Role of Glycogen Synthase Kinase-3β in Neuronal Apoptosis Induced by Trophic Withdrawal

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Glycogen synthase kinase-3β (GSK3β) activity is negatively regulated by several signal transduction cascades that protect neurons against apoptosis, including the phosphatidylinositol-3 kinase (PI-3 kinase) pathway. This suggests the interesting possibility that activation of GSK3β may contribute to neuronal apoptosis. Consequently, we evaluated the role of GSK3β in apoptosis in cultured cortical neurons induced by trophic factor withdrawal or by PI-3 kinase inhibition. Neurons were subjected to several apoptotic paradigms, including serum deprivation, serum deprivation combined with exposure to NMDA receptor antagonists, or treatment with PI-3 kinase inhibitors. These treatments all led to stimulation of GSK3β activity in cortical neurons, which preceded the induction of apoptosis. Expression of an inhibitory GSK3β binding protein or a dominant interfering form of GSK3β reduced neuronal apoptosis, suggesting that GSK3β contributes to trophic factor withdrawal-induced apoptosis. Furthermore, overexpression of GSK3β in neurons increased apoptosis, indicating that activation of this enzyme is sufficient to trigger programmed cell death. Although destabilization of β-catenin is an important physiological effect of GSK3β activation, expression of a mutant β-catenin that is not destabilized by GSK3β did not protect against apoptosis. We conclude that inhibition of GSK3β is one of the mechanisms by which PI-3 kinase activation protects neurons from programmed cell death.

Key words: cortical neurons; glycogen synthase kinase-3β; phosphatidylinositol-3 kinase; Akt; β-catenin; NMDA; glutamate; brain-derived neurotrophic factor; apoptosis; signal transduction

Differentiated cells, including neurons in the CNS, require the presence of survival factors to suppress the intrinsic cell death machinery and thereby avoid apoptosis (Raff et al., 1993; Park et al., 1997). The regulation of apoptosis by survival factors is therefore critical for normal development and proper functioning of multicellular organisms. In addition, abnormal apoptosis in CNS neurons may play a significant role in neurodegenerative diseases (Raff et al., 1993; Stefanis et al., 1997; Estus, 1998).

A number of survival factors for neurons have been identified, including serum, insulin-like growth factor-1, neurotrophins, and NMDA (Levi-Montalcini and Booker, 1960; D'Mello et al., 1993; Koh et al., 1995; Dutta and Greenberg, 1998; F. X. Zhang et al., 1998). These factors activate the phosphatidylinositol-3 kinase (PI-3 kinase) pathway, which is one of several signal transduction pathways implicated in the survival of neurons (Yao and Cooper, 1995; Castellino and Chao, 1996; D’Mello et al., 1997; Dudek et al., 1997; Miller et al., 1997; Parrizas et al., 1997; Philpott et al., 1997; Yamada et al., 1997; Chao et al., 1998; Crowder and Freeman, 1998; F. X. Zhang et al., 1998; Hetman et al., 1999).

Although effectors downstream from PI-3 kinase that mediate neuron survival have not been completely identified, one likely candidate is protein kinase Akt (also known as PKB or RAC), a Ser/Thr-protein kinase that is activated by PI-3 kinase (Dudek et al., 1997; Franke et al., 1997; Hemmings, 1997; Kaufmann-Zeh et al., 1997; Kulik et al., 1997; Philpott et al., 1997; Crowder and Freeman, 1998; Murga et al., 1998). Akt phosphorylates and inhibits glycogen synthase kinase-3β (GSK3β) (Cross et al., 1995; Moule et al., 1997), although GSK3β may also be regulated by other PI-3 kinase-dependent, but Akt-independent, pathways (Delcommenne et al., 1998; Kobayashi and Cohen, 1999). Because the PI-3 kinase–Akt pathway is neural-protective and negatively regulates GSK3β activity, GSK3β may be an important downstream proapoptotic target that contributes to apoptosis in neurons. Consistent with this hypothesis, Pap and Cooper (1998) demonstrated that GSK3β activity is required for apoptosis induced by inhibition of PI-3 kinase in Rat1 fibroblasts and neuronal-like PC12 cells. However, the role of GSK3β in apoptosis in primary cultured CNS neurons has not been evaluated. Here, we examined the activity of GSK3β after trophic withdrawal and quantitated the effect of inhibiting or stimulating the GSK3β pathway on induction of apoptosis. Our data indicate that this pathway plays an important role in the regulation of neuronal apoptosis.

MATERIALS AND METHODS

Materials. The following plasmids have been described previously: pON260 (Cherrington and Mocarski, 1989), expression constructs for Xenopus GSK3β binding protein (GBP) (Yost et al., 1998), rat GSK3β (Dominguez et al., 1995), and Myc-tagged expression vectors for wild-type and mutant Xenopus β-catenin (Pierce and Kimelman, 1995). The
Figure 1. Withdrawal of trophic support induces apoptosis in cortical neurons. A. Representative photomicrographs of cortical neurons (6 DIV) treated for 24 hr with control wash (+S), serum deprivation (−S), or serum deprivation together with exposure to the NMDA receptor antagonist MK-801 (−S+MK). The top panels are phase contrast photomicrographs (Phase), and the bottom panels are Hoechst-stained nuclei to visualize nuclear morphology (Hoechst). Arrows indicate healthy cells with uniformly stained nuclei. Arrowheads identify cells with apoptotic morphology, including shrunken cell bodies, fragmented processes, and condensed or fragmented nuclei. B. DNA fragmentation manifested as a DNA ladder. Cortical neurons were treated with serum deprivation with exposure to MK-801 (−S+MK) for 24 hr. Washed cells exposed to serum were used as control (+S). Positions of molecular size markers (MW) are indicated on the left in base pairs. Similar results were obtained with serum deprivation alone or with LY294002 treatment.

RESULTS
Cortical neurons undergo apoptosis after serum withdrawal, serum withdrawal with inhibition of NMDA receptors, or after treatment with LY294002

We have shown previously that cultured rat cortical neurons undergo apoptosis after serum withdrawal and that activation of the PI-3 kinase pathway is the dominant mechanism for serum-dependent survival in these neurons (Hetman et al., 1999). The objective of this study was to test the hypothesis that inhibition of GSK3β through the PI-3 kinase–Akt pathway is one of the downstream mechanisms that mediate PI-3 kinase-dependent neuronal survival. Cortical neuron apoptosis was induced by serum deprivation, serum deprivation together with exposure to NMDA receptor antagonists MK-801 or APV, or treatment with LY294002, a pharmacological PI-3 kinase inhibitor (Vlahos et al., 1994). These three apoptotic paradigms are all expected to decrease PI-3 kinase signaling and were chosen to test the generality of our hypothesis.

Apoptosis was measured by monitoring neurite degeneration, cell body shrinkage, nuclei fragmentation or condensation, and DNA cleavage into oligonucleosome fragments manifested as “DNA laddering,” hallmarks of apoptosis (Raff et al., 1993; Stefanis et al., 1997). Consistent with our previous report (Hetman et al., 1999), serum withdrawal or LY294002 treatment...
induced apoptosis in cortical neurons (Figs. 1, 2). Because activation of NMDA receptors promotes survival in cultured cerebellar neurons (F. X. Zhang et al., 1998; Bhave et al., 1999), we also monitored apoptosis when serum withdrawal was combined with NMDA receptor antagonists MK-801 or APV (Watkins and Collingridge, 1989). Although MK-801 or APV treatment alone had little effect on neuronal survival in the presence of serum, they both greatly potentiated serum deprivation-induced apoptosis (Figs. 1, 2). These data suggest that both serum and NMDA receptor activity are required for optimal survival of cortical neurons. Furthermore, serum withdrawal combined with NMDA receptor antagonists should be a useful model to study mechanisms that regulate activity-dependent neuronal survival.

**Trophic withdrawal inhibits PI-3 kinase and activates GSK3β**

To evaluate the effect of trophic withdrawal on PI-3 kinase, we assayed its activity by Western analysis using an antibody that only recognizes phosphorylated Akt because PI-3 kinase phosphorylates and activates Akt (Franke et al., 1997). Phosphorylation of Akt on Ser-473 is primarily dependent on PI-3 kinase activity (Franke et al., 1997). As anticipated, serum deprivation or serum deprivation plus MK-801 reduced Akt phosphorylation, indicative of PI-3 kinase inactivation (Fig. 3). LY294002 at 30 μM almost completely inhibited PI-3 kinase (Fig. 3).

Inhibition of PI-3 kinase leads to activation of GSK3β in PC12 and other non-neuronal cells (Cross et al., 1995; Moule et al., 1997; Pap and Cooper, 1998). To determine whether this is also the case in primary cultured postmitotic neurons, GSK3β activity was measured by an immune complex kinase assay. Serum deprivation or serum deprivation plus MK-801 caused a statistically significant (p < 0.0001) activation of GSK3β compared with control washed neurons (Fig. 4A). Similarly, direct inhibition of PI-3 kinase by LY294002 also activated GSK3β in cortical neurons (Fig. 4B). In all three paradigms, GSK3β was activated 75–100% relative to control treated neurons, its activity remained elevated for at least 6 hr after the initial treatment, and GSK3β activation preceded the peak of morphological changes associated with apoptosis (36–72 hr). These data suggest that inhibition of PI-3 kinase and subsequent activation of GSK3β may contribute to neuronal apoptosis induced by trophic withdrawal.
BDNF suppresses GSK3β activation and protects cortical neurons from apoptosis after trophic withdrawal

If trophic withdrawal-induced apoptosis is mediated by inhibition of PI-3 kinase and subsequent activation of GSK3β, then factors that activate PI-3 kinase might prevent GSK3β activation and apoptosis. We reported earlier that brain-derived neurotrophic factor (BDNF) activates PI-3 kinase in cultured cortical neurons and protects these neurons from serum withdrawal-induced apoptosis (Hetzman et al., 1999). Here, we tested whether BDNF can also block cortical neuron apoptosis after serum deprivation plus MK-801 and whether the neuroprotective effect of BDNF correlated with activation of PI-3 kinase and inhibition of GSK3β.

Cortical neurons were treated with serum deprivation plus 10 μM MK-801 to induce apoptosis (Fig. 5A). Approximately 50% of cells underwent apoptosis 24 hr after the treatment. However, only 20% of the cells were apoptotic in the presence of BDNF; the neural protection afforded by BDNF was primarily reversed by cotreatment with LY294002, suggesting a role for the PI-3 kinase in BDNF protection against apoptosis induced by serum deprivation plus MK-801 (Fig. 5A). Moreover, BDNF increased Akt phosphorylation (Fig. 5B, C) and prevented GSK3β activation (Fig. 5D) after serum deprivation plus MK-801 treatment. These data are consistent with the hypothesis that inhibition of GSK3β activity may be one of the anti-apoptotic mechanisms used by the PI-3 kinase pathway.

Direct inhibition of GSK3β protects cortical neurons from trophic withdrawal-induced apoptosis

To determine whether GSK3β activation is necessary for trophic withdrawal-induced apoptosis, we transiently transfected cortical neurons with an inhibitory GSK3β binding protein (GBPwt) (Yost et al., 1998) and examined the effect of blocking GSK3β activation on neuronal apoptosis (Fig. 6). In control experiments, neurons were transfected with an inactive GBP mutant (GBPmt), which does not bind or inhibit GSK3β (Yost et al., 1998). Expression of the wild-type or mutant GBP was confirmed by immunocytochemistry (data not shown). Expression of the wild-type or mutant GBP did not significantly affect the rate of basal cell death without apoptotic treatment (Fig. 6). However, transfection of neurons with GBPwt significantly protected them from apoptosis induced by serum deprivation, serum deprivation together with MK-801 treatment, or treatment with LY294002 (Fig. 6B).

We also transiently transfected cortical neurons with a kinase dead dominant negative form of GSK3β (GSK3βdn) (Domínguez et al., 1995) and examined its effect on neuronal apoptosis (Fig. 7). In control experiments, neurons were transfected with the cloning vector pEF1α. Transfection with GSK3βdn or the vector had no effect on basal cell death without any treatment. In contrast, expression of GSK3βdn caused a statistically significant reduction in apoptosis triggered by serum deprivation, serum deprivation plus MK-801 treatment, or treatment with LY294002 (*p < 0.01; **p < 0.0001). These data suggest that activation of GSK3β contributes to apoptosis induced by trophic deprivation.

Activation of GSK3β is sufficient to induce cortical neuron apoptosis

If trophic withdrawal-induced apoptosis in cortical neurons is mediated by activation of GSK3β, then direct and selective activation of GSK3β might be sufficient to induce apoptosis. Therefore, cortical neurons were transfected with plasmids encoding either the wild-type or a kinase dead dominant negative mutant of GSK3β (Domínguez et al., 1995). The corresponding cloning vector (pEF1α) was used as a control. Transfection of neurons with the wild-type but not the kinase dead mutant form of GSK3β significantly increased apoptosis (Fig. 8). Furthermore, apoptosis induced by expression of GSK3β was inhibited by BDNF in a PI3 kinase-dependent manner (data not shown). Thus, direct and selective activation of GSK3β is sufficient to induce apoptosis in cortical neurons.

Degradation of β-catenin is not critical for cortical neuron apoptosis induced by withdrawal of trophic support

Activated GSK3β leads to phosphorylation and subsequent degradation of β-catenin (Miller and Moon, 1996). This is one of the best characterized biochemical events downstream of GSK3β activation (Miller and Moon, 1996). To determine whether β-catenin degradation is important for GSK3β-induced apoptosis, we transiently transfected cortical neurons with either wild-type or a mutant form of β-catenin in which all four GSK3β-targeted serine residues were mutated to alanines (Miller and Moon, 1996). This mutant β-catenin is very stable and is resistant to GSK3β-induced degradation (Miller and Moon, 1996). Our results confirmed this observation. Expression of the wild-type or mutant β-catenin before apoptotic treatment was demonstrated by immunocytochemistry (Fig. 9A). Furthermore, the mutant β-catenin protein was indeed stable, whereas the wild-type
β-catenin protein was undetectable in these neurons after any of the three apoptotic treatments (data not shown). These results are consistent with the notion that apoptotic treatments cause activation of endogenous GSK3β and subsequent degradation of the wild-type but not mutant β-catenin. If β-catenin degradation is important for GSK3β-induced apoptosis, then overexpression of the stable β-catenin mutant might protect neurons from apoptosis. However, expression of either the wild-type or the mutant β-catenin had no significant effect on cortical neuron apoptosis after serum deprivation, serum deprivation together with MK-801 treatment, or treatment with LY294002 (Fig. 9B). Therefore, stabilization of β-catenin is ineffective in blocking GSK3β-mediated apoptosis, suggesting that β-catenin is not the critical substrate by which GSK3β triggers neuron death.

**DISCUSSION**

The goal of this study was to test the hypothesis that inhibition of GSK3β through PI-3 kinase pathway is one of the downstream mechanisms that mediate PI-3 kinase-dependent neuronal survival. Using cultured cortical neurons, we identified three apoptotic paradigms in which PI-3 kinase activity was suppressed and correlated with inhibition of GSK3β. GSK3β is the substrate of Akt (Cross et al., 1995; Moule et al., 1997), although it might also be inhibited by other PI-3 kinase-dependent pathways (Delcommenne et al., 1998; Kobayashi and Cohen, 1999). Although we cannot distinguish between these two possibilities, our results strongly suggest that GSK3β is an important inducer of neuronal apoptosis and that PI-3 kinase-promoted neuronal survival may involve negative regulation of GSK3β.

Pap and Cooper (1998) reported that nerve growth factor leads to GSK3β inactivation via the PI-3 kinase pathway. Furthermore, inhibition of PI-3 kinase by either LY294002 treatment or expression of dominant negative mutants interfering with PI-3 kinase–Akt pathway induces apoptosis in Rat1 fibroblasts and neuronal-like PC12 cells, whereas inhibition of GSK3β prevented apoptosis (Pap and Cooper, 1998). Together with our findings, these data suggest that inhibition of GSK3β activity by the PI-3 kinase signaling pathway may be a general mechanism for survival of neurons and non-neuronal cells. Activation of the PI-3 kinase–Akt signal transduction system may promote survival through several mechanisms, including inhibition of GSK3β, phosphorylation of proapoptotic proteins Bad, FKHR1 (Datta et al., 1997; del Peso et al., 1997; Brunet et al., 1999), and phosphorylation of caspase-9 (Cardone et al., 1998). This suggests that multiple mechanisms may work in parallel downstream from PI-3 kinase to suppress apoptosis.

The mechanism for induction of apoptosis by GSK3β remains undefined. GSK3β phosphorylates four serine residues at the N-terminal region of β-catenin and causes β-catenin degradation (Miller and Moon, 1996; Yost et al., 1996). It has been proposed that destabilization of β-catenin potentiates neuronal apoptosis induced by β-amyloid peptide (Z. Zhang et al., 1998). This suggested the interesting hypothesis that GSK3β-induced apoptosis involves degradation of β-catenin. However, our data do
serum deprivation (2°C after transfection, neurons were either fixed directly (C) or treated with serum deprivation (−S), serum deprivation with 10 μM MK-801 (−S+MK), or 30 μM LY294002 (LY) for 24 hr. A. Representative immunofluorescence photomicrographs of cortical neurons. Transfected cells were identified by β-galactosidase immunostaining (red cells). To reveal nuclear morphology, cells were counter-stained with Hoechst 33258 (blue). Arrows indicate transfected cells with healthy morphology. The arrowhead identifies a transfected cell with apoptotic morphology after serum deprivation combined with MK-801 treatment. B. Expression of the wild-type but not the mutant GBP inhibited apoptosis. Apoptosis in the transfected cell population was quantitated. Data are averages of duplicate determinations in three independent experiments. At least 1000 transfected cells were scored for each data point. Error bars are SEM. Statistically significant differences are indicated (*p < 0.01; **p < 0.0001, one-way ANOVA, factor-transfected plasmid).

not support this general hypothesis because overexpression of wild-type or a stable mutant form of β-catenin did not rescue cortical neuron from trophic deprivation-induced apoptosis.

In addition to β-catenin and glycogen synthase, several other substrates for GSK3β have been identified, some of which are worth consideration as candidate mediators of GSK3β-induced cell death. For example, mitochondrial pyruvate dehydrogenase is phosphorylated and inhibited by GSK3β; the subsequent metabolic failure might cause neuron death (Hoshi et al., 1996). GSK3β also phosphorylates insulin receptor substrate 1 (IRS-1) and converts IRS-1 into an inhibitor of insulin receptor tyrosine kinase activity (Eldar-Finkelman and Krebs, 1997). Because IRS-1 is critical in the signaling of insulin and insulin-like growth factor and both factors promote neuronal survival, phosphorylation of IRS-1 by GSK3β may contribute to GSK3β-induced cell death. Furthermore, GSK3β phosphorylates microtubule-associated protein tau into Alzheimer’s disease-like forms (PH-tau) found in tangles (Hanger et al., 1992; Mandelkow et al., 1992; Hanger et al., 1992).

Figure 7. Expression of a dominant negative mutant form of GSK3β protects trophic-deprived neurons from apoptosis. Cortical neurons were transfected with 4 μg of plasmid DNA encoding a dominant negative form of rat GSK3β (GSKdn). The empty cloning vector pEF1α was used as control (vector). Cells were also cotransfected with an expression vector encoding β-galactosidase (2 μg) as a marker for transfection. Three days after transfection, neurons were either fixed directly (C) or treated with serum deprivation (−S), serum deprivation with 10 μM MK-801 (−S+MK), or 30 μM LY294002 (LY) for 24 hr. Apoptosis in the transfected cell population was quantitated. Expression of the dominant negative GSK3β inhibited apoptosis. Data are averages of duplicate determinations in three independent experiments. At least 1000 transfected cells were scored for each data point. Error bars are SEM. Statistically significant differences are indicated (*p < 0.01; **p < 0.0001, one-way ANOVA, factor-transfected plasmid).

Figure 8. Activation of GSK3β is sufficient to induce neuronal apoptosis. Cortical neurons were transfected with 1 or 4 μg of plasmid DNA encoding either wild-type (wt) or a dominant negative mutant of GSK3β (dn). Cells were also cotransfected with 2 μg of plasmid DNA encoding β-galactosidase as a marker for transfection. The cloning vector pEF1α was used as a control (vector) and to supplement the total DNA to 6 μg in each case. Three days after transfection, cells were fixed and immunostained. Apoptosis in transfected cell population (β-galactosidase-positive) was scored. Data are averages of duplicate determinations in three independent experiments. At least 1000 transfected cells were scored for each data point. Error bars are SEM. Statistical analysis was performed as described in Figure 7 (*p < 0.0001).
mutant form (\( \beta \)-catenin). Regardless of the downstream target, our data indicate determinations. Similar results were obtained in two independent experiments. At least 350 transfected cells were scored for each condition. Error bars are SEM.

Ishiguro et al., 1993; Mulot et al., 1994). The appearance of PH-tau is associated with early alterations in neurites associated with Alzheimer's disease (Goedert et al., 1995). Tau phosphorylation by GSK3\( \beta \) may cause axonal dysfunction and trigger neuronal apoptosis. Regardless of the downstream target, our data strongly indicate that inhibition of GSK3\( \beta \) by PI-3 kinase is an important mechanism for neuronal survival.

Our finding that serum withdrawal-induced apoptosis of cortical neurons is potentiated by NMDA antagonists suggests that optimal cortical neuron survival requires both trophic factors and NMDA receptor activity. The role of NMDA receptor activity in supporting neuronal survival has also been demonstrated in cultured cerebellar granule neurons (F. X. Zhang et al., 1998; Bhave et al., 1999) and in forebrain cortical neurons in vivo (Ikonomidou et al., 1999). Collectively, these findings support the notion that neuronal survival depends on both the availability of peptide trophic factors and neuronal activity.

In summary, we have discovered that cortical neuron apoptosis induced by trophic withdrawal is mediated in part by GSK3\( \beta \) activation. Our findings add GSK3\( \beta \) to the list of potential drug targets for pharmacotherapy of neurodegenerative disorders and suggest that GSK3\( \beta \) may play a critical role in neuronal apoptosis.

REFERENCES


