Fibroblast Growth Factor 1 Regulates Signaling via the Glycogen Synthase Kinase-3β Pathway

IMPLICATIONS FOR NEUROPROTECTION

We hypothesize that in neurodegenerative disorders such as Alzheimer’s disease and human immunodeficiency virus encephalitis the neuroprotective activity of fibroblast growth factor 1 (FGF1) against several neurotoxic agents might involve regulation of glycogen synthase kinase-3β (GSK3β), a pathway important in determining cell fate. In primary rat neuronal and HT22 cells, FGF1 promoted a time-dependent inactivation of GSK3β by phosphorylation at serine 9. Blocking FGF1 receptors with heparinase reduced this effect. The effects of FGF1 on GSK3β were dependent on phosphatidylinositol 3-kinase (PI3K)-protein kinase B (Akt) because inhibitors of this pathway or infection with dominant negative Akt adenovirus blocked inactivation. Furthermore, treatment of neuronal cells with FGF1 resulted in ERK-independent Akt phosphorylation and β-catenin translocation into the nucleus. On the other hand, infection with wild-type GSK3β recombinant adenovirus-associated virus increased activity of GSK3β and cell death, both of which were reduced by FGF1 treatment. Moreover, FGF1 protection against glutamate toxicity was dependent on GSK3β inactivation by the PI3K-Akt but was independent of ERK. Taken together these results suggest that neuroprotective effects of FGF1 might involve inactivation of GSK3β by a pathway involving activation of the PI3K-Akt cascades.

Neurotrophic factors are capable of maintaining particular neuronal populations during cellular stress. While some factors, such as nerve growth factor, support a narrowly defined neuronal population (e.g. cholinergic neurons), other factors such as fibroblast growth factor (FGF) support more diverse populations (1). Among the more than 20 members of the FGF family (2, 3), FGF1 (or acidic FGF) is abundant in sensory and motor neurons, and FGF2 (or basic FGF) is primarily produced by astrocytes, although it can be taken up by neurons and translocated to the nucleus (4). Of the four FGF receptors (FGFRs), three are found in the brain: FGFR1 is mainly expressed on neurons, while FGFR2 and FGFR3 are found on glial cells (1, 2, 6, 7). Binding of FGF leads to dimerization of FGFR followed by tyrosine kinase activation (2). FGF2 promotes survival of cortical and hippocampal neurons (8, 9) and is also capable of rescuing neurons from denervation and injury (1). Similarly, FGF1 protects selective neuronal populations against the neurotoxic effects of molecules involved in the pathogenesis of neurodegenerative disorders such as Alzheimer’s disease (10, 11) and HIV encephalitis (12).

FGF1 and -2 are potent regulators of central nervous system development (13, 14) and maintenance after neuronal injury (1). However, there is no consensus as to the signal transduction pathways initiated by FGF during neuronal differentiation or during neuroprotection. Some studies suggest that during FGF2-induced neuronal differentiation (i) activation of a mitogen-activated protein kinase, such as extracellular signal-regulated kinases (ERK1 and ERK2), is neither necessary nor sufficient, (ii) activation of Src kinases is necessary but not sufficient, and (iii) FGF2 requires at least two signaling pathways activated by Ras and Src (15–17). These results indicate that the neuroactive effects of FGF depend on signaling pathways other than ERK. Most studies have focused on the neurotrophic effects of FGF2, while fewer studies have investigated the effects of FGF1. We hypothesize that the neurotrophic effects of FGF1 might involve regulation of other signaling cascades such as the glycogen synthase kinase-3β (GSK3β) pathway, which is important in determining cell fate (18, 19). Supporting this possibility, a recent study showed that FGF2-mediated tau hyperphosphorylation was inhibited by lithium, an inhibitor of GSK3β, but not by inhibitors of ERK or the cyclin-dependent kinases (20). For the present study, we investigated the effects of FGF1 on the GSK3β pathway in rat primary neuronal and HT22 cells. Our results suggest that the neuroprotective properties of FGF1 might involve phosphorylation-mediated inactivation of GSK3β via the phosphatidylinositol 3-kinase (PI3K)-Akt signaling pathway.

EXPERIMENTAL PROCEDURES

Cell Culture and Treatments—All experiments were performed with rat primary cortical neurons prepared from embryonic day 17 Sprague-Dawley rats as described previously (21) and with HT22 cells (22), mouse hippocampal cells derived from the HT4 cell line (23). Briefly, primary cortical neurons were dissociated from the cortex and maintained in tissue culture dishes coated with 100 μg/ml poly-b-lysine in
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minimum Eagle’s medium supplemented with 30 mM glucose, 2 mM glutamine, 1 mM pyruvate, and 10% fetal bovine serum. Cultures were used within 1 week after preparation. HT22 cells were maintained at no greater than 70% confluence in 10% fetal bovine serum in Dulbecco’s modified Eagle’s medium (high glucose, Irvine Scientific, Irvine, CA) with 1% penicillin/streptomycin (Invitrogen).

To analyze the effects of FGF1 on neurons, cells were placed in N-2-supplemented serum-free medium (Invitrogen) 24 h before treatment. Neurons were then exposed to FGF1 (10 ng/ml, Sigma) for 6 h. After washing with PBS, cells were incubated at 37°C with 5, 10, 30, and 60 min and analyzed by immunoblot for levels of GSK3β. Neurons were then exposed to FGF1 (10 ng/ml, Sigma) for 0, 1, 2, and 3 cycles. The lysates were centrifuged at 10,000 rpm, and protein concentration was determined using the BCA reagent (Pierce). Two hundred micrograms of the supernatant were preabsorbed with a protein G-Sepharose (Amersham Biosciences) for 1 h, and the precleared lysates were incubated with anti-GSK3β monoclonal antibody (1:50, Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4°C followed by incubation with protein G-Sepharose for 2 h at 4°C. The immune complexes were then washed twice with the lysis buffer and twice with kinase buffer (20 mM HEPES, pH 7.2, 0.1 mM Na3VO4, 10 mM glycerophosphate, 10 mM MgCl2, 1 mM dithiothreitol, 5 μg/ml aprotinin, and 5 μg/ml leupeptin). The cell lysates were then centrifuged for 10 min at 14,000 rpm, and protein concentration was determined using the BCA reagent (Pierce). Two hundred micrograms of the supernatant were preabsorbed with a protein G-Sepharose (Amersham Biosciences) for 1 h, and the precleared lysates were incubated with anti-GSK3β monoclonal antibody (1:50, Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4°C followed by incubation with protein G-Sepharose for 2 h at 4°C. The immune complexes were then washed twice with the lysis buffer and twice with kinase buffer (20 mM HEPES, pH 7.2, 0.1 mM Na3VO4, 10 mM glycerophosphate, 10 mM MgCl2, 1 mM dithiothreitol, 0.1 mM EDTA). Finally, the immune complexes were incubated in 30 μl of the kinase buffer containing either 2.5 μg of phosphoglycerokinase synthase-2 peptides or the Ala-21 mutant peptides (Upstate Biotechnology, Lake Placid NY) and 10 μCi of [γ-32P]ATP (6000 Ci/mmol, PerkinElmer Life Sciences) for 20 min at 30°C. Reactions were terminated by the addition of 5 μl of 50% PPO in 5 μl of 0.3 M ATP. Samples were then spotted onto Whatman P81 phosphocellulose filter paper. The filters were washed with 180 mM phosphoric acid, dried with acetone, and scintillation counting.

Immune complex kinase assays for Akt were performed essentially as described previously with some modifications (18). Briefly, for the GSK3β assay, cells were rinsed twice with cold PBS and incubated for 20 min on ice in lysis buffer (1% Triton X-100, 10% glycerol, 50 mM HEPES, pH 7.4, 140 mM NaCl, 1 mM EDTA, 1 mM Na3VO4, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 5 μg/ml aprotinin, and 5 μg/ml leupeptin). The cell lysates were then centrifuged for 10 min at 14,000 rpm, and protein concentration was determined using the BCA reagent (Pierce). Two hundred micrograms of the supernatant were preabsorbed with a protein G-Sepharose (Amersham Biosciences) for 1 h, and the precleared lysates were incubated with anti-GSK3β monoclonal antibody (1:50, Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4°C followed by incubation with protein G-Sepharose for 2 h at 4°C. The immune complexes were then washed twice with the lysis buffer and twice with kinase buffer (20 mM HEPES, pH 7.2, 0.1 mM Na3VO4, 10 mM glycerophosphate, 10 mM MgCl2, 1 mM dithiothreitol, 0.1 mM EDTA). Finally, the immune complexes were incubated in 30 μl of the kinase buffer containing either 2.5 μg of phosphoglycerokinase synthase-2 peptides or the Ala-21 mutant peptides (Upstate Biotechnology, Lake Placid NY) and 10 μCi of [γ-32P]ATP (6000 Ci/mmol, PerkinElmer Life Sciences) for 20 min at 30°C. Reactions were terminated by the addition of 5 μl of 50% PPO in 5 μl of 0.3 M ATP. Samples were then spotted onto Whatman P81 phosphocellulose filter paper. The filters were washed with 180 mM phosphoric acid, dried with acetone, and scintillation counting.

Immune complex kinase assays for Akt were performed essentially as described previously with some modifications. Briefly, the precleared lysates were incubated with polyclonal anti-human Akt antibody (anti-protein kinase B-(88–126) (1 μg/sample) (Calbiochem) followed by incubation in protein G-Sepharose. After washing, immune complex assays were performed in the presence of 1.0 μg of GSK3β fusion protein (Cell Signaling, Beverly, MA) as substrate. Reactions were terminated by the addition of the SDS sample buffer. The samples were then subjected to SDS-PAGE (15%) analysis followed by autoradiography. Quantification was performed with the PhosphoImager using the ImageQuant software (Amersham Biosciences).

Immunocytochemical Analysis of β-Catenin by Laser Scanning Confocal Microscopy—Further analysis of catalytically active GSK3β was carried out in neuronal cultures by immunocytochemical visualization of the cellular localization of β-catenin. Using this method, inactivation of GSK3β is associated with nuclear translocation of β-catenin (32, 33). Briefly, cells were plated on coverslips, exposed to FGF1 in the presence or absence of inhibitors as described above, and fixed with 10 min of 4% paraformaldehyde in PBS. Cells were then immunolabeled with the mouse monoclonal antibody against β-catenin (1:1000, Transduction Laboratories) followed by incubation with fluorescein isothiocyanate-conjugated horse anti-mouse IgG (1:75) (Vector Laboratories, Inc.). Cells on coverslips were analyzed with a laser
FGF1 Promotes Akt, GSK3β, and ERK Phosphorylation in Neuronal Cells—To investigate the effects of FGF1 on Akt, GSK3β, and ERK, we first determined the time course for phosphorylation of these protein kinases in rat cortical neurons and HT22 cells. Western blot analysis showed that stimulation with FGF1 resulted in maximum Akt, GSK3β, and ERK phosphorylation at 10–15 min followed by a progressive decrease reaching non-detectable levels at 60 min (Fig. 1). Since FGF1 signal transduction involves binding both at the high affinity FGF receptor and the low affinity heparin sulfate receptor, optimizing the effects of FGF (24), experiments were performed with heparin and heparinase. While pretreatment with heparin enhanced FGF1-mediated phosphorylation of Akt and GSK3β and ERK by 24-h treatment with FGF1 resulted in maximum Akt, GSK3β, and ERK phosphorylation at 10–15 min followed by a progressive decrease reaching non-detectable levels at 60 min (Fig. 1). Since FGF1 signal transduction involves binding both at the high affinity FGF receptor and the low affinity heparin sulfate receptor, optimizing the effects of FGF (24), experiments were performed with heparin and heparinase. While pretreatment with heparin enhanced FGF1-mediated phosphorylation of all three kinases (not shown).

FGF1 Regulation of GSK3β Activity Is Dependent on the PI3K-Akt Pathway—Since time course experiments (Fig. 1) suggested that the effects of FGF1 on GSK3β might be mediated via either the PI3K-Akt or the ERK signaling pathways, further analysis of the molecular events was conducted by treating cells with the PI3K inhibitor LY294002 or the ERK inhibitor PD98059. Inhibition of PI3K, which is upstream of Akt (18), resulted in

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**Fig. 1.** Time course of FGF1 effects on GSK3β, Akt, and ERK phosphorylation in primary cortical neurons. A Western blot demonstrates the inducible expression of phospho-Akt, -GSK3β, and -ERK and constitutive expression of total Akt, GSK3β, and ERK by exposure to FGF1, 1–15 minutes.

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**Fig. 2.** Effects of heparinase on FGF1-mediated Akt, GSK3β, and ERK phosphorylation in primary cortical neurons. Western blot analysis showed that pretreatment of neuronal cells with heparinase (1 unit/ml, 1 h) reduced the effects of FGF1 in promoting phosphorylation of Akt and GSK3β but had no effect on ERK phosphorylation.

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**Fig. 3.** Effects of inhibitors on FGF1-mediated phosphorylation on Akt, GSK3β, and ERK in primary cortical neurons. The inhibitor of PI3K-Akt (LY294002) abolished expression of phospho-Akt and phospho-GSK3β, while neither of the ERK inhibitors (U0126 and PD98059) suppressed expression of phospho-Akt or phospho-GSK3β.
decreased phosphorylation of Akt and GSK3β but had no effect on ERK phosphorylation (Fig. 3). In contrast, inhibitors of the ERK pathway completely blocked FGF1-mediated ERK phosphorylation with only slight effects on Akt and GSK3β phosphorylation (Fig. 3). Taken together, these results indicate that phosphorylation of GSK3β by FGF1 stimulation is mediated via the PI3K-Akt pathway but is independent of ERK.

To further confirm that FGF1-mediated phosphorylation of GSK3β via PI3K-Akt resulted in inactivation of GSK3β, immunocomplex kinase assays were performed. FGF1 treatment decreased GSK3β activity by 40%, while inhibition of the PI3K-Akt pathway with LY294002 re-established GSK3β activity even in the presence of FGF1 (Fig. 4A). Inhibition of the ERK pathway with U0126 did not interfere with the FGF1-mediated effect on GSK3β activity (Fig. 4A). Moreover, while transfection of neuronal cells with dominant negative Akt re-established GSK3β activity and blocked FGF1 effects on GSK3β (Fig. 4B), constitutively active Akt reduced GSK3β activity in a similar fashion to FGF1 (Fig. 4B). Immunocomplex activity assays for Akt confirmed that dominant negative Akt blocked FGF1 effects on Akt (Fig. 4C), and constitutively active Akt increased basal Akt activity levels (Fig. 4C). Consistent with these results, Western blot analysis showed that dominant negative Akt resulted in reduced Akt and GSK3β phosphorylation, while constitutively active Akt increased Akt and GSK3β phosphorylation (Fig. 4D). Furthermore, these effects were enhanced by FGF1 (Fig. 4D).

Neuronal cells were also infected with a rAAV-expressing wild-type GSK3β or with a control vector (AdV-GFP). In cells transfected with rAAV wild-type GSK3β, the activity of this enzyme (Fig. 5A) as well as cell death (not shown) increased over basal levels compared with vector control. In contrast, pretreatment with FGF1 decreased GSK3β activity in neuronal cells infected with rAAV wild-type GSK3β (Fig. 5A). Western blot analysis using antibodies against phosphorylated and total GSK3β (Fig. 5B) confirmed these findings. Taken together, these results support the notion that FGF1 might regulate GSK3β activity via PI3K-Akt activation.

Inactivation (Phosphorylation) of GSK3β by FGF1 Signaling Is Associated with β-Catenin Translocation to the Nucleus—Since previous studies show that inactivation of GSK3β by phosphorylation at serine 9 results in translocation of β-catenin to the nucleus (33), immunocytochemical analyses with β-catenin antibodies were performed in neurons treated with FGF1 with or without pharmacological inhibitors. Laser scanning confocal microscopy showed that under basal conditions β-catenin immunoreactivity was primarily in the cytoplasm and, to a lesser extent, in the nucleus (Fig. 6, A and B). After stimulation with FGF1, β-catenin labeling in the nucleus was significantly increased (Fig. 6, C and D). Consistent with Western blot studies (Fig. 3) and kinase assays (Fig. 4), the effects of FGF1 on β-catenin nuclear localization were blocked by LY294002 (PI3K inhibitor) (Fig. 6E) but not by the ERK inhibitors PD98059 (Fig. 6F) and U0126 (Fig. 6G), supporting the idea that FGF1 blocks GSK3β activation leading to β-catenin degradation.

Neuroprotective Effects of FGF1 against Glutamate Are Mediated by GSK3β—To investigate the physiological relevance of FGF1 on the PI3K-Akt, GSK3β, and ERK pathways, cell viability was measured by DNA fragmentation, the MTT assay, and trypan blue exclusion in cells pretreated with FGF1 (10 ng/ml, 24 h) and challenged with glutamate (5 mM, 24 h) in the presence or absence of specific inhibitors. Consistent with results from the MTT assay and trypan blue exclusion (not shown), DNA fragmentation studies showed that FGF1 protected neurons against neurotoxic effects of glutamate and that these effects were blocked by LY294002 but not by U0126 (Fig. 7). Analysis of DNA fragmentation by fluorescence-activated cell sorting showed that treatment of the neuronal cells with glutamate resulted in the formation of apoptotic bodies (Fig. 7), whereas pretreatment with FGF1 reduced the formation of apoptotic bodies in the cells. Treatment with LY294002 (PI3K inhibitor) (Fig. 7A), but not U0126 (Fig. 7B), blocked the neuroprotective effects of FGF1 against glutamate. Similarly, dominant negative Akt blocked the neuroprotective effects of FGF1, while constitutively active Akt was protective against glutamate toxicity in the absence of FGF1 (Fig. 7C).
DISCUSSION

The present study shows that the neurotrophic effects of FGF1 involve signaling via the PI3K-Akt and GSK3β pathways. The FGFR tyrosine kinase is linked to the G-protein Ras, which stimulates the ERK signal transduction cascade (15, 25) (Fig. 8). In this context, since ERK plays a central role in mediating cellular responses to a variety of signaling molecules (36, 37), most studies in both neuronal and non-neuronal cells have concentrated on characterizing the effects of FGF2 (rather than FGF1) on the ERK pathway (16, 17, 25, 36, 38–42).

Although ERK plays an important role in regulating the trophic effects of FGF, other pathways may also be involved (Fig. 8). For example, the neurotrophic activity of FGF1 is dependent on endogenous FGF1 expression but independent of ERK (43). In agreement with this finding, we showed that stimulation of neuronal cells with FGF1 resulted in GSK3β inactivation and β-catenin translocation to the nucleus independent of ERK. Trophic factors such as insulin also promote GSK3β inactivation by phosphorylation at serine 9 (44), facilitating β-catenin nuclear localization (45). While inactivation of
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GSK3β correlates with cell survival, activation of GSK3β results in cell death (18). These effects are important for understanding the neurotrophic activity of FGF1 because the GSK3β signaling pathway has been shown to play an important role in regulating central nervous system development (32, 46) and cell fate (18, 19). Phosphorylation of GSK3β sere 9 by the dishevelled signal (Wnt) through the frizzled receptor and activated through the Notch receptor results in its inactivation (32) with subsequent β-catenin translocation to the nucleus (45). Alterations of this pathway are also currently being recognized as important in the pathogenesis of neurodegenerative disorders such as Alzheimer’s disease (47, 48) and HIV encephalitis (49). For example, activation of GSK3β might facilitate HIV-mediated neurotoxicity since recent studies have shown that the HIV protein Tat may activate GSK3β (49). In contrast, the neuroprotective effects of FGF1 against neurotoxins such as HIV-derived proteins and the amyloid β protein of Alzheimer’s disease might be associated with its ability to block GSKβ. In support of this possibility, the present study shows that protection against glutamate toxicity is associated with inactivation of GSKβ. Furthermore, the neurotoxic effects of gp120 (12) and amyloid β (10) are blocked by inhibitors of GSK3β such as LiCl (60–52). In addition, in individuals with HIV encephalitis high levels of neuronal FGF1 expression correlate with improved cognitive performance and preservation of the dendritic integrity (12). Similarly, neurons that express high levels of FGF1, such as motor neurons, are resistant to amyloid β toxicity (11).

As to the potential mechanisms mediating the effects of FGF1 on GSK3β, the present study shows that inhibitors of the PI3K-Akt pathway block the effects of FGF1 on GSK3β, while ERK inhibitors have no effect. Further confirming the involvement of the PI3K pathway, FGF1 treatment of neuronal cells resulted in Akt phosphorylation independent of ERK activation. This is consistent with previous studies showing that the PI3K-Akt signaling inactivates GSK3β, which is important for cell survival (53, 54). Both PI3K and Akt are activated by other growth factors including platelet-derived growth factor (55), insulin (56), and brain-derived neurotrophic factor (57). Growth factor-induced cell survival is dependent on the activation of PI3K and its downstream effector, Akt (18). Akt phosphorylates several intracellular substrates, thus affecting cell survival and programmed cell death (5, 18). GSK3β has been previously identified as one of the main substrates for the PI3K-Akt pathway (18, 54). Overexpression of catalytically active GSK3β in neuronal cell lines results in apoptosis, while dominant negative GSK3β prevents cell death following the phosphorylation-mediated inhibition by the PI3K-Akt cascade (18). Similarly, recent studies have shown that, in primary neuronal cultures, apoptosis induced by withdrawal of trophic factors or by PI3K inhibition is dependent on GSK3β activation (57). These studies also show that both the expression of an inhibitory GSK3β-binding protein or a dominant-interfering form of GSK3β reduced neuronal apoptosis (57). In contrast, expression of a mutant β-catenin, not affected by GSK3β activity, did not protect against apoptosis. Thus, although stabilization of β-catenin as a result of GSK3β inactivation is an important physiological effect, several other pathways downstream of GSK3β might regulate cell fate (57). Taken together these results suggest that the neuroprotective effects of FGF1 involve a PI3K-Akt-mediated inactivation of GSK3β.

In conclusion, FGF1 neuroprotective effects might involve inactivation of GSK3β by a pathway involving activation of PI3K-Akt cascades (Fig. 8). To the best of our knowledge, this is the first study showing that FGF1 is capable of acting on these intracellular pathways to enhance cell viability. This finding is significant because alterations in FGF1 and GSK3β are being recognized as important in the pathogenesis of neurodegenerative disorders such as Alzheimer’s disease and HIV encephalitis.

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