Numerous studies reveal that phosphatidylinositol (PI) 3-kinase and Akt protein kinase are important mediators of cell survival. However, the survival-promoting mechanisms downstream of these enzymes remain uncharacterized. Glycogen synthase kinase-3β (GSK-3β), which is inhibited upon phosphorylation by Akt, was recently shown to function during cell death induced by PI 3-kinase inhibitors. In this study, we tested whether GSK-3β is critical for the death of sympathetic neurons caused by the withdrawal of their physiological survival factor, the nerve growth factor (NGF). Stimulation with NGF resulted in PI 3-kinase-dependent phosphorylation of GSK-3β and inhibition of its protein kinase activity, indicating that GSK-3β is targeted by PI 3-kinase/Akt in these neurons. Expression of the GSK-3β inhibitor Frat1, but not a mutant Frat1 protein that does not bind GSK-3β, rescued neurons from death caused by inhibiting PI 3-kinase. Similarly, expression of Frat1 or kinase-deficient GSK-3β reduced death caused by inhibiting Akt. In NGF-maintained neurons, overexpression of GSK-3β caused a small but significant decrease in survival. However, expression of neither Frat1, kinase-deficient GSK-3β, nor GSK-3-binding protein inhibited NGF withdrawal-induced death. Thus, although GSK-3β function is required for death caused by inactivation of PI 3-kinase and Akt, neuronal death caused by NGF withdrawal can proceed through GSK-3β-independent pathways.

The survival of developing neurons and perhaps most cells requires extracellular cues to actively prevent programmed cell death. In the nervous system such cues are provided in part by neurotrophic factors, such as the nerve growth factor (NGF), that bind to tyrosine protein kinase receptors and activate intracellular signaling pathways (1). One such pathway initiated by NGF involves the generation of 3'-phosphorylated phosphoinositides via the activation of phosphatidylinositol (PI) 3-kinase (2–4). This pathway has recently emerged as an important mechanism by which NGF and other neurotrophins promote cell survival (1). The primary effector of PI 3-kinase for cell survival is the Akt serine/threonine protein kinase, which is activated in cells stimulated with NGF in a PI 3-kinase-dependent manner (5, 6). Importantly, inhibitors of PI 3-kinase and Akt block the survival-promoting effects of NGF and other neurotrophic factors, and activated forms of PI 3-kinase and Akt promote neuronal survival in the absence of external survival factors (6–13).

Despite the significance of the PI 3-kinase/Akt pathway for neuronal survival, the mechanisms that lie downstream of Akt and mediate survival remain obscure. Recent findings in neurons and nonneuronal cells suggest that Akt may function by inhibiting death-promoting proteins (14). For example, Akt can phosphorylate the pro-apoptotic Bcl-2 family member, BAD, as well as members of the Forkhead family of transcriptional regulators. Phosphorylation of BAD or the Forkhead transcription factor FKHR1 by Akt suppresses the death-promoting activity of these and other proteins (15–17), suggesting at least two ways that Akt can contribute to cell survival.

Glycogen synthase kinase-3β (GSK-3β) was the first Akt substrate shown to be inhibited upon phosphorylation by Akt (18). By expressing a catalytically inactive form of GSK-3β in Rat-1 fibroblasts and in undifferentiated pheochromocytoma PC12 cells, Pap and Cooper (19) showed that GSK-3β function is required for cell death that is caused by the inhibition of PI 3-kinase. Stimulation of PC12 cells with NGF caused a PI 3-kinase-dependent reduction in GSK-3β activity, raising the possibility that inhibition of GSK-3β may be a critical component of NGF-dependent survival in PC12 cells. More recently, inhibitors of GSK-3β were shown to reduce cell death in rat cortical neurons when death was initiated by the PI 3-kinase inhibitor LY294002, serum deprivation, or serum deprivation combined with exposure to an N-methyl-D-aspartate receptor antagonist (20). Although these studies suggest a role for GSK-3β in neuronal death, the importance of regulating GSK-3β activity for the survival of neurons by their physiological neurotrophic factor has not been examined.

In this study, we have used NGF-dependent rat sympathetic neurons to compare the role of GSK-3β in NGF withdrawal-induced death with its role in death caused by inhibiting PI 3-kinase or Akt. Our results indicate that GSK-3β is a target of PI 3-kinase and Akt in sympathetic neurons but that inhibition of GSK-3β activity is not sufficient to block apoptosis caused by NGF withdrawal.

**EXPERIMENTAL PROCEDURES**

Primary Neuronal Culture—Primary cultures of sympathetic neurons were prepared from superior cervical ganglia of embryonic day 21

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*This work was supported in part by a generous contribution from the Paul Stark Endowment at the University of Rochester and by National Institutes of Health Grant NS34400. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Supported by Predoctoral Training Program Grant AG00107 from the National Institutes of Health.

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The abbreviations used are: NGF, nerve growth factor; PI, phosphatidylinositol; GSK-3β, glycogen synthase kinase-3β; GBP, Xenopus GSK-3-binding protein; PBS, phosphate-buffered saline; GSK-3βKM, kinase-deficient glycogen synthase kinase-3β mutant; TBST, Tris-buffered saline containing Tween 20; IRS-1, insulin receptor substrate 1.
rats and maintained in vitro for 5–7 days in medium containing 50 ng/ml NGF (Harlan Bioproducts, Madison, WI) as described previously (6). For depriving neurons of NGF, the cultures were rinsed with NGF-free medium and then switched into NGF-free medium containing neutralizing anti-NGF antiserum (Harlan Bioproducts).

**Protein Expression Vectors—Plasmids for expressing β-galactosidase (LacZ), Myr-Akt, Frat1, FratN, Xenopus GSK-3-binding protein (GBP), and Bax under the control of the cytomegalovirus promoter are described elsewhere (6, 21–24). GSK-3β and kinase-deficient GSK-3β mutant (GSK-3βK) cDNAs (25) were expressed using the plasmid PCS2+ (6). The AH-Akt expression vector was constructed by subcloning the AH-Akt cDNA from pCDNA3-AH-Akt-FLAG (6) into the multiple cloning site of pNF-EB-d2EGFP (CLONTECH, Palo Alto, CA).

**Intracellular Microinjections—Procedures for the microinjection of neurons and the assessment of neuronal viability are described in detail elsewhere (6, 26). Plasmid DNAs (each at 50 μg/ml) were injected into P1 neurons using a Nanoject II microinjector (Drummond, Broomall, PA) in a solution containing 1% sterile glycerol and 1% sterile PBS. The volume of injected solution was 5 μl. At least 24 h after injection, the number of injected neurons was assessed by phase-contrast microscopy. Neurons were considered to have been successfully transfected if at least 80% of the membranes showed clear staining.

**Immunofluorescence—Indirect immunofluorescence was performed as described elsewhere to confirm the expression of injected cDNAs (6). The following antibodies were used at the indicated concentrations: anti-GSK-3β monoclonal antibody (10 μg/ml, Transduction Laboratories, Lexington, KY); anti-T7/Tag monoclonal antibody for detecting Myr-Akt (5 μg/ml, Novagen, Madison, WI); anti-β-galactosidase monoclonal antibody (10 μg/ml, Promega, Madison, WI); anti-Akt/PKB PH-domain polyclonal antibody for detecting AH-Akt (1:50 dilution, Upstate Biotechnologies, Lake Placid, NY); anti-FLAG M2 antibody for detecting Bax, Frat1, and GBP (10 μg/ml, Sigma); and anti-hemagglutinin monoclonal antibody for detecting FratN (10 μg/ml, Sigma). In each experiment, greater than 80% of microinjected neurons expressed the appropriate protein(s).

**GSK-3β Kinase Assays—After the indicated treatments, neurons were rinsed twice with cold phosphate-buffered saline and then incubated for 15 min on ice in lysis buffer (1% Triton X-100, 10% glycerol, 20 mM Tris, 100 mM NaCl, 1 mM EDTA, 200 μg/ml Na3VO4, 1 mM microcystin LR (Biomol, Plymouth Meeting, PA), 1 mM phenylmethylsulfonyl fluoride, 5 μg/ml aprotinin, 5 μg/ml leupeptin, 1 mM dithiothreitol). The lysates were centrifuged (5 min) and were incubated 45 min in TBST/1% nonfat dry milk containing a 1:100,000 dilution of a horse radish peroxidase-conjugated goat anti-rabbit antibody (Bio-Rad). This procedure was followed by six washes in TBST and three washes in TBST without detergent. Blots were developed using SuperSignal (Pierce) and exposed to CL-XPosure film (Pierce). To control for variability in sample loading, membranes were stripped and reprobed with a 1:1000 dilution of anti-GSK-3β antibody.

**RESULTS**

**Inhibition of GSK-3β Activity by NGF Is PI 3-Kinase-dependent—NGF stimulation activates Akt via PI 3-kinase in PC12 cells and rat sympathetic neurons (5, 6, 12, 13, 27). Because Akt can inhibit GSK-3β (18), we tested whether NGF stimulation would inhibit GSK-3β kinase activity in sympathetic neurons. NGF stimulation of sympathetic neurons caused a 26% decrease in in vitro kinase activity of immunoprecipitated GSK-3β (Fig. 1), similar to results obtained in NGF-stimulated PC12 cells (19). Treatment with NGF in the presence of the PI 3-kinase inhibitors LY294002 or wortmannin restored GSK-3β activity to that of unstimulated neurons, indicating that NGF-induced inhibition of GSK-3β was largely due to activation of the PI 3-kinase pathway.

**Phosphorylation of GSK-3β on serine 9 by Akt inhibits GSK-3β kinase activity (18). To determine whether inhibition of GSK-3β activity by NGF might involve Akt-dependent phosphorylation of GSK-3β, we prepared immunoblots of GSK-3β from sympathetic neurons using an antibody that recognizes serine 9-phosphorylated GSK-3β. In these experiments, NGF stimulation led to an increase in phosphorylation on serine 9 (Fig. 2). Treatment with LY294002 or wortmannin reduced the NGF-induced phosphorylation of GSK-3β on serine 9, indicating that NGF negatively regulates GSK-3β activity in sympathetic neurons via a PI 3-kinase-dependent mechanism that probably involves Akt.

**GSK-3β Activity Is Important for Death of Sympathetic Neurons Caused by PI 3-Kinase or Akt Inhibition—**To determine whether GSK-3β contributes to the death of sympathetic neurons that occurs after PI 3-kinase inhibition, we microinjected neurons with expression plasmids encoding proteins that inhibit GSK-3β function. The various expression vectors were injected in a solution containing rhodamine-labeled dextran to facilitate visualization of the injected cells. The injected neurons were treated for 48 h with the PI 3-kinase inhibitor LY294002 in the presence of NGF and then scored for the existence of condensed or degraded chromatin, a characteristic of apoptotic cell death, using the DNA-binding dye Hoechst.
We next examined whether inhibition of GSK-3β could prevent neuronal death caused by Akt inhibition. Frat1, FratN, a kinase-deficient GSK-3β mutant (GSK-3βKM) (25), or LacZ was co-expressed in neurons with a dominant inhibitory form of Akt (AH-Akt) (6, 8), and survival was evaluated 72 h later. In each microinjection experiment, greater than 80% of the injected neurons expressed both target proteins as determined by Western blot analysis of whole cell lysates (data not shown). Approximately half of the neurons co-injected with AH-Akt and LacZ underwent cell death (Fig. 4). Expression of either Frat1 or GSK-3βKM partially rescued neurons from AH-Akt-induced death. In contrast, expression of FratN did not protect against cell death caused by Akt inhibition. GSK-3β function therefore appears to be important for cell death caused by either dominant-negative Akt or a PI 3-kinase inhibitor. Taken together, these results suggest that NGF-induced inhibition of GSK-3β kinase activity by a PI 3-kinase and Akt-dependent mechanism may contribute to the survival-promoting effects of NGF on sympathetic neurons.

**Ectopic GSK-3β Partially Blocks the Survival of NGF-maintained Neurons**—Overexpression of GSK-3β has been shown to reduce the survival of undifferentiated PC12 cells and cortical neurons maintained in serum-containing medium (19, 20). Accordingly, we tested whether microinjection of neurons with a GSK-3β expression vector was sufficient to override NGF-promoted survival signals in sympathetic neurons. Ectopic expression of GSK-3β produced a small but significant decrease in the survival of NGF-maintained neurons compared with the survival of neurons expressing LacZ (Fig. 5A). Expression of GSK-3βKM did not decrease survival compared with neurons expressing LacZ, indicating that the death caused by GSK-3β required a functional protein kinase domain. In comparison with GSK-3β, expression of the Bcl-2-related protein Bax, an inducer of cell death in these neurons (24), resulted in the death of greater than 60% of the injected neurons. Thus, overexpression of GSK-3β can partially override the survival-promoting effects of NGF on sympathetic neurons.
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neurons (Fig. 5B). Expression of the Frat1-related GSK-3-binding protein (23) also failed to inhibit the death of neurons deprived of NGF. The GSK-3β inhibitors had no effect on cell death at earlier times after NGF withdrawal (24 and 36 h), and overexpression of wild-type GSK-3β did not enhance the rate of NGF withdrawal-induced cell death (data not shown). Consistent with the microinjection results, treatment of neurons with 1–10 mM LiCl, a pharmacological inhibitor of GSK-3β (32, 33), did not significantly inhibit the death of NGF-deprived neurons (data not shown). These data indicate that inhibition of GSK-3β activity is not sufficient to protect neurons from death induced by NGF withdrawal.

**DISCUSSION**

Inhibitors of GSK-3β activity have recently been shown to reduce cell death caused by PI 3-kinase inhibition or serum withdrawal in PC12 cells and cortical neurons (19, 20). However, the importance of GSK-3β for cell death in neurons deprived of their physiological survival factor has not been tested previously. Sympathetic neurons from neonatal rat superior cervical ganglia, which require NGF for survival in vivo and in vitro, provide a powerful model for studying neurotrophic factor dependence (34). We used these cells to compare the role of GSK-3β in neuronal death caused by inhibition of the PI 3-kinase/Akt pathway with its role in death caused by NGF withdrawal. Our results corroborate previous findings that GSK-3β is a target of the PI 3-kinase/Akt pathway in primary neurons and that inhibition of GSK-3β can protect neurons from death induced by PI 3-kinase inhibitors. In addition, we demonstrate that inhibition of GSK-3β reduces neuronal death caused by the inhibition of Akt, suggesting that endogenous Akt acts to inhibit GSK-3β in neurons. Finally, our results provide evidence that inhibiting GSK-3β is not sufficient to block cell death after NGF withdrawal.

Stimulation of sympathetic neurons with NGF caused a PI 3-kinase-dependent increase in GSK-3β phosphorylation on serine 9 and a parallel decrease in GSK-3β kinase activity. Because Akt can phosphorylate serine 9 and inactivate GSK-3β after insulin stimulation (18), it is probable that the NGF-induced inhibition of GSK-3β activity seen here is mediated by a similar Akt-dependent mechanism. Nonetheless, it remains possible that regulation of GSK-3β by NGF may involve other PI 3-kinase effectors (35). Our observation that both Frat1 and kinase-defective GSK-3β provide protection against AH-Akt-induced death suggests that GSK-3β is at least partly regulated by Akt in response to NGF treatment.

A role for GSK-3β activity in neuronal death has recently been demonstrated in cortical neurons. Withdrawal of serum from cortical neurons caused an increase in GSK-3β activity that preceded the onset of cell death and that was reversed by the addition of brain-derived neurotrophic factor (20). When cortical neurons were transiently transfected with expression vectors for GBP or kinase-deficient GSK-3β, apoptosis caused by serum deprivation or by PI 3-kinase inhibition was significantly reduced. Overexpression of wild-type GSK-3β in cortical neurons caused a modest increase in neuronal death. These data are consistent with a role for GSK-3β in the death of neurons deprived of survival factors. In agreement with such a role, we found that overexpression of GSK-3β in sympathetic neurons could partially override the survival signals initiated by NGF, resulting in a 20–30% increase in cell death. However, none of the GSK-3β inhibitors that we tested (Frat1, GBP, or GSK-3β KM) was sufficient to prevent the death of NGF-deprived sympathetic neurons. This lack of protection was not simply a consequence of inadequate expression of these proteins as both Frat1 and GSK-3β KM were expressed sufficiently in neurons to inhibit death caused by blocking Akt function.

**GSK-3β Inhibitors Do Not Prevent the Death of NGF-deprived Neurons**—Because the results described above suggest the possibility that inhibition of GSK-3β may be important for NGF-promoted survival, we tested whether GSK-3β has an essential role in the death of NGF-deprived sympathetic neurons. Neurons were microinjected with vectors encoding inhibitors of GSK-3β and then deprived of NGF for 48 h. In contrast to their protective effects on AH-Akt-induced death, neither Frat1 nor GSK-3β KM inhibited the death of NGF-deprived neurons (Fig. 5B). Expression of the Frat1-related GSK-3-binding protein (23) also failed to inhibit the death of neurons deprived of NGF. The GSK-3β inhibitors had no effect on cell death at earlier times after NGF withdrawal (24 and 36 h), and overexpression of wild-type GSK-3β did not enhance the rate of NGF withdrawal-induced cell death (data not shown). Consistent with the microinjection results, treatment of neurons with 1–10 mM LiCl, a pharmacological inhibitor of GSK-3β (32, 33), did not significantly inhibit the death of NGF-deprived neurons (data not shown). These data indicate that inhibition of GSK-3β activity is not sufficient to protect neurons from death induced by NGF withdrawal.
Why do GSK-3β inhibitors prevent death of serum-deprived cortical neurons but not NGF-deprived sympathetic neurons? GSK-3β activity is regulated not only by phosphorylation on serine residues but also through its association with proteins, such as Frat1, Axin, and Dishevelled (22). GSK-3β-binding proteins appear to inhibit GSK-3β kinase activity toward certain substrates but not other substrates (31), and recent reports suggest that kinase-deficient GSK-3β mutants might not block all downstream pathways regulated by GSK-3 (30, 36). Consequently, the precise effects of overexpressing GSK-3β-binding proteins and kinase-deficient GSK-3β mutants are difficult to ascertain and may vary in different cell types. In addition, different cells may vary in their dependence on the function of various GSK-3β substrates, such as glycoxygen thase, eIF2B, Tau, and β-catenin (37). For example, destabilization of β-catenin, which occurs as a consequence of its phosphorylation by GSK-3β (38), has been reported to enhance cell death in hippocampal neurons (39). This observation suggests that stabilization of β-catenin through down-regulation of GSK-3β activity could be one mechanism by which neurotrophins promote survival in neurons. However, expression of a stabilized form of β-catenin failed to protect cortical neurons from death caused by serum withdrawal or inhibition of PI 3-kinase, indicating that at least in some cases GSK-3β-mediated destabilization of β-catenin is not necessary for neuronal death (20). GSK-3-mediated phosphorylation of insulin receptor substrate 1 (IRS-1) has been shown to inhibit insulin signaling in nonneuronal cells (40), and by analogy it could play a similar role in negatively regulating neurotrophin-derived survival signals in neurons. A report that IRS-1 can mediate the brain-derived neurotrophic factor-dependent activation of PI 3-kinase in cortical neurons is consistent with this possibility (41). However, a role for IRS-1 in NGF signaling has not been reported. Whereas IRS-1 may be regulated by GSK-3β in brain-derived neurotrophic factor-responsive cortical neurons, it seems unlikely to be an important GSK-3β target in NGF-dependent neurons.

Although inhibitors of GSK-3β failed to prevent death caused by NGF withdrawal, they did protect neurons from death caused by the inhibition of PI 3-kinase and Akt. One explanation for this apparent discrepancy could be that NGF withdrawal leads to higher levels of GSK-3β activity than does PI 3-kinase or Akt inhibition. In this case, the GSK-3β inhibitors might not be expressed sufficiently to inhibit the potentially higher level of GSK-3β activity in NGF-deprived neurons. This scenario seems unlikely because the level of GSK-3β kinase activity detected in PI 3-kinase inhibitor-treated neurons was not reduced to that of NGF-deprived neurons (Fig. 1). Moreover, treatment with LiCl at concentrations at and above those known to inhibit GSK-3β kinase activity (32, 33, 42) failed to provide protection from NGF withdrawal. Our results favor a model in which NGF withdrawal leads to the activation of all death pathways in addition to those activated by PI 3-kinase inhibitors. Although pro-apoptotic proteins, such as BAD, Caspase-9 (human), and FKHR1L1 can be negatively regulated by Akt (15–17), they can also be regulated by additional pathways (43, 44). Therefore, removal of NGF might result in their activation to a level beyond that achieved by inhibition of PI 3-kinase and Akt alone. In addition, other pro-apoptotic mechanisms are up-regulated after NGF withdrawal including c-Jun NH2-terminal kinase activation, translocation of Bax to mitochondria, and release of cytochrome c from mitochondria (45–50). Activation of one or more of these pathways after NGF withdrawal could result in cell death that is independent of GSK-3β activity. Finally, an alternative explanation for these results is that NGF withdrawal, but not PI 3-kinase/Akt inhibition, could conceivably activate GSK-3β functions that are not targeted by the inhibitors used in this study.

NGF activates proteins in addition to PI 3-kinase and Akt that can function to promote cell survival. For example, the transcription factors NF-κB (51, 52) and cAMP-response element-binding protein (53) are activated by NGF and have recently been shown to contribute to NGF-dependent survival in neurons (26, 54). Decreased activity of these transcription factors after NGF withdrawal might lead to cell death even in the presence of GSK-3β inhibitors, possibly via decreased expression of anti-apoptotic Bcl-2 family members (54–57).

Although our results indicate that GSK-3β inhibition by itself is not sufficient to maintain the survival of NGF-deprived neurons, the inhibition of GSK-3β activity is clearly an important aspect of the survival mechanisms mediated by PI 3-kinase and Akt. Exactly how GSK-3β activity influences the many other effectors of cell death and survival remains to be determined.
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Glycogen Synthase Kinase-3β Activity Is Critical for Neuronal Death Caused by Inhibiting Phosphatidylinositol 3-Kinase or Akt but Not for Death Caused by Nerve Growth Factor Withdrawal

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doi: 10.1074/jbc.M006160200 originally published online August 22, 2000

Access the most updated version of this article at doi: 10.1074/jbc.M006160200

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