Differential Modulation of Akt/Glycogen Synthase Kinase-3β Pathway Regulates Apoptotic and Cytoprotective Signaling Responses

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We have previously reported that specific dopamine agonists mediate protection against apoptosis induced by oxidative stress by activating the D₂ receptor-coupled phosphoinositide 3-kinase (PI-3K)/Akt pathway. In the present study we examined the downstream effectors of PI-3K/Akt signaling and their role in cell death after oxidative stress and protection provided by ropinirole, a D₂ receptor agonist in PC12 cells and primary cultures of dopamine neurons. Ropinirole treatment was associated with rapid translocation and phosphorylation of the PI-3K substrate Akt and phosphorylation of Akt substrates. One of these Akt downstream substrates was identified as the pro-apoptotic factor glycogen synthase kinase-3β (GSK-3β). Ropinirole-induced protection was associated with phosphorylation of GSK-3β (inactivation). In contrast, inhibition of PI-3K blocked the phosphorylation of Akt and GSK-3β (activation) and prevented the protection mediated by ropinirole. Suppression of Akt with specific short hairpin RNA in normal PC12 cells caused cell death, which was associated with reduced phosphorylation of GSK-3β and reduced levels of β-catenin, a transcriptional activator that is regulated by GSK-3β. Knock-out of GSK-3β expression with a short hairpin RNA alone was itself sufficient to cause cell death. We further demonstrated that oxidative stress induced by hydrogen peroxide (H₂O₂) dephosphorylates Akt and GSK-3β, increases GSK-3β activity, and promotes an interaction with β-catenin and its degradation. Inhibition of GSK-3β activity by inhibitor VIII protects cells from H₂O₂ similar to ropinirole. These results indicate that GSK-3β downstream of Akt plays a critical role in cell death and survival in these models.

The characteristic pathology of Parkinson disease (PD) is degeneration of dopaminergic neurons coupled with Lewy body inclusions in the substantia nigra pars compacta (1). The mechanism underlying dopaminergic cell death in PD has not been elucidated. A variety of cellular and molecular changes indicative of mitochondrial dysfunction, oxidative stress, pro- teaseal dysfunction, and apoptosis have been identified in the parkinsonian brain (for review, see Refs. 2 and 3). Specifically, a large body of evidence suggests that oxidative stress or reactive oxygen species-mediated apoptosis may contribute to the progressive and selective neuronal degeneration observed in PD (4). Brains of PD patients have increased iron, which promotes free radical formation, decreased levels of reduced glutathione, which is the major anti-oxidant in the brain, and evidence of oxidative damage to DNA, lipids, and proteins (5). Furthermore, in the substantia nigra pars compacta of PD patients there are increased levels of cyclooxygenase, which contribute to formation of the oxidant species dopamine-quinone (5), and reduced mitochondrial complex I activity, which promotes free radical formation (6–8).

Current therapies for PD are primarily based on a dopamine replacement strategy. Although they provide effective anti-parkinsonian effects, particularly in the early stages of the disease, PD patients eventually develop potentially disabling features such as falling, freezing, and dementia that are not satisfactorily controlled with available therapies (9). As a consequence, there has been an intensive search for therapies that might protect or restore function to neurons that would otherwise undergo degeneration in PD and thereby stop or slow the rate of disease progression.

Dopamine agonists that activate D₂ receptors are widely used to treat PD based on their capacity to provide short-term symptomatic improvements. Recent interest has also focused on the potential of dopamine agonists to provide neuroprotective effects and slow the rate of PD progression (18). Ropinirole and other dopamine agonists have been found to be capable of protecting dopamine neurons from a variety of toxins in both in vitro and in vivo models (10–15). Furthermore, in clinical trials in PD patients, ropinirole delayed the rate of decline of a neuroimaging surrogate biomarker of nigrostriatal function in comparison to levodopa (16, 17). These findings raise the possibility that ropinirole may be neuroprotective and slow the rate of PD progression. Although several mechanisms have been proposed to account for how these agents might provide neuroprotection (18), most interest has focused on the potential of their capacity to provide anti-apoptotic effects. However, the precise signaling mechanism whereby ropinirole induces anti-apoptotic effects is not known.

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2 The abbreviations used are: PD, Parkinson disease; PI-3K, phosphoinositide 3-kinase; Akt, protein kinase B; PH, pleckstrin homology; DAPI, 4′,6-diamidino-2-phenylindole dihydrochloride; GSK-3β, glycogen synthase kinase-3β; shRNA, short hairpin RNA; FKHR, fork head transcription factor; 6-OHDA, 6-hydroxydopamine; ERK, extracellular-regulated kinase; GTPγS, guanosine 5′-3-O-(thio)triphosphate; GFP, green fluorescent protein; GST, glutathione S-transferase; D₂R, D₂ receptor.
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Previously, we have demonstrated that some dopamine agonists protect PC12 cells from oxidative stress by activating a D_2 receptor-dependent PI-3K/Akt signaling pathway independent of their ability to activate GTP\_\gamma\_S binding (14, 15). To elucidate the downstream effectors of the PI-3K/Akt signaling pathway that mediates the agonist-specific modulation of cell survival, we have investigated the anti-apoptotic signaling pathway activated by the dopamine agonist ropinirole. We now report that ropinirole-mediated protection against oxidative stress involves activation of PI-3K/Akt-mediated phosphorylation (deactivation) of GSK-3β and that oxidative stress has opposing effects on the modulation of this pathway.

**EXPERIMENTAL PROCEDURES**

**Chemicals**—Dulbecco’s modified Eagle’s medium (DMEM), Opti-MEM1, DMEM/F-12, N2 supplements, and fetal calf serum were obtained from Invitrogen. Ropinirole was obtained from GlaxoSmithKline Inc (Research Triangle Park, NC). PI-3K inhibitor LY294002 and GSK-3β inhibitor VIII were from Calbiochem. CellTiter-Blue assay kits were from Promega (Madison, WI). An Amersham Biosciences enhanced chemiluminescence lighting (ECL) Western blotting detection reagent kit was from GE Healthcare. Haloperidol, eticlopride, hydrogen peroxide (H₂O₂), poly-D-lysine, and β-actin antibody were from Sigma. Antibodies specific to phospho-Akt, Akt, phospho-GSK-3β, phospho-p70S6 kinase, phospho-β-catenin, and β-catenin were from Cell Signaling Technology (Beverly, MA). Phospho-Forkhead transcription factor (FKHR), and GSK-3β antibodies were from Santa Cruz Biotechnology (Santa Clara, CA). Tyrosine hydroxylase antibody was from Chemicon (Temecula, CA). Neural Transient transfection reagent was from Mirus (Madison, WI). Alexa Fluor 488 donkey anti-sheep IgG and Alexa Fluor 592 chicken anti-rabbit IgG were from Molecular Probes (Invitrogen). [3H]Dopamine (40 μCi/mm) was purchased from Amersham Biosciences.

**Cell Cultures**—PC12 and PC12-D2R cells were cultured as previously described in a humidified atmosphere containing 5% CO₂ at 37 °C (14, 15, 19–21). Medium was replaced with Opti-MEM 3 h before various treatments of the cells. Dopaminergic neuronal cultures were prepared from embryonic day 14 rat fetuses (E14; Charles River Laboratories, Wilmington, MA) as previously described (22). Briefly, the ventral portion of the midbrain was removed in sterile ice-cold Ca²⁺ and Mg²⁺-free HBSS/HEPES solution, cleaned free of meningeal tissue, and mechanically dissociated by passage through a flame-polished Pasteur pipette. Dissociated cells were plated at a density of ~1.2 × 10⁵ cells per cm² on poly-D-lysine-coated 24-well plates or glass coverslips. The neurons were maintained in a chemically defined medium consisting of Dulbecco’s modified Eagle’s medium/F-12 medium with N2 supplements, l-glutamine (0.5 mM), and penicillin/streptomycin (serum-free medium). Half of the culture medium was replaced every 2 days. Approximately 5–7-day-old cultures were used for experiments.

**CellTiter-Blue Cytotoxicity Assays**—For analysis of cell survival, cells were plated at a density of 10⁴ cells/well on 96-microwell cell culture plates (in 100 μl of medium) and grown for 24 h. Thereafter, 200 μM H₂O₂ was added either with or without ropinirole at the indicated concentrations, and cells were incubated for another 24 h. After treatments, 20 μl of the CellTiter-Blue reagent was added to each well, and the plates were incubated for 2–3 h. The conversion of non-fluorescent CellTiter-Blue reagent to highly fluorescent substrate by living cells was quantified using a spectrofluorometer (Spectra Max Gemini XS, Molecular Devices, Sunnyvale, CA) as described (19, 20). The data are expressed as percentages of the vehicle-treated controls, and the values represent the means ± S.E. from eight microwells from each of three independent experiments (n = 24).

**Uptake of [3H]Dopamine**—[3H]Dopamine uptake by primary mesencephalic neurons was carried out as described previously (19). The results were expressed as percentages of vehicle-treated control culture response.

**Transfections and DNA Constructs**—PC12-D2R cells (1 × 10⁵) were plated into 60-mm culture dishes with complete medium. After 24 h, the cells were transfected with 2.5 μg of the plasmid DNA encoding the pleckstrin homology (PH) domain of Akt protein kinase (1–167 amino acids) tagged with green fluorescent protein (PH-Akt-GFP) (15) or shRNA constructs of Akt protein kinase (1–167 amino acids) tagged with green fluorescent protein (PH-Akt-GFP) (15) or shRNA constructs such as U6-XASH3 HP, U6-Akt1 HP3, and U6-GSK-3β HP1 (generously provided by D. L. Turner (23)) with the use of the TransIT-Neural transfection reagent as described previously (19).

**Immunocytochemistry**—For immunofluorescence detection, PC12-D2R cells expressing PH-Akt-GFP cells were treated with ropinirole for 15 min. The cells were fixed, and GFP was visualized under an Olympus (BX65) upright epifluorescence microscope. To detect endogenous phospho-Akt, phospho-GSK-3β, GSK-3β, and β-catenin, PC12-D2R cells were immunostained with antibodies to phospho-Akt, phospho-GSK-3β, GSK-3β, or β-catenin and visualized using Alexa Fluor 592-conjugated chicken anti-rabbit IgG as described previously (20). To detect phospho-GSK-3β in dopamine neurons, primary mesencephalic cultures were stained with antibodies specific to tyrosine hydroxylase and phospho-GSK-3β and visualized using Alexa Fluor 488 donkey anti-sheep IgG and 592-conjugated chicken anti-rabbit IgG. The nuclei were stained with fluorescent DNA dye 4',6-diamidino-2-phenylindole dihydrochloride (DAPI). The slides were mounted in Vectashield (Vector Laboratories, Burlingame, CA) mounting medium and examined under an Olympus (BX65) upright epifluorescence microscope.

**Immunoblotting and Immunoprecipitation**—Cells (3 × 10⁶ cells/100-mm plate) were grown for 24 h, and after respective treatments, the cells were washed twice with ice-cold phosphate-buffered saline and lysed in buffer 20 mm Tris-HCl, pH 7.5, 150 mm NaCl, 1% IGEPSAL C630, 1 mm phenylmethylsulfonyl fluoride, 1 mm sodium orthovanadate, 5 μg/ml aprotinin, and a mixture of protease inhibitors (Roche Diagnostics) at 4 °C for 20 min. After centrifugation at 14,000 × g for 20 min at 4 °C, equal amounts of proteins were resolved by SDS-polyacrylamide gel electrophoresis. The resolved proteins were electrophoresed into nitrocellulose membrane and incubated with phosphorylated Akt, Akt substrates, GSK-3β, p70S6 kinase, FKHR, or β-catenin and then detected with peroxidase-conjugated secondary antibodies and ECL reagent. The blots were then stripped in stripping buffer containing 62.5 mm Tris-HCl,
pH 6.7, 2% SDS, 100 μg β-mercaptoethanol, and probed for total Akt, GSK-3β, or β-actin proteins.

For immunoprecipitation, the protein extract was incubated sequentially (2 h for each incubation at 4 °C) with anti-GSK-3β antibody and anti-rabbit IgG beads (eBioscience, San Diego, CA). Bacterial lysate for GST-β-catenin fusion protein (24) was pre-bound to GST-Sepharose beads (Amersham Biosciences) at room temperature. Beads were then incubated with GSK-3β immunoprecipitate in assay buffer (20 mM Tris, pH 7.5, 10 mM MgCl₂, 5 mM dithiothreitol, 0.2 mM ATP) for 30 min at 30 °C. Reactions were stopped by the addition of Laemmli loading buffer, boiled for 5 min, and resolved on SDS-PAGE. Western immunoblotting was carried out using Rabbit IgG TrueBlot Set reagents (eBioscience). Anti-β-catenin and anti-phospho-β-catenin antibodies were used to detect GST-β-catenin. To detect GSK-3β in the reaction mixture, anti-GSK-3β antibody was used.

Statistical Analysis—Data were analyzed by either two-tailed t test or analysis of variance followed by the Tukey’s test to correct for multiple comparisons.

RESULTS

The Dopamine Agonist Ropinirole Protects PC12-D2R Cells and Primary Mesencephalic Neurons from Cell Death Induced by Oxidative Stress via Activation of the D₂ Receptors—We studied the potential of the dopamine agonist ropinirole to protect against apoptosis induced by H₂O₂ in PC12 cells that express D₂ receptors (PC12-D₂R). Cells were preincubated (1 h) with varying concentrations of ropinirole (10⁻¹¹ to 10⁻³ M) before the addition of H₂O₂ (200 μM). After 24 h of incubation, cell viability was assessed using the CellTiter-Blue cell death assay. The administration of 200 μM of H₂O₂ induced a 52.9 ± 5.0% reduction in cell survival in comparison to controls. Ropinirole protected PC12-D₂R cells from H₂O₂-induced apoptosis in a robust and concentration-dependent manner (Fig. 1A). In contrast, ropinirole did not protect PC12 cells that lacked D₂ receptors from exposure to H₂O₂. Ropinirole also did not protect against H₂O₂ when PC12-D₂R cells were pretreated with the dopamine antagonist haloperidol (10 μM) before the addition of ropinirole (Fig. 1B).

Although PC12 cells are used as a good model to study dopaminergic function, they are non-neuronal cells derived from adrenal pheochromocytomas. Therefore, to further determine whether ropinirole protects dopamine neurons, we used primary rat mesencephalic neuronal cultures treated with 6-hydroxydopamine (6-OHDA). As shown in Fig. 1, C and D, ropinirole (1 μM) offered significant neuroprotection against 6-OHDA-induced neuronal loss. Our results are consistent with the report that ropinirole protects the primary mesencephalic neurons from 1-methyl-4-phenylpyridinium, the active metabolite of the neurotoxin 1-methyl-4-phenyl-1,2,5,6-tetrahydroxydipyrindine toxicity (10). Ropinirole did not protect against 6-OHDA toxicity when primary mesencephalic neurons were co-incubated with ropinirole plus the dopamine antagonist eticlopride (10 μM) (Fig. 1B). These results indicate that ropinirole protects dopamine neurons against cell death induced by H₂O₂ and 6-OHDA and that this protection occurs by way of functional D₂ receptors.

FIGURE 1. Ropinirole protects PC12-D₂R cells and primary mesencephalic dopamine neurons from cell death induced by oxidative stress by activating Akt signaling pathway. A, ropinirole increased cell survival of undifferentiated PC12-D₂R cells exposed to H₂O₂; PC12 cells that did not express dopamine receptors and PC12 cells engineered to express the D₂ receptor were exposed to 200 μM H₂O₂ for 24 h in the presence of increasing concentrations of ropinirole, and cell viability was assessed by a CellTiter-Blue assay. The data are expressed as percentages of the vehicle-treated controls, and the values represent the means ± S.E. from eight microwells from each of four independent experiments (n = 8). Note that ropinirole provides a dose-dependent increase in cell survival in PC12 cells that express D₂ receptors but not in subclones that do not express these receptors. B, ropinirole-mediated protection depends on interaction with the D₂ receptor. PC12-D₂R cells were treated for 24 h with 200 μM H₂O₂ plus 1 μM ropinirole in the presence and absence of 10 μM haloperidol or pretreated (30 min) with 10 μM PI-3K inhibitor LY294002. The protective effect of ropinirole against H₂O₂-induced apoptosis was eliminated in the presence of the D₂ receptor antagonist haloperidol and pretreatment with the PI-3K inhibitor LY294002. The data represent the means ± S.E. of three independent experiments (n = 8). *, p < 0.001 compared with vehicle; #, p < 0.001 versus H₂O₂; *#, p < 0.001 versus ropinirole plus H₂O₂. C and D, effect of ropinirole on survival and uptake rate of [³H]dopamine uptake by primary mesencephalic neurons. Cultures derived from the mesencephalon of E14 rat embryos were pretreated with 1 μM ropinirole (30 min), ropinirole plus 10 μM eticlopride (30 min), or ropinirole plus 10 μM LY294002 (30 min). The cultures were then treated with 100 μM 6-OHDA for 1 h, replaced with fresh medium containing respective drugs (without 6-OHDA), and incubated for 24 h. Neuronal viability was assessed by CellTiter-Blue assay (C) or by [³H]dopamine uptake assay (D). Values from each treatment were expressed as a percentage over the untreated control (100%). Pretreatment with eticlopride or LY294002 (10 μM) eliminates the protective effect of ropinirole on 6-OHDA-induced neuronal loss. Data are plotted from one experiment (mean ± S.E., n = 8) representative of two independent experiments. *, p < 0.001 compared with vehicle; #, p < 0.001 versus 6-OHDA; *#, p < 0.001 versus ropinirole plus 6-OHDA.
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Ropinirole Induced Protection in PC12-D2R Cells, and Primary Mesencephalic Neurons Involves the PI-3K/Akt Signaling Pathway—We have previously reported that the increase in cell survival mediated by D2 receptor activation is abolished by inhibitors of PI-3K (14). We, therefore, studied whether PI-3K signaling is modulated by the D2 receptor when complexed with ropinirole. To determine whether PI-3K signaling is involved in ropinirole-mediated protection, we tested the effect of the PI-3K inhibitor LY294002 (10 μM). Inhibition of PI-3K completely abolished the capacity of ropinirole to protect against cell death induced by oxidative stress in PC12 cells and by 6-OHDA in primary mesencephalic neuronal cultures (Fig. 1, B–D). However, LY294002 by itself had no effect on cell survival (Fig. 1B). These data suggest that activation of the PI-3K pathway contributes to the protective effects of ropinirole against apoptosis induced by oxidative stress in PC12-D2R cells and by 6-OHDA in primary mesencephalic neuronal cultures.

To examine if Akt, the principle downstream target of PI-3K (25), is implicated in ropinirole-mediated neuroprotection, we measured the translocation and phosphorylation of Akt after ropinirole administration. Akt phosphorylation and its protective effects occur after it translocates to the plasma membrane through an interaction of its N-terminal PH domain with phosphatidylinositol 3,4,5-triphosphate (PIP3) (26), thereby bringing the enzyme into the proximity of additional PIP3-dependent and -independent protein kinases (27). The distribution of Akt was assessed using a PH-Akt-GFP (15). In unstimulated cells, phosphorylated Akt was mainly localized in the perikarya (supplemental Fig. 1). In contrast, D2 receptor activation by ropinirole caused a rapid (15 min) translocation of PH-Akt-GFP to peripheral membrane regions (Fig. 2A, right panel). Ropinirole-induced translocation was similarly demonstrated using antibodies to endogenous phospho-Akt (Fig. 2, B and C). The addition of ropinirole to normal PC12-D2R cells was also associated with a significant increase in phosphorylated Akt, with phosphorylation occurring at the serine 473 (Ser473) site. These results were also observed 15 min after the addition of the drug and returned to basal levels at 60 min (Fig. 2, D and E). Levels of total Akt protein remained unchanged. Thus, D2 receptor stimulation by ropinirole in PC12-D2R cells causes rapid translocation and phosphorylation of Akt. These changes in Akt translocation and phosphorylation were prevented by co-administration of the PI-3K inhibitor LY294002 (data not shown), indicating that ropinirole induces Akt activation through a PI-3K signaling pathway.

Effects of Ropinirole on Downstream Effectors of PI-3K/Akt Signaling Pathway—The PI-3K/Akt pathway is known to promote cell survival by inactivating pro-apoptotic factors and activating anti-apoptotic factors by phosphorylation-dependent mechanisms (28–30). To determine whether ropinirole induces the activation of any specific effectors of the PI-3K/Akt pathway, we examined the effect of ropinirole on the phosphorylation at Ser/Thr of Akt substrate proteins (28) when admin-
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A

FIGURE 3. Downstream effectors of Akt signaling pathway activated by ropinirole. A, PC12-D2R cells were stimulated with 1 µM ropinirole for the periods of time indicated. After stimulation, cells lysates were prepared and analyzed by Western blotting with specific antibodies to anti-phospho-(Ser/Thr) Akt substrates (arrowheads) or anti-β-actin antibodies. B, GSK-3β is one of the downstream effectors of ropinirole. PC12-D2R cells were treated with ropinirole (1 µM) for the periods of time indicated, and cell lysates were analyzed by Western immunoblotting using anti-phospho (p)-p70S6 kinase, anti-phospho-FKHR, and phospho-GSK-3β (Ser9) antibodies. Phospho-GSK-3β blot was stripped and reprobed with anti-GSK-3β antibody. Experiments were repeated three times with similar results. Note the increased phosphorylation of GSK-3β (p-GSK-3β) after ropinirole treatment. The graphs show the mean ± S.D. from three experiments. The phosphorylation of Ser9 of GSK-3β was calculated as the ratio between phospho-GSK-3β total GSK-3β and expressed as fold difference between treatment periods.

B

C

FIGURE 4. Inhibition of PI 3-kinase abolished the phosphorylation of Akt and effectors of Akt by ropinirole. PC12-D2R cells were pretreated with PI-3K inhibitor LY294002 (10 µM) for 30 min and incubated with 1 µM of ropinirole for 15 min. After stimulation, cells lysates were prepared and analyzed by Western immunoblotting using anti-phospho (p)-Akt, phospho-(Ser/Thr) Akt substrates, and phospho-GSK-3β (Ser9) antibodies. Phospho-GSK-3β blot was stripped and reprobed with anti-β-actin antibody. Experiments were repeated three times with similar results. Note that ropinirole induces phosphorylation of Akt and GSK-3β and that these effects are reversed with the PI-3K inhibitor LY294002.

Ropinirole
LY294002

- + + - - + p-Akt p-Akt-substrates p-GSK-3β p-GSK-3β β-actin β-actin

A

Ly294002

- - - + + +

B

C

with transfection of the U6-XASH3 HP control vector, transfection of hairpin small interfering RNA expression vectors against Akt and GSK-3β reduced the expression of Akt and GSK-3β, respectively (Fig. 5, A and B). Because GSK-3β is a downstream effector of Akt, we examined the phosphorylation of GSK-3β in Akt knockdown cells. As shown in Fig. 5C, Akt suppression reduced phosphorylation of GSK-3β and the levels of the GSK-3β target β-catenin (32). To further confirm the role of Akt in cell survival, we have assessed the levels of Akt in cells transfected with Akt1 shRNA. We find that Akt levels are preserved in cells showing normal nuclear morphology and markedly reduced in cells showing condensed nuclei, indicating a strong correlation between cell death and low levels of Akt (Fig. 5D). These results suggest that Akt acting through GSK-3β signaling is necessary for the survival of PC12-D2R cells. Previously we have demonstrated that p53 and extracellular-regulated kinase (ERK) signaling plays an important role in mediating cell death and survival, respectively, in these cells (19–21). A significant increase in the levels of phosphorylated p53 and decreased ERK was observed in Akt knockdown cells (Fig. 5C), suggesting a cross-talk between Akt and pro- and anti-apoptotic signaling pathways.

Oxidative Stress Causes, and Ropinirole Prevents, Dephosphorylation of Akt/GSK-3β—It has previously been reported that activated (dephosphorylated) GSK-3β promotes cell death, whereas N-terminal serine phosphorylation deactivates GSK-3β and promotes cell survival (33–36). We examined the phosphorylation states of Akt and GSK-3β after exposure to H2O2. We found that H2O2 caused dephosphorylation of Ser473 of Akt (which causes it to become inactive) and Ser9 of GSK-3β (which causes it to become active) (Fig. 6, A and B). However, H2O2 treatment had no effect on the total levels of Akt or GSK-3β proteins (Fig. 6A).

To determine the effect of oxidative stress on the activation state of GSK-3β, we examined the levels of β-catenin, a transcriptional activator that is regulated by GSK-3β. Phosphorylation of β-catenin by activated GSK-3β leads to its rapid degradation (for review, see Ref. 32). H2O2 treatment of PC12-D2R

D

ropinirole-induced phosphorylation of Akt substrates including GSK-3β (Fig. 4).

Akt and GSK-3β Are Essential for Cell Survival—To investigate the role of Akt/GSK-3β signaling in cell survival, shRNA specific to Akt1 and GSK-3β were used (23). We used a PH-Akt-GFP construct to determine the transfection efficiency of PC12-D2R cells. Transient expression of PH-Akt-GFP yielded ~50–60% GFP-positive cells after 48 h of transfection (data not shown). When normal PC12-D2R cells were transfected with either Akt shRNA or GSK-3β shRNA, markedly reduced cell viability was observed 48 h after transfection (Fig. 5, A and B). When compared

FIGURE 5. RTPCR analysis of Akt knockdown cells. A, transfection efficiency was determined by transfecting normal PC12-D2R cells with 100 nM Akt shRNA or GSK-3β shRNA for 24 h and analyzed by RTPCR. B, cell survival assay was performed with transfection of Akt shRNA. C, cell survival assay was performed with transfection of GSK-3β shRNA. D, cell survival assay was performed with transfection of both Akt and GSK-3β shRNA.
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FIGURE 5. Loss of Akt and GSK-3β induces cell death in PC12-D2R cells. Effects of Akt (A)- and GSK-3β (B)-specific shRNA constructs on PC12-D2R cell survival. Cells were transfected with Akt (A)- or GSK-3β (B)-specific shRNA constructs, and cell viability was determined by CellTiter-Blue fluorescent assay after 48 h of transfection. A control shRNA construct XASH3 HP was used as a control. GSK-3β protein level was determined by Western immunoblotting in GSK-3β-suppressed cells (B). Data are expressed as a percentage of non-transfected controls, and the values represent the mean ± S.E. from 12 microwells from each of two independent experiments. In A and B, p < 0.001 compared with XASH3 HP3. C, cells were transfected with Akt1 HP3 shRNA construct. After 48 h, total cell lysates were prepared, and Akt, GSK-3β, phospho (p)-GSK-3β, β-catenin, p53, and ERK levels were determined by Western immunoblotting. A shRNA construct XASH3 HP was used as a control. β-Actin was a loading control. Note that knockdown of Akt induces down-regulation of phosphorylated (deactivation) GSK-3β, β-catenin, with increased levels of the p-p53 and reduced levels of ERK. D, immunocytochemical analysis of Akt levels in Akt knockdown cells. PC12-D2R cells were transfected with XASH3 or Akt1 shRNA and 44 h later labeled with anti-Akt antibody (red). In all, nuclei were stained with DAPI (blue). Note that Akt1 shRNA causes a reduction in the levels of Akt and that co-localizes in cells with condensed nuclei (arrows).

cells leads to dephosphorylation of Ser9 of GSK-3β (activation) (Fig. 6, A and B) and increased degradation of β-catenin (Fig. 6C). Immunocytochemical staining of phospho-Ser9 of GSK-3β and total β-catenin further confirm that treatment of PC12-D2R cells with H2O2 dephosphorylates GSK-3β (Fig. 6D, top left panel) and promotes degradation of β-catenin (Fig. 6D, bottom left panel).

Because ropinirole protected primary mesencephalic neurons, we hypothesized that the increase in phospho-GSK-3β Ser9 levels, indicative of decreased GSK-3β activity, might contribute to neuroprotection against 6-OHDA toxicity. We, thus, investigated the effects of ropinirole and 6-OHDA on the phosphorylation of GSK-3β in primary mesencephalic neurons. Ropinirole caused an increase in Ser9 phosphorylation of GSK-3β, specifically in tyrosine hydroxylase-positive dopaminergic neurons (Fig. 7 and supplemental Fig. 2). In contrast, 6-OHDA caused small but significant decrease in the levels of Ser9 of GSK-3β in primary mesencephalic neurons (Fig. 7 and supplemental Fig. 2). These results are compatible with our hypothesis that the activation state of GSK-3β contributes to the cell survival state and that increased serine phosphorylation of GSK-3β protein induced by ropinirole contributes to dopaminergic neuronal survival against oxidative stress.

To further examine the role of GSK-3β on cell death induced by oxidative stress, we examined the effects of the GSK-3β inhibitor VIII. Pretreatment (1 h) of PC12-D2R cells with the GSK-3β inhibitor VIII induced a significant increase in cell survival after H2O2 treatment (Fig. 8). Significant neuroprotection was observed at a concentration of 0.5 μM, and the maximal response was observed at a concentration of 1 μM.

We next investigated the mechanism by which GSK-3β promotes cell survival and cell death. It is known that phosphorylation of β-catenin by GSK-3β causes its degradation by the ubiquitin proteasome system; therefore, we examined the activity of GSK-3β in cells treated with H2O2 with or without ropinirole or GSK-3β inhibitor VIII pretreatment. GSK-3β has been shown to phosphorylate at Ser33, Ser37, and Thr41 of β-catenin in vivo and in vitro (37). Thus, we used GST-tagged β-catenin protein as a substrate to measure the kinase activity of GSK-3β. The kinase assay indicated that the ability of
H2O2 increased interaction between GSK-3β in H2O2-treated cells as compared with vehicle-treated cells or in H2O2-treated cells that were pretreated with ropinirole or haloperidol. Loss of neuroprotection observed with the D2/D3 antagonist eticlopride in primary cultures of mesencephalic neurons treated with ropinirole similarly suggests that D2 receptors are involved in mediating the neuroprotective effect of ropinirole. Furthermore, we show that these protective effects are dependent on the phosphorylation and deactivation of the Akt substrate GSK-3β. Protective effects of ropinirole were lost with PI-3K inhibition in both PC12 cells and primary mesencephalic neurons. The phosphorylation of Akt and its translocation to the cell membrane in both its native and GFP-linked forms are also lost with PI-3K inhibition (which prevents phosphorylation and activation of Akt). Functional readout of Akt activation was demonstrated by an increase in the phosphorylation of downstream Akt substrates at an Akt-specific phosphorylation site. Activated Akt is known to promote cell survival by inactivating its pro-apoptotic substrates, such as FKHR, Bcl-2-associated death protein (BAD), caspase-9, and GSK-3β (30, 32).

Specifically, in our study we found that treatment with ropinirole and cell protection were associated with Akt-mediated phosphorylation at Ser9 of GSK-3β, which down-regulates its pro-apoptotic activity (39). The capacity of ropinirole to phosphorylate Ser9 of GSK-3β (deactivation) and the loss of GSK-3β phosphorylation (activation) in the presence of a chemical inhibitor of PI-3K suggest that GSK-3β is a downstream inhibitor of PI-3K/Akt signaling pathway. Akt suppression by modulation of the Akt/GSK-3β signaling pathway leads to cytoprotective or pro-apoptotic signaling responses.

We have previously reported that activation of D2 receptors by some (but not all) experimental dopamine agonists can induce a PI-3K/Akt pro-survival signaling pathway in PC12-D2R cells that is linked to transactivation of the epidermal growth factor receptor. This effect is independent of activation of G proteins and varies in degree with different dopamine agonists (14, 15). In the present study, we examined the signaling mechanisms whereby ropinirole might mediate a neuroprotective effect. We find no evidence for receptor-independent cytoprotective activities of ropinirole against oxidative stress in PC12 cells and 6-OHDA toxicity in primary mesencephalic neurons. Rather, we find that the activation of dopamine D2 receptors is required for the prevention of apoptosis induced by H2O2 in this model. This conclusion is based on the observations that similar protective effects could not be obtained in PC12 cells that lacked D2 receptors or that were pretreated with haloperidol.

GSK-3β phosphorylates β-catenin was significantly higher in H2O2-treated cells as compared with vehicle-treated cells or in H2O2-treated cells that were pretreated with ropinirole or GSK-3β inhibitor (Fig. 9A).

Consistent with these observations, we found that H2O2 dephosphorylates GSK-3β at Ser9 residue (Fig. 6, A and B), which is known to activate GSK-3β kinase activity (38). Treatment with H2O2 increased interaction between GSK-3β and β-catenin and promoted its degradation, whereas in vehicle-treated cells very little association of β-catenin/GSK-3β could be detected (Fig. 9B).

To verify the expression of these β-catenin and GSK-3β proteins, input control of total cell lysate was blotted with antibodies against β-catenin, GSK-3β, and β-actin (Fig. 9B). Taken together, these data suggest that oxidative stress induces β-catenin degradation through activation of GSK-3β.

**DISCUSSION**

We have determined that GSK-3β is a downstream effector of the D2 receptor-activated PI-3K/Akt signaling pathway that mediates neuroprotection against oxidative stress by the D2 agonist ropinirole in dopaminergic PC12 cells and in primary mesencephalic cultures. We show that oxidative stress activates GSK-3β and inactivates Akt with resultant cell death. In contrast, ropinirole activates Akt and deactivates GSK-3β through activation of the D2 receptor/PI-3K signaling pathway to mediate cell survival. Our results demonstrate that the differential effects of ropinirole might mediate a neuroprotective effect. Rather, we find that the activation of dopamine D2 receptors is required for the prevention of apoptosis induced by H2O2 in this model. This conclusion is based on the observations that similar protective effects could not be obtained in PC12 cells that lacked D2 receptors or that were pretreated with haloperidol.
shRNA resulted in cell death, indicating that Akt is necessary for cell survival. Down-regulation of GSK-3β/β catenin in Akt knockdown cells further confirm that GSK-3β is downstream of Akt in PC12 cells. Activated Akt is known to promote cell survival by inactivating the pro-apoptotic protein p53 and activating anti-apoptotic ERK (30, 32). The level of p53 is regulated by proteasomal degradation after its ubiquitination, which is mediated by the E3 ubiquitin ligase Mdm2. Akt plays a critical role in controlling Mdm2 activity (40). We have previously demonstrated the existence of a negative signaling cross-talk pathway from p53 to ERK in these cells (21). This ERK suppression pathway most likely contributes to the low levels of ERK observed in Akt knockdown cells.

GSK-3β activity is negatively regulated by Akt-mediated phosphorylation at Ser9 in the pseudosubstrate domain (32). This pathway is activated in response to growth factors and neurotrophins (41, 42) as well as ropinirole as shown in our study. Our data demonstrate that oxidative stress induced by H₂O₂ leads to dephosphorylation of Akt (deactivation) and Ser9 of GSK-3β, indicative of activation of this pro-apoptotic molecule. The activation of GSK-3β by H₂O₂ was confirmed by our finding of increased phosphorylation of GST-β catenin in H₂O₂-treated cells and of an interaction between GSK-3β and β-catenin with evidence of its degradation (Fig. 9). Chemical inhibition of GSK-3β activity by GSK-3β inhibitor VIII also prevented cell death caused by H₂O₂, further suggesting that Akt/GSK-3β signaling plays a critical role in cell death induced by oxidative stress. The critical role of GSK-3β in cell survival was further illustrated by evidence that a reduction of GSK-3β expression using a specific shRNA leads to cell death. Our results are consistent with the reports that GSK-3β plays a crit-

FIGURE 7. Increased levels of phosphorylated GSK-3β in tyrosine hydroxylase-positive neurons after ropinirole treatment. Primary cultures of mesencephalic neurons were treated with 1 µM ropinirole for 30 min or 100 µM 6-OHDA for 1 h. Cultures were double-immunolabeled with an anti-tyrosine hydroxylase (TH, green) and an anti-phospho (p)-GSK-3β (red) antibodies as described under "Experimental Procedures." Nuclei were stained with DAPI (blue). Cultures then were examined by using fluorescent microscope. Experiments were repeated three times, and representative images are shown. Bottom panel, quantification of phospho-GSK-3β immunofluorescence signal in dopamine neurons. To measure the relative increase in cytoplasmic phospho-Ser9 of GSK-3β, line profiles that transected the neuron but avoided the nucleus were used to assess fluorescence intensity (see supplemental Fig. 2). Six to eight-line cells per group were assessed using ImageJ (NIH) to obtain an average profile of fluorescence intensity for each of the treatment groups. Note the marked increase (p < 0.001) in phospho-GSK-3β in ropinirole-treated dopamine neurons and a small but significant decrease (p < 0.05) in 6-OHDA-treated cells.

FIGURE 8. Inhibition of GSK-3β and D₂ receptor-stimulation prevented cell death and dephosphorylation of GSK-3β by H₂O₂. PC12-D₂R cells were pretreated with 1 µM GSK-3β inhibitor VIII for 1 h before the addition of 200 µM H₂O₂. The cell viability was assessed after 24 h by CellTiter-Blue fluorescent assay. Data are expressed as the percentage of the vehicle treated controls, and the values represent the mean ± S.E. from eight microwells from each of three independent experiments. The value for H₂O₂ alone was 56.61 ± 4.35 as compared with control, *, p < 0.001 compared with vehicle; #, p < 0.001 versus H₂O₂. inh. VIII, inhibitor VIII.
Dopamine Agonist-mediated Anti-apoptosis

It is noteworthy that deactivation of GSK-3β protects cells from H₂O₂ toxicity but that complete knockdown of GSK-3β is not compatible with cell survival. In fact, it has been shown that inhibition of GSK-3β activity by either overexpression of the GSK-3β-binding protein, FRAT-1, the use of a kinase-dead dominant negative mutant of GSK-3β, or pharmacological inhibitors such as lithium, each, protects cortical neurons from trophic withdrawal (50). It has also been reported that marked chemical inhibition of GSK-3β prevents apoptosis induced by H₂O₂ in neuronally differentiated PC12 cells (33). There is, thus, strong evidence indicating that activation of GSK-3β promotes apoptosis, but normal GSK-3β activity appears to be essential for cell survival. It is possible that GSK-3β acts through multiple pathways and that RNA inhibition interferes with its activity in a different way than the inhibitor. It appears that although GSK-3β in its inactive form can induce cell death, some level of GSK-3β activity is necessary for cell survival.

Taken together, our findings suggest that intracellular GSK-3β is a downstream target of the PI-3K/Akt signaling pathway and plays a major role in mediating the cell death induced by oxidative stress and the protection mediated by the dopamine agonist ropinirole. There are multiple downstream targets for GSK-3β, some of which are involved in controlling cell survival against oxidative stress. To maintain redox homeostasis, aerobic cells have developed an antioxidant mechanism that includes a group of antixenobiotic genes termed phase II detoxification genes such as NAD(P)H:quinone oxidoreductase 1, glutathione S-transferases, glutamate-cysteine ligase, glutathione peroxidases, and heme oxygenase (51–54). The transcription factor nuclear factor E2-related factor 2 regulates the expression of antioxidant phase II genes and contributes to preserve redox homeostasis and cell viability in response to oxidant insults (55, 56). Recent reports suggest that a survival signal elicited by PI-3K/Akt acting through GSK-3β is the key mediator of the antioxidant phase II cell response (57, 58). It is widely accepted that in PD, dopaminergic neurons have compromised antioxidant mechanisms. It will be interesting to investigate the role of antioxidant phase II genes in mediating the cell survival and death in experimental models of PD. It has been shown that there is a loss of oxidative stress tolerance with aging that is linked to a parallel reduction in Akt activity (59) and to an increase in GSK-3β activity (60).

It is not yet known if the Akt/GSK-3β signaling pathway is involved in the cell death process that occurs in PD. However, ropinirole has been shown to have protective effects in experimental models of PD, and a clinical trial shows positive effects of the drug on a biomarker of nigrostriatal function consistent with a protective effect (10, 13, 16, 17). The molecular mechanisms described here could, thus, explain how ropinirole might provide neuroprotective effects in PD.

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REFERENCES

1. Forno, L. S. (1996) J. Neuropathol. Exp. Neurol. 55, 259–272
2. Moore, D. J., West, A. B., Dawson, V. L., and Dawson, T. M. (2005) Annu. Rev. Neurosci. 28, 57–87
3. Olanow, C. W. (2004) Annu. Rev. Med. 55, 41–60
