2003). Belonging to the family of growth factors, is also another factor, platelet derived growth factor (PDGF), that is widely expressed in both embryonic and adult CNS, where it is known to exert neurotrophic effects (Pietz *et al.*, 1996). It is an essential factor that is involved in neuroprotection, promotes neuronal differentiation (Williams *et al.*, 1997; Erlandsson *et al.*, 2001), and modulates synaptic transmission (Valenzuela *et al.*, 1997). Its protective role in HIV toxicity, however, has not been explored in detail and is the subject of this study. Based on our earlier findings demonstrating the protective effect of PDGF against gp120 toxicity in SH-SY5Y cells (Peng *et al.*, 2008), in the present study we sought to explore in depth the mechanism(s) of PDGF-mediated neuroprotection. Since control of cell survival by growth factors can be achieved either through the inhibition of apoptosis and/or the activation of survival signals, we examined whether PDGF-mediated protection involved caspase inactivation and activation of survival signal Akt. Our findings suggest the roles of both PI3K/Akt and Bcl family pathways in PDGF-mediated neuroprotection in SH-SY5Y cells. Detailed understanding of these pathways will prove useful in development of treatment modalities for HIV-dementia.

Materials and Methods

Materials

Human recombinant PDGF-BB was purchased from R&D Systems (Minneapolis, MN, USA) and viral gp120 (IIIB strain) was obtained from the AIDS Research and Reference Reagent Program of National Institutes of Health. The specific PI3-kinase inhibitor, LY294002 and MEK1/2 inhibitor, U0126 were purchased from Calbiochem (San Diego, CA). STI-571, an inhibitor of PDGF receptortyrosine kinase was obtained from Novartis, Basel, Switzerland and N-a-tosyl-L- phenyl alanine chloromethyl ketone (TPCK) was purchased from Sigma Chemicals (St. Louis, MO).

Cell culture and Treatments

Human neuroblastoma cells (SH-SY5Y) were purchased from American Type Culture Collection (Manassas, VA). Cells were cultured in a 1:1 mixture of Eagle's Minimum Essential Medium containing non-essential amino acids (Gibco, Gaithersburg, MD) and F12 Medium (Gibco) supplemented with heat inactivated fetal bovine serum (10% v/v), 2mM glutamine at 37°C in 5% CO₂. Confluent cells were replated at 1×10⁵ cells/ml and differentiated by

treatment with 10 μ M retinoic acid (Sigma) for 7 days with medium changes every two days. In all of the experiments cells were serum-starved for 24h prior to treatment with PDGF-BB for 30 min followed by addition of gp120 IIIB since serum contains growth factors that can mask the effects of the treatments. In the experiments involving pharmacological inhibitors, cells were pre-cultured with the corresponding inhibitor(s) for 1h prior to treatment with gp120 &/or PDGF-BB.

Rat Cortical Neuronal Cultures

All the animal experiments were conducted according to the guidelines of the Institutional Animal Care & Use Committee of Kansas University. Primary cultures of embryonic rat cortical neurons were prepared from 18-day-old fetuses of Sprague-Dawley rats as previously described (Brewer *et al.*, 1993). Briefly, pregnant rats were injected with an over dose of pentobarbital (50mg/rat), and pups were then removed and decapitated. Fetal rat brain cortices were harvested by removal of brainstem and hippocampi followed by mechanical trituration. Cells were then suspended in Neurobasal medium (Gibco) supplemented with 2 mM glutamine, 2% B-27 supplement, and 1% antibiotic and seeded at a density of 40,000 cells per well in 96 well plate or 5 $\times$ 10⁵ cells per well in 6-well plates (all plates were pre-coated with poly-D-lysine) and maintained at 37°C with 5% CO₂. At day 3 of incubation, half the medium was changed and the cells were cultured for additional 3 days. Cell purity was determined by immunocytochemistry using anti-MAP-2 antibody (Chemicon, Temecula, CA) and were found to be >95% pure.

Western blot Analysis

Embryonic rat cortical neuronal cultures or SH-SY5Y cells were treated according to the experimental setup followed by rinsing in chilled PBS and lysis in mammalian lysis buffer (Sigma) containing protease and phosphatase inhibitors (Pierce Chemical Co, Rockford, IL). Protein estimation was performed using the micro-BCA method (Pierce). Cell lysates were subjected to the separation by 12% SDS-PAGE electrophoresis (about 20 μ g protein per well) and transferred to PVDF membrane. Western blots were performed using the appropriate dilution of primary antibodies against the corresponding antigen (1:500 phospho-Akt, 1:200 phospho-PDGFR β , 1:200 phospho-GSK, 1:200 Bcl-xL, 1:200 Bax (all from Cell Signaling, Danvers, MA); 1:200 phospho-Bad ser136 (Santa Cruz Biotechnology, Santa Cruz, CA); 1:500 NF- κ B (Abcam, Cambridge, MA); and 1:4000 β -actin (Sigma). Each experiment was repeated at least three times.

Cell Transfections

SH-SY5Y cells were transfected with dominant negative Akt vector or Wild type Akt vector (gift from Dr. Scott Russo, University of Texas South Western Medical Center) using ExGene500 (Fermentas, Glen Burnie, MD) according to the manufacturer's instructions. Briefly, SH-SY5Y cells were seeded in 96-well plate at a density of 3 $\times$ 10⁴ cells per well. Twenty four hours later the medium was replaced with serum-free medium. To prepare the combination of Akt vectors and the transgenic reagent for each well, 0.3 μ g vector DNA was mixed with 20 μ l 150mM NaCl and 0.99 μ l ExGene500. The DNA mixtures were kept at room temperature for 10 min and were then added to the cell cultures. After gentle shaking and spinning at 1200g for 5 min, the transfected SH-SY5Y cells were then cultured at 37°C for 24h.

Lactate Dehydrogenase (LDH) Assay

The LDH release assay was performed using a CytoTox96 non-radioactive cytotoxicity assay (Promega, Madison, MI). The ratio of LDH released into the culture medium and total LDH of lysed cells were evaluated by this assay. Briefly, the SH-SY5Y cells were seeded at a density

of 5×10^4 cells per well in a 96-well microplate. Following the respective treatments, 50 μ l of medium was removed to a new multi-well plate to evaluate the LDH released into the medium. The cells were lysed by adding 15 μ l of the lysis solution (9% Triton X-100 in water) and incubated for 45 min at 37°C. After incubation, the plate was centrifuged (250 g for 4 min) and 50 μ l of the supernatant was transferred to another plate. Thereafter, 50 μ l of Substrate Mix solution provided in the kit was added to each well (both the supernatant and lysed cell fractions) and incubated at room temperature for 30 min. The reaction was stopped with 50 μ l of stop solution (1 M acetic acid) and the plates read at 490 nm. The ratio, released LDH: total LDH was then calculated as a measure of cell death. Each experiment consisted of three replicates and was repeated at least three times.

Caspase-3 activity assay

Activity of caspase 3 was analyzed using the Caspase 3 Colorimetric Assay Kit Systems (R&D) following the manufacturer's instructions. Briefly, SH-SY5Y cells were plated at 2×10^6 cells per well in 6-well plates. Following treatment, cells were collected and lysed with 50 μ l lysis buffer for 10 min on ice. The lysate was centrifuged at 200g for 5min and was incubated with 50 μ l of 2 $\times$ reaction buffer containing 0.5 μ l DTT and 5 μ l of the caspase-3 colorimetric substrate, DEVD-pNA. Two hour post-incubation at 37°C, caspase-3 protease activity was measured in a spectrophotometer at a wavelength of 405 nm. Absorbance was normalized to the protein concentration of each lysate, which was determined using the BCA Protein Assay Reagent (Pierce). The changes in caspase-3 activity in treated cells were presented relative to the values obtained from the untreated samples. Each experiment consisted of three replicates and was repeated at least three times.

Analysis of Mitochondria Membrane Depolarization

The change of mitochondrial membrane potential in the neurons was monitored using the mitochondrial membrane potential detection kit (Cell Technology, Mountain View, CA) according to the manufacturer's instructions. SH-SY5Y neuroblastoma cells, cultured in 24-well plate (1×10^5 per well) or 96-well plate (3×10^4 per well) were treated with gp120 and/or PDGF followed by treatment with 1X JC-1 reagent diluted in serum free culture medium for 20 min at 37°C, 5% CO₂. Thereafter, cells were rinsed once by 1X rinsing buffer provided in the kit. Uptake of dye was then assessed using Nikon inverted fluorescence microscope TE2000-E (Nikon, Tokyo, JAPAN), or fluorescence was measured using the FL600 fluorescent plate reader (Bio-Tek Instruments, Winooski, VT) at the excitation wavelengths of 485 nm and 535 nm.

Isolation of Cytochrome C and Cytochrome C ELISA Assay

The isolation of cytochrome C in the cytosolic fraction was performed using the Mitochondrial Fractionation Kit (Active motif, Carlsbad, CA) according to the manufacturer's instructions with slight modifications. Briefly, treated cells (2×10^7) were harvested following washing with ice-cold PBS. The cells were then lysed in cytosolic buffer and kept in ice for 15min. The lysates were homogenized using a Dounce pestle homogenizer for 40 strokes, and were then centrifuged at 600g for 5 min at 4°C. Cell supernatants were re-centrifuged at 11,000g for 20 min twice and the supernatant fractions were stored at -80°C for cytochrome C ELISA using the FunctionELISA™ Cytochrome C kit (Active motif). Briefly, 100 μ l samples were diluted to 50 μ g/ml in blocking buffer and were added to each well followed by incubation for 2hrs at room temperature. The samples were then rinsed with wash buffer three times followed by addition of 100 μ l of 1:1000 diluted detecting antibody and subsequent incubation for 1 hour at room temperature. One hundred microliter of HRP-conjugated streptavidin was then added following the removal of the detecting antibody and incubation continued for 1 hour. After the

incubation the plate was rinsed with wash buffer followed by addition of developing solution and reading of the plate at 450 nm in a spectrophotometer.

Co-immunoprecipitation

The procedure for immunoprecipitation was performed as described previously with slight modifications (Springer *et al.*, 2000). SH-SY5Y cells were treated with PDGF-BB and gp120 for varying times (0h, 2h, 6h and 12h), followed by lysis in RIPA buffer (50mM Tris, pH 8.0, 150 mM NaCl, 0.1% SDS, 1.0% NP-40, 0.5% Sodium Deoxycholate) containing proteinase and phosphatase inhibitors. For each sample, 200 µg of protein was used for coimmunoprecipitation. The sample protein was incubated with 1:100 diluted anti-14-3-3 antibody (Santa Cruz Biotechnology) overnight at 4°C followed by incubation with 20µl of protein A-Sepharose for 3h at 4°C. The mixture was then centrifuged (at 6,000g, 30 sec) and the cell pellets were rinsed twice with RIPA, followed by boiling in 2× Western blot loading buffer for 4 min. After spinning (at 6,000g, 30 sec), the supernatants were subjected to Western blot as described above.

Statistical Analysis

Statistical analysis was performed using one-way analysis of variance with a post hoc Student t test. Results were judged statistically significant if $p < 0.05$ by analysis of variance.

Results

Functional role of Akt in PDGF-BB mediated neuroprotection

To gain insights into the role of Akt in protection conferred by PDGF-BB, we resorted to a pharmacological approach using inhibitors specific for PDGF-βR as well as Akt. Since binding of PDGF-BB to its cognate tyrosine kinase receptor results in receptor phosphorylation, we first wanted to investigate the kinetics of this process. As shown in Fig 1A, 15 min PDGF-BB treatment of neuronal SH-SY5Y cells resulted in the phosphorylation of PDGF-βR. This effect was blocked by pretreatment of the cells with the tyrosine kinase antagonist, STI-571. Upon binding to its receptor, PDGF-BB can regulate cell proliferation and differentiation via activation of both PI3K/Akt and MEK/Erk pathways (Chang *et al.*, 2003; Song *et al.*, 2005). We explored the activation of Akt in SH-SY5Y cells exposed to PDGF-BB, and did detect phosphorylation of Akt that could also be blocked by STI-571.

Confirmation of the functional role of AKt was further elucidated by LDH toxicity assays. Pre-treatment of SH-SY5Y cells with PDGF-βR antagonist (STI-571) prior to PDGF exposure resulted in complete abrogation of Akt activation as expected (Fig 1A) and also resulted in loss of protection mediated by PDGF-BB, thus underscoring the role of PDGF/PDGF-βR axis in regulating neuroprotection. To assess the role of Akt in protection by PDGF, we next pre-treated neuronal cells with the PI3K inhibitor, LY294002. Since PI3K lies upstream of Akt, pre-treating cells with LY294002 would be expected to result in abrogation of Akt activation. As shown in Fig. 1B, in cells pretreated with LY294002 there was abrogation of PDGF-BB protective effect, thus emphasizing the role of Akt in neuroprotection.

Since gp120 is known to induce caspase 3-mediated apoptosis in neurons (Bodner *et al.*, 2002; Castedo *et al.*, 2003), we next sought to determine whether blocking Akt activation in PDGF and gp120 treated cells could lead to induction of apoptosis via caspase-3 activation. As shown in Fig. 1C, in the absence of PI3K inhibition, PDGF suppressed caspase-3 activation as expected and, this effect was abrogated in the presence of LY294002, thus highlighting the role of Akt in PDGF-mediated neuroprotection.

Further confirmation of the role of Akt in PDGF-mediated neuroprotection was demonstrated by transfecting SH-SY5Y cells either with the dominant negative (DN) or the wild type (WT) constructs of Akt prior to PDGF and/or gp120 exposure. As shown in Fig. 1E in cells transfected with DN Akt, PDGF did not mediate protection against gp120. In contrast, cells transfected with WT Akt were responsive to PDGF protection.

Bad but not NF- κ B plays a role in PDGF-mediated neuroprotection

GSK3 β which lies downstream of Akt is an important signaling protein implicated in the translocation of several transcription factors involved in the proliferation and differentiation of the cells (Brunet *et al.*, 1999; Kane *et al.*, 1999). We therefore sought to explore the role of GSK3 β in PDGF-mediated neuroprotection. Western blot analysis demonstrated that in both PDGF-BB treated SH-SY5Y and rat primary neurons there was time dependent phosphorylation of GSK3 β . Interestingly, however, the downstream transcription factor, NF- κ B remained unaffected in cells treated with PDGF-BB (Figs. 2A & B). This was further confirmed using the pharmacological inhibitor of NF- κ B, TPCK, which failed to block PDGF-BB mediated protection against gp120, thus negating its role in PDGF-neuroprotection (Fig. 2C).

Recent studies have indicated that the inactivation of the pro-apoptotic mediator Bad by Akt is critical for cell survival (Kennedy *et al.*, 1999). In the present study, we next sought to examine whether PDGF-BB could phosphorylate and thus, inactivate Bad in SH-SY5Y cells. As shown in Fig. 3A, PDGF-BB treatment of SH-SY5Y cells resulted in phosphorylation of Bad at Serine 136 and, this effect could be blocked by the PI3K inhibitor, but not by the Erk inhibitor. Furthermore, since Bad inactivation leads to its uncoupling from the Bcl-xL and a concomitant coupling with 14-3-3 protein, we next examined whether PDGF-BB pretreatment of gp120-exposed SH-SY5Y cells resulted in binding of Bad with the protein 14-3-3 using immunoprecipitation followed by subsequent Western blot assays. As shown in Fig. 3B PDGF-BB treatment resulted in inactivation of Bad with its coupling to protein 14-3-3.

Regulation of Bax and Bcl-xL expression by PDGF-BB

Bax and Bcl-xL, the critical pro- and anti-apoptotic proteins (Tamatani *et al.*, 1998) respectively, play key roles in maintaining mitochondrial membrane integrity thus regulating the release of cytochrome C into the cytoplasm. Ratio of the levels of Bax/Bcl-xL proteins is considered an indicator of cell apoptosis. To gain insights into the role of these complementary proteins, we examined time-dependent expression of these proteins in SH-SY5Y cells treated with PDGF-BB and/or gp120. As shown in Fig. 4A, treatment of cells with gp120 resulted in increased and sustained time-dependent expression of Bax beginning around 9h post gp120 exposure. Levels of Bcl-xL, on the other hand, remained unaffected following gp120 treatment. Interestingly, unlike the gp120-treated cells, SH-SY5Y cells pretreated with PDGF-BB demonstrated time-dependent decrease in the expression of Bax protein (Fig. 4C). Furthermore, treatment of PDGF-BB exposed cells with the PI3K inhibitor abrogated PDGF-mediated down-regulation of Bax (Fig. 4E).

Release of Cytochrome C from mitochondria is inhibited by PDGF-BB

Mitochondrion-mediated pathway is involved in HIV-protein mediated neuronal apoptosis (Corasaniti *et al.*, 2005). To explore whether the PDGF mediated protection against gp120 reversed the mitochondrial damage, we performed experiments to examine the membrane potential and the release of the pro-apoptotic molecule, cytochrome C in the cytoplasm, following treatment of the cells with gp120 and/or PDGF-BB. We sought to explore neuronal mitochondrial membrane depolarization after 8hrs exposure of cells to PDGF-BB &/or to gp120 using JC-1, a fluorescent lipophilic cationic dye that accumulates in mitochondria in proportion to the electrical potential differential ($\delta\psi$) that normally exists across the inner

mitochondrial membrane (Cossarizza *et al.*, 1993). As shown in Fig. 5A & B, there was increased membrane depolarization in the presence of gp120, and this was reversed in the presence of PDGF-BB. Further we observed an increase in cytochrome C levels in the cytosolic fractions following incubation of SH-SY5Y cells with gp120 for 24 hr, as evidenced in Fig. 5C. Pre-treatment of cells with PDGF-BB however, reversed this effect ($p < 0.05$). Interestingly, in the presence of PI3K inhibitor, PDGF-BB could not reverse the gp120-induced elevation of cytosolic cytochrome C. These findings thus suggest that activation of PI3K or its downstream signal(s) are critical for the maintenance of mitochondrial integrity.

Discussion

A large number of HIV-1-infected individuals develop varying degrees of neurological deterioration collectively termed as HIV-associated neurocognitive disorder (HAND) (Ances & Ellis, 2007). Although the syndrome is characterized by neuronal dysfunction and/or death, neurons are not infected by the virus. The current thinking about the model of HIV-1 neuropathogenesis is thus that indirect consequences of virus-infected mononuclear phagocytes lead to neuronal degeneration (Nath, 1999). Based on this model it has been suggested that virus infected and/or activated cells contribute to neuropathogenesis through secretion of viral (gp120, Tat and Nef) and cellular (cytokines, chemokines, and nitric oxide) neurotoxic products (Nath, 1999). Among the various HIV-1 proteins, neurotoxic potential of the viral envelope gp120 has been well documented both *in vitro* (Brenneman *et al.*, 1988; Dreyer *et al.*, 1990) and *in vivo* (Bageetta *et al.*, 1996; Tabatabaie *et al.*, 1996).

We have previously reported that PDGF protects neurons against gp120 toxicity, with possible involvement of the Akt survival pathway (Peng *et al.*, 2008). In the current study, we provide evidence for the direct roles of PI3K, and its downstream signals Akt and the Bcl family proteins as key signaling players contributing to PDGF/PDGF-receptor axis-mediated neuronal survival against gp120 toxicity. We demonstrate that treatment of neuroblastoma cell line, SH-SY5Y cells with the pharmacological inhibitor of PI3K inhibitor or transfection of cells with the dominant negative Akt vector abrogated PDGF-mediated protection against gp120 toxicity. Role of PI3K/Akt pathway in cell survival has also been demonstrated in protection by other growth factors as well (Hashimoto *et al.*, 2002; Almeida *et al.*, 2005; Langford *et al.*, 2005). For example, Akt has been shown to be involved in protection of neurons by brain-derived growth factor (Almeida *et al.*, 2005).

Activation of the PI3K/Akt pathway results in phosphorylation of the downstream GSK-3 β , a physiologically relevant principal regulatory target of the Akt pathway (Facci *et al.*, 2003). Phosphorylation of GSK-3 β often leads to its inactivation, which consequently results in nuclear translocation of key downstream transcription factors such as β -catenin and NF- κ B (Romashkova & Makarov, 1999; Fang *et al.*, 2007). Interestingly in the present study, similar to that seen with fibroblast growth factor (FGF) (Hashimoto *et al.*, 2002), PDGF could also induce phosphorylation of GSK-3 β . However, unlike FGF, PDGF did not induce nuclear translocation of either NF- κ B or β -catenin (data not shown). Thus contrary to neuroprotection mediated by other relevant growth factors (Hashimoto *et al.*, 2002), PDGF-mediated protection of neurons was independent of either NF- κ B or β -catenin translocation.

PI3K/Akt signal can also be transduced through alternative pathways, such as Bad or Bax, which belong to the Bcl family of proteins that are substrates of Akt. In the case of pro-apoptotic Bad protein, Akt mediated cell survival involves phosphorylation of Bad resulting in its inactivation and subsequently its coupling with another partner protein 14-3-3. Partnering of Bad with 14-3-3 can facilitate cell survival. Additionally, sequestration of Bad by 14-3-3 and its concomitant uncoupling from Bcl-2 or Bcl-xL, can also promote cell survival by maintaining mitochondrial integrity (Datta *et al.*, 1997; Yin *et al.*, 2005). In the present study we found that

PDGF treatment of SH-SY5Y cells resulted in time-dependent phosphorylation of Bad that could be blocked by PI3K inhibitor. Furthermore, we also demonstrated the coupling of Bad with 14-3-3 in gp120 exposed cells pretreated with PDGF, thus underpinning the role of these proteins in PDGF-mediated neuroprotection.

Akt can also regulate cell survival by modulating the levels of pro-apoptotic and anti-apoptotic proteins, Bax and Bcl-xL respectively, the ratios of which determine the stability of the mitochondrial membrane (Vogelbaum *et al.*, 1998). We have clearly shown that in the presence of gp120, there was increased expression of the pro-apoptotic Bax, while the levels of Bcl-xL remained unaffected resulting in increased Bax to Bcl-xL ratio, thus exemplifying apoptosis. In contrast, PDGF pretreatment of neurons resulted in down-regulation of the of the pro-apoptotic Bax, leading to a decrease in Bax to Bcl-xL ratio, thus exemplifying cell survival.

Work by various groups has suggested the mitochondria as a link between the initial apoptotic signal and the endpoint biochemical reactions leading to caspase activation (Kluck *et al.*, 1997; Yang *et al.*, 1997). It is suggested that in response to death stimuli, mitochondrial membranes are permeabilized (Newmeyer *et al.*, 1994; Kroemer, 1999), thereby causing the release of cytochrome C (Kluck *et al.*, 1997; Green & Reed, 1998). Mitochondria play a central role in programmed cell death, and dissipation of the mitochondrial transmembrane potential is a critical early event of the lethal process, resulting in release of cytochrome C and other caspase-activating factors into the cytosol. We observed increase in neuronal mitochondrial membrane depolarization on exposure of cells to gp120 using JC-1 dye and this increase in membrane potential was reversed with PDGF-BB treatment. This technique has been successfully used to assess the functional status of mitochondria in cultured neurons (White & Reynolds, 1996). In addition, we also saw evidence of mitochondrial release of cytochrome C following gp120 treatment of SH-SY5Y neurons, an effect that was reversed by PDGF pretreatment. These findings are in contrast to the studies by Singh *et al.* wherein they demonstrated gp120-mediated apoptosis of rat striatal neurons via a cytochrome C independent pathway (Singh *et al.*, 2004). Such a discrepancy could be attributed to the cell types used in the respective studies.

Based on our current findings we suggest a potential survival cascade that is initiated by PDGF/PDGF-R axis in gp120-exposed neurons. As shown in Fig. 6, PDGF-BB binding to its cognate PDGF- β R, results in receptor phosphorylation with transduction of signal via activation of the PI3K-Akt pathway. This in turn, leads to phosphorylation of several downstream targets including GSK3 β and Bad. The protective effect of PDGF against gp120-induced apoptosis was mediated by activation of Akt involving the inhibition of Bad translocation from the cytosol to the mitochondria by the induction of protein protein interactions between 14-3-3 and phospho-Bad. It has been reported that the phosphorylation of Bad at either Ser-112 or Ser-136 facilitates formation of a complex between Bad and 14-3-3 in the cytosol, blocking its interaction with Bcl-xL at the mitochondrial level (Datta *et al.*, 1997). Our results showed that PDGF treatment increased the phosphorylation of Bad in gp120-treated SH-SY5Y cells. Thus PDGF inhibited gp120-induced apoptosis by increasing the interaction between 14-3-3 and phospho-BAD, thus preventing Bad interaction at the mitochondria to initiate apoptosis. Furthermore, PDGF treatment not only increased the protein protein interactions between 14-3-3 and phospho-Bad, but also decreased the release of cytochrome C from mitochondria into the cytosol.

Additionally, PDGF also modulated Akt-mediated survival by regulating the levels of pro and anti-apoptotic Bax and Bcl-xL respectively. PDGF can also protect neurons against gp120 toxicity by down-regulating the expression of pro-apoptotic Bax, thus resulting in decreased Bax to Bcl-xL ratio, a process that can inhibit mitochondrial permeability. Overall, this then results in reduced cytosolic cytochrome C.

In conclusion, we have demonstrated that the molecular action of PDGF-BB against gp120-induced apoptosis is mediated by activation of Akt and its downstream targets, specifically, partnering of 14-3-3 with phospho-Bad and down-regulation of pro-apoptotic protein, Bax. These results could provide possible therapeutic options for the neurodegeneration observed in HIV-infected patients with AIDS.

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Abbreviations

PDGF	Platelet-derived growth factor
PI3K	phosphatidylinositol 3-kinase
HAD	Human immunodeficiency virus type 1-associated dementia
SH-SY5Y	Human neuroblastoma cells
TPCK	N-a-tosyl-L- phenyl alanine chloromethyl ketone
Jc-1	5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide

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Figure 1.

Platelet-derived growth factor (PDGF)-BB mediated neuroprotection against gp120 involves PI3K-Akt pathway. **(A)** Human neuroblastoma cells (SH-SY5Y) cells exposed to PDGF-BB (20ng/ml) and gp120 (200ng/ml) for 15 min demonstrate increased phosphorylation of PDGF-βR and Akt. Pre-treatment of SH-SY5Y cells with PDGF receptor antagonist, STI-571 (1μM) inhibited PDGF-BB-mediated activation of PDGF-βR and Akt. **(B)** Cell death in SH-SY5Y cells exposed to PDGF &/or gp120 was analyzed by LDH release assay. Gp120 treatment of SH-SY5Y neuroblastoma resulted in increased toxicity, an effect that was abrogated by PDGF-BB. Pre-treatment of neuroblastoma with either the PI3K inhibitor (LY294002; 10μM) or the tyrosine kinase antagonist (STI-571; 1μM) mitigated PDGF-mediated neuroprotection. **(C)** PDGF exposure inactivates gp120-induced activation of caspase-3. Gp120 induced caspase-3 activation, which was mitigated by PDGF-BB exposure of neurons. This effect involved PI3K pathway since pretreatment of neuroblastoma with LY294002 followed by PDGF exposure resulted in activation of caspase-3. **(D)** SH-SY5Y cells were transfected with or without the dominant negative Akt or Wild type Akt constructs, and subsequently treated with PDGF-BB and/or gp120. Cells transfected with the dominant negative Akt construct demonstrated inhibition of PDGF-mediated activation of Akt. This also resulted in the corresponding increased toxicity in neuroblastoma exposed to PDGF and gp120. **(E)** Wild-type Akt construct, on the other hand, did not block PDGF-mediated neuroprotection against gp120. The figures shown are representatives of three independent experiments and the data are presented as mean ± SEM. * $p < 0.05$, *** $p < 0.001$ versus control.

Figure 2.

NF-κB is not involved in Platelet-derived growth factor (PDGF)-mediated neuroprotection. Human neuroblastoma cells (SH-SY5Y) cells or rat primary neurons were exposed to gp120 (200ng/ml) and/or PDGF-BB (20ng/ml) for varying times and cell lysates were prepared. Western blot (**A and B**) analysis was then performed on cell lysates to test the role of GSK3β and NF-κB in PDGF-mediated neuroprotection. The immunoblots showed that GSK3β was inactivated through phosphorylation induced by PDGF and gp120. The cytosolic levels of p65 subunit of NF-κB however, remained unchanged in both SH-SY5Y and rat primary neurons. (C) To confirm that NF-κB did not play a role in PDGF-mediated protection, we next pretreated rat primary neurons with TPCK (N-α-tosyl-L-phenyl alanine chloromethyl ketone; 2μM) prior to exposure with gp120 and/or PDGF. TPCK did not impact the protective effect of PDGF against gp120 neurotoxicity. The figures are representatives of three independent experiments and the data are presented as mean ± SEM. * p<0.05 versus control.

Figure 3.

Platelet-derived growth factor (PDGF) inactivates pro-apoptotic protein Bad. Human neuroblastoma cells (SH-SY5Y) cells pretreated with or without the PI3-K or MEK inhibitors (LY294002 or U0126, respectively; both at 10 μ M) and were subsequently exposed to PDGF-BB and gp120 for varying times. Following treatment cells were lysed and were subjected to Western blot analysis for detecting the phosphorylation of Bad at serine 136 position. **(A)** Exposure of cells to both PDGF and gp120 resulted in time-dependent phosphorylation of Bad at serine 136 position. This effect could be blocked by PI3K inhibitor but not by the MEK inhibitor. **(B)** Parallel cell lysates were also used in co-immunoprecipitation assays to detect the coupling of Bad with its binding partner 14-3-3. Co-immunoprecipitation results using immunoprecipitation with 14-3-3 antibody followed by Western blotting of the complex using phospho-Bad antibody demonstrated that as early as 2h following PDGF and gp120 exposure, Bad protein bound to its partner 14-3-3. The figure shown is a representative of three independent experiments. N indicates antibody negative control.

Figure 4.

Platelet-derived growth factor (PDGF)-mediates neuroprotection by regulating expression of Bax and Bcl-xL. (**A & C**) Human neuroblastoma cells (SH-SY5Y) cells were treated with PDGF-BB and/or gp120 for varying times from 0 to 24 hours as indicated and cell lysates were run on a Western blot for detection of levels of pro-apoptotic (Bax) and anti-apoptotic proteins (Bcl-xL). In the presence of gp120 there is increased time-dependent expression the pro-apoptotic protein Bax. In the presence of PDGF and gp120, there is a time-dependent downregulation of Bax resulting in decreased Bax to Bcl ratio, thus indicating cell survival. Panels **B & D** are densitometric scans of levels of Bax and Bcl in **A & C**, respectively. (**E & F**) Cells were treated with PDGF & gp120 for 12 hrs and cell lysates run on a Western blot for detection of Bax and Bcl proteins. In the presence of PI3K inhibitor, PDGF-mediated reduction of pro-apoptotic Bax is inhibited. The immunoblots are a representative of three independent blots and the data are presented as mean $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus untreated.

Figure 5. Mitochondrial membrane depolarization and cytochrome C release from mitochondria. **(A and B)** Human neuroblastoma cells (SH-SY5Y) cells were treated with gp120 IIIB (200ng/ml) &/ or PDGF-BB (20ng/ml) for 8hrs, or staurosporine (1 μ M) for 4h, followed by staining with JC-1 dye. The gp120 exposure resulted in reduction of the aggregation of JC-1 dye in the mitochondria (red fluorescence) and decreased ratio of the aggregate (red fluorescence) to monomer JC-1 (green fluorescence) in the cells as shown in **B**, which could be reversed by PDGF-BB; **(C)** SH-SY5Y cells were pretreated with or without PI3K inhibitor in the presence of platelet-derived growth factor (PDGF) &/or gp120 for 1h and assessed for cytosolic cytochrome C using the cytochrome C ELISA kit. Gp120-mediated up-regulation of cytochrome C release was significantly inhibited by pretreatment of cells with PDGF. In the presence of PI3K inhibitor, PDGF was not able to down-regulate the release of cytochrome C. Data are mean $\pm$ SEM from three independent experiments. * $p < 0.05$ versus control, # $p < 0.05$ versus gp120 alone.

Figure 6.

Schematic illustration demonstrating the roles of Akt and Bcl pathways in platelet-derived growth factor (PDGF)-mediated neuroprotection. PDGF binding to its cognate PDGFR-β can result in activation of Akt and Erk1/2. Activation of Akt results in phosphorylation of activation of several downstream substrates, such as Bad, Bax and GSK3β. PDGF-mediated inactivation of GSK3β however, does not result in translocation of NF-κB to the nucleus. On the other hand, Akt phosphorylation resulting in inactivation of pro-apoptotic protein Bad leads to the association of Bad with 14-3-3. Another pathway by which PDGF-mediate neuroprotection is through down-regulation of expression of Bax induced by gp120, inhibition of release of mitochondrial cytochrome C thus culminating in neuronal survival,