Antiapoptotic Effects of Erythropoietin in Differentiated Neuroblastoma SH-SY5Y Cells Require Activation of Both the STAT5 and AKT Signaling Pathways*

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The hematopoietic cytokine erythropoietin (Epo), a glycoprotein hormone produced primarily in the fetal liver and in the adult kidney, supports survival of erythroid progenitor cells and is essential for their proliferation and differentiation (1, 2). Epo has been thought to act in hematopoietic cells.

The hematopoietic cytokine erythropoietin (Epo),1 a glycoprotein hormone produced primarily in the fetal liver and in the adult kidney, supports survival of erythroid progenitor cells and is essential for their proliferation and differentiation (1, 2). Epo has been thought to act exclusively on erythroid progenitor cells. However, recent evidence indicates that Epo provides neuroprotective effects in the damaged brain during ischemic events and neurodegenerative diseases; the EpoR is expressed in several neuronal cell lines and in hippocampal and cortical neurons of rodent and human brains (3–6). Expression of Epo also occurs in the brain and in in vitro cultured astrocytes and neurons (4, 7–10). Together, these results suggest that Epo may function in the brain in a paracrine and/or autocrine fashion. Indeed, Epo reduces neuronal damage from ischemia in the brain of rodent models of stroke and also has a neuroprotective role against mechanical trauma, excitotoxins, neuroinflammation, and in an animal model of Parkinsonism (7, 11–15). Several studies show that Epo prevents apoptosis caused by cerebral ischemia in in vivo systems and by a variety of insults to in vitro cultured neuronal cell lines and primary cultured neurons (6, 12, 16–20). These results suggest that, like its role in erythroid progenitor cells, Epo supports survival of neuronal cells through antiapoptotic effects.

Mice null for the EpoR (EpoR−/−) or Epo or Jak2 genes die at embryonic day 13 because of a deficiency in erythropoiesis that leads to severe anemia (1, 2, 21, 22). No gross morphological abnormalities were identified in the brain of these mice. Suzuki et al. (23) generated a transgenically rescued EpoR−/− mouse by expression of the EpoR exclusively in the hematopoietic lineage using a GATA-1 minigene cassette. These mice are grossly normal despite the fact that they do not express EpoR in nonhematopoietic tissues, including the brain. This suggests that Epo signaling might not be necessary for normal brain development but could be required to respond to stresses in adults.

In the hematopoietic system, the mechanisms by which the Epo signal is linked to gene transcription have been extensively investigated. The EpoR is a member of the type I cytokine receptor family that does not contain intrinsic tyrosine kinase activity (24). Instead, the signal is mediated by the Janus family protein-tyrosine kinase, JAK2. Once Epo binds to the EpoR, JAK2 associated with the EpoR cytoplasmic domain rapidly becomes activated by transphosphorylation. Subsequently, several tyrosine residues in the EpoR become phosphorylated and recruit multiple signaling molecules that contain Src homology 2 domains, including signal transducers and activators of transcription 5 (STAT5), phosphatidylinositol 3-kinase (PI 3-kinase), and phospholipase C-γ.

One major EpoR intracellular signaling pathway involves STAT5. After binding to the activated EpoR, STAT5 becomes phosphorylated, dissociates from the EpoR, homodimerizes, and translocates to the nucleus, where it up-regulates expression of the antiapoptotic gene Bcl-X (25–28). Socolowsky et al. (27, 28) reported that mouse embryos and adults null for both Stat5a and Stat5b genes (Stat5a−/−; Stat5b−/−) are severely anemic and show increased levels of apoptosis in erythroid progenitors, demonstrating that STAT5 is one of the major antiapoptotic pathways activated by the EpoR.

Activation of PI 3-kinase through recruitment to the activated EpoR

1 The abbreviations used are: Epo, erythropoietin; EpoR, erythropoietin receptor; PrlR, prolactin receptor; Prl, prolactin; JAK2, Janus kinase 2; STAT5, signal transducer and activator of transcription 5; PI, phosphatidylinositol; MAPK, mitogen-activated protein kinase; PI(4,5)P2, phosphatidylinositol 4,5-bisphosphate; PI(3,4,5)P3, phosphatidylinositol 3,4,5-trisphosphate; GFP, green fluorescent protein; FACS, fluorescence-activated cell sorting; PBS, phosphate-buffered saline; BSA, bovine serum albumin; APC, allophycocyanin; 7-AAD, 7-aminoactinomycin D; p-NFH, phosphorylated Neu-

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results in conversion of the plasma membrane lipid phosphatidylinositol 4,5-bisphosphate (PI(4,5)P$_2$) to phosphatidylinositol 3,4,5-trisphosphate (PI(3,4,5)P$_3$) (29–33). PI(3,4,5)P$_3$ anchors the serine/threonine kinase AKT to the plasma membrane and induces a conformational change, which consequently allows phosphorylation of AKT by two other kinases. Activation of AKT requires phosphorylation of two regulatory residues, a threonine residue on the kinase domain and a serine residue on the C-terminal hydrophobic domain (Thr$^{308}$ and Ser$^{473}$ for AKT1; Thr$^{309}$ and Ser$^{473}$ for AKT2; and Thr$^{305}$ and Ser$^{472}$ for AKT3). Phosphorylation of the threonine residue, mediated by phosphoinositide-dependent kinase 1, is essential for AKT activation, whereas phosphorylation of the serine residue enhances the AKT activity about 10-fold. Several kinases, including phosphoinositide-dependent kinase 2, integrin-linked kinase, DNA-dependent protein kinase, protein kinase C$_{\text{mu}}$, and the Rictor-mTOR complex, were suggested to phosphorylate the serine residue of AKT (32–34). However, which kinase is primarily responsible for the phosphorylation of the serine residue has not been clarified. Once activated, AKT phosphorylates and inactivates members of the Forkhead transcription factor family. Subsequently, this leads to reduced expression of several apoptotic proteins that are normally activated by the Forkhead transcription factors. In hematopoietic cells, the EpoR also activates other signaling pathways, including the phospholipase C-\gamma and Ras/MAPK pathways (24, 35–43). These multiple pathways involved in Epo signaling provide the same output, namely antiapoptosis. Studies using Epo mutants overexpressed in EpoR$^{-/-}$ erythroid progenitor showed that normal erythroid differentiation was fully supported when only some of these signal proteins were activated (44), suggesting that these signaling pathways may play redundant roles.

The detailed molecular mechanisms by which Epo exerts its neuroprotective effects have not been clarified. Recent papers have suggested that the antiapoptotic activity of Epo in neuronal cells is mediated by activation of the PI 3-kinase/AKT signaling pathways (16, 18, 45); inactivation of the pro-apoptotic gene Bad (18); inhibition of glutamate secretion (17); and activation of Bel-2 (20). Digicaylioglu and Lipton (12) suggested that activation of the EpoR in neurons triggers cross-talk between the Jak2 and Nf-\kappaB signaling pathways. Nevertheless, the contribution of each signaling pathway downstream of the EpoR to the neuroprotective activity of Epo in the brain is not clarified and may differ in different types of neurons. We demonstrate that activation of at least the STAT5 and AKT signaling pathways is essential for the antiapoptotic activity of Epo in neuronal cells.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—SH-SYSY cells were maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum using collagen-coated plates (BD Biosciences). Differentiation was induced with 5 \( \mu \text{M} \) all-trans-retinoic acid (Sigma) in Neurobasal-A medium (Invitrogen) for 7 days.

**Generation of Ecotropic Receptor-overexpressing SH-SYSY Cell Lines**—SH-SYSY cells were transfected with the plasmid pM5-EcoNeo that contains the murine ecotropic receptor cDNA and a neomycin resistance gene (46) using FuGENE (Roche Applied Science). Clones stably expressing the murine ecotropic receptor were isolated by drug selection using 1 mg/ml G418 (Invitrogen) for 2 weeks. Ten isolated clones were expanded and tested for growth rate, morphology, infection efficiency, differentiation by trypsin treatment. The cell population was then analyzed by FACS. Median fluorescence intensities of APC for GFP-positive cells that correspond to the cells expressing PrlR/EpoR chimeras or the HA-EpoR were used for quantification.

**Apoptosis Analysis**—Apoptosis was induced by treatment with 0.1 \( \mu \text{M} \) staurosporine (Calbiochem). For pretreatment, 30 \( \mu \text{M} \) Prl (National Hormone and Peptide Program) was added to cells 24 h prior to addition of staurosporine and removed when staurosporine was added. For concomitant treatment, 30 \( \mu \text{M} \) Prl was added at the same time as staurosporine. At 24 h after addition of staurosporine, cells were harvested by trypsin treatment. The cell population was then analyzed by FACS using APC-conjugated annexin V binding and 7-aminoactinomycin D (7-AAD) staining (BD Biosciences). For quantification of the “apoptosis proportion,” both dead cell fraction and apoptotic cell fraction were calculated. Apoptosis induced by staurosporine was normalized by removing the background apoptosis detected in control differentiation medium. To facilitate comparisons, apoptosis induced by 0.1 \( \mu \text{M} \) staurosporine in each type of cells without Prl treatment was set equal to 1.0 (relative apoptosis proportion). For the EpoR-overexpressing cells, 25 \( \mu \text{M} \) Epo (Epogen; Amgen) was used instead of Prl with the same procedures. For experiments involving LY294002 or wortmannin tests, 10 \( \mu \text{M} \) LY294002 (Calbiochem) or 100 \( \mu \text{M} \) wortmannin (Calbiochem) was used.
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added at the same time as Prl. All subsequent procedures were as described above.

Immuno precipitation and Western Blot Analysis—SH-SY5Y cell lysates were prepared as described (52) with minor modifications. In detail, SH-SY5Y cells differentiated for 7 days were growth factor-starved for 5 h, harvested with 10 mM EDTA/PBS, treated with Prl (or Epo for the EpoR-overexpressing cells) for 10 min, and lysed in Nonidet P-40 Lysis buffer (5 mM Tris, pH 7.4, 150 mM NaCl, 1% Nonidet P-40) with protease inhibitors (Roche Applied Science) and 1 mM sodium vanadate. The soluble fractions of the lysates were incubated with anti-STAT5 (Santa Cruz Biotechnology) or anti-AKT (Santa Cruz Biotechnology) or anti-ERK 1/2 (Cell Signaling Technology) antibodies. The immune complexes were recovered using protein-A-Sepharose beads and eluted with NuPAGE buffer (Invitrogen) containing 1% β-mercaptoethanol. Proteins from these immunoprecipitates were separated on NuPAGE gels (Invitrogen), transferred to nitrocellulose membranes (Schleicher & Schuell), and Western-blotted using anti-phospho-STAT5 (p-STAT5, Cell Signaling Technology) or anti-phosphoserine-AKT (p-Ser-AKT, Cell Signaling Technology) or anti-phospho-ERK 1/2 (p-ERK 1/2, Cell Signaling Technology) antibodies. For experiments in Fig. 7, anti-phosphothreonine-AKT (p-Thr-AKT, Cell Signaling Technology) antibodies were used instead of anti-p-Ser-AKT antibody. For quantification of protein loading, the membranes were stripped with 0.2M Glycine buffer (pH 2.6) and reprobed with anti-STAT5 or anti-AKT or anti-ERK 1/2 antibodies. For cells overexpressing the EpoR, cells were treated with Epo instead of Prl, and subsequent procedures were as described above.

To confirm expression of FLAG-tagged PrlR/EpoR chimeras and the exogenously expressed HA-tagged EpoR, SH-SY5Y cells overexpressing PrlR/EpoR chimeras or the HA-EpoRs were harvested with 10 mM EDTA/PBS and lysed in Nonidet P-40 lysis buffer with protease inhibitors. The lysates were analyzed by Western blotting using anti-FLAG or anti-HA antibodies (Covance).

Nuclear Extract Preparation and Electrophoretic Mobility Shift Assay—SH-SY5Y cells differentiated for 7 days were growth factor-starved for 5 h, harvested with 10 mM EDTA/PBS, and treated with Prl (or Epo for the EpoR-overexpressing cells) for the times indicated in the figure legends. To isolate nuclear fractions, cells were resuspended in hypotonic buffer (20 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 1 mM Na3V2O4, 1 mM DTT, protease inhibitor (Roche Applied Science)), incubated for 15 min on ice, treated with Nonidet P-40 (0.5% final concentration), and mixed vigorously. The pellets were recovered by centrifugation. Nuclear extracts were made by resuspending the nuclear pellets in hypertonic buffer (20 mM HEPES, pH 7.9, 400 mM NaCl, 1 mM EDTA, 1 mM DTT, protease inhibitor (Roche Applied Science)). After 20 min of incubation on ice, the supernatants were collected by centrifugation and stored at −80 °C. As a positive control for NF-κB activation, nuclear extracts were made from SH-SY5Y cells treated with 50 ng/ml TNF-α (53, 54) under the same procedures.

The nuclear extracts were analyzed by Western blotting using anti-NF-κB p65 (Santa Cruz Biotechnology) or anti-TATA-binding protein (Santa Cruz Biotechnology) antibodies. For electrophoretic mobility shift assays, consensus NF-κB binding oligonucleotides (Santa Cruz Biotechnology) were labeled with 32P and used as a DNA probe. SH-SY5Y nuclear extracts were incubated with 0.25 ng of 32P-labeled oligonucleotide probe in binding buffer (20 mM HEPES, pH 7.9, 0.5 mM dithiothreitol, 20% glycerol, 20 mM KCl, 5 mM MgCl2) with 5 μg of poly(dI-dC) at room temperature for 20 min. The protein-DNA complexes were resolved by electrophoresis using 4% native polyacrylamide gel (30:0.8, acrylamide:bisacrylamide) at 4 °C and visualized by autoradiography. The autoradiogram was analyzed by a PhosphorImager (Fujifilm, BAS2500).

Immunofluorescence Microscopy—Immunofluorescence staining for phosphorylated Neurofilament-H (p-NFH), glial fibrillary acidic protein (GFAP), and GFP was performed as described (55) with minor modifications. A panel of SH-SY5Y cells was plated on 22-mm collagen-coated coverslips (BD Biosciences). After 7 days of differentiation with 5 μM all-trans-retinoic acid in Neurobasal-A medium, cells were fixed with 4% paraformaldehyde in 0.1M phosphate buffer, pH 7.2, for 30 min, washed three times with PBS, permeabilized with Blocking solution (1% BSA in PBS containing 0.1% Triton X), and incubated with anti-p-NFH (Chemicon International Inc.) or anti-GFAP antibodies (Chemicon International Inc.) in Blocking solution (1:200 dilution) for 1 h. After washing three times with PBS, cells were incubated with secondary antibodies conjugated with either Alexa Fluor 405 or Alexa Fluor 594 (1:100 dilution; Molecular Probes) in Blocking solution (1:200 dilution) for 1 h. For GFP expression, cells were incubated only with the fluorescein isothiocyanate-conjugated anti-GFP antibody (1:200 dilution; Santa Cruz Biotechnology). After incubation, cells were washed three times with PBS, mounted using the Mount media (Vector Laboratories), and analyzed by immunofluorescence microscopy (Nikon Eclipse Inverted TE300) using Openlab (Improvement) software.

RESULTS

Generation of PrlR/Epo Chimera- and EpoR-overexpressing SH-SY5Y Cells—The EpoR contains eight cytoplasmic tyrosine residues (Fig. 1). When specific tyrosine residues are mutated to phenylalanine, specific signal transduction pathways downstream of the EpoR are individually and selectively inactivated in erythroid progenitors (25, 26, 44, 47, 56).

To study how Epo prevents apoptosis of neuronal cells, we used differentiated human cholinergic neuroblastoma SH-SY5Y cells. These can be efficiently differentiated to neuron-like cells by retinoic acid. To verify that the antiapoptotic activity of Epo in SH-SY5Y cells is through prevention of apoptosis, but not through other effects such as promotion of cell growth, apoptosis was induced by staurosporine, a potent
protein kinase inhibitor that induces apoptosis in many cell types, including SH-SY5Y cells (57, 58). Using these differentiated SH-SY5Y cells, we showed that Epo through binding to the EpoR significantly alleviates apoptosis induced by staurosporine.4

To explore the contribution of different signaling pathways to Epo-mediated antiapoptosis in neuronal cells, we used EpoR mutants in which a limiting component of a downstream signaling pathway is either selectively retained or inhibited. A PrlR/EpoR chimera combining the extracellular domain of the PrlR and the transmembrane and the intracellular domains of the EpoR was employed to distinguish the effects of the endogenous EpoR versus the introduced EpoR mutants (Fig. 1) (48). Binding of Prl to the extracellular domain of the PrlR/EpoR chimera activates EpoR signaling in the cytosol. A FLAG epitope tag was added at the N terminus of the extracellular domain of the PrlR to enable detection of the PrlR/EpoR chimera. In addition to the chimera of the PrlR and the wild-type EpoR (PrlR/EpoR-WT), we made mutant PrlR/ EpoR chimeras in which activation of only the STAT5 signaling pathway (PrlR/EpoR-F7Y343) or only the PI 3-kinase/AKT signaling pathway (PrlR/EpoR-F7Y479) was retained (25, 26, 47, 48, 60, 61). As a control, a mutant that activates neither the STAT5 nor the AKT signaling pathway (PrlR/EpoR-F8) was also used. Conversely, we also constructed a mutant PrlR/EpoR chimera with a single tyrosine to phenylalanine change, which is unable to activate the STAT5 signaling pathway (PrlR/EpoR-Y343F). As controls for overexpression of receptors, we generated SH-SY5Y cells that overexpress the wild-type murine or human EpoRs. Exogenously expressed EpoR is HA epitope-tagged at the N terminus of the extracellular domain to differentiate it from the endogenous EpoR.

PrlR/EpoR chimeras- or the HA-EpoR-overexpressing SH-SY5Y cells were generated by infection of the bicistronic GFP-expressing retrovirus. However, the transfection and retroviral infection efficiencies of SH-SY5Y cells were insufficient to generate enough cells expressing PrlR/EpoR chimeras or the HA-EpoR to conduct experiments. Therefore, we first generated SH-SY5Y cell lines that overexpress the murine ectropic receptor, similar to the method previously applied by our laboratory to PC12 cells (49). Ten clonal SH-SY5Y cell lines that overexpress the murine ectropic receptor were obtained by transfection and drug selection. Each was tested for growth rate, morphology, infection efficiency, differentiation by retinoic acid, and response to Epo upon induction of apoptosis (data not shown). One cell line (Eco-SH-SY5Y 7) was very similar to parental SH-SY5Y cells in these assays, yet showed a reasonably high infection efficiency (~50%). It was used in further experiments.

Infected cells were isolated by FACS based on the top 50% level of GFP expression (data not shown). Control cells, where the empty vector was introduced so that only GFP but not any receptor was expressed, were also generated (SH-SY5Y-GFP; data not shown). Expression of each PrlR/EpoR chimera and each HA-EpoR in infected cells was confirmed by Western blotting, using anti-FLAG and anti-HA antibodies, respectively (Fig. 2A). All PrlR/EpoR chimeras and the HA-EpoR were expressed at comparable levels. Parental SH-SY5Y cells and cells infected with an empty vector (SH-SY5Y-GFP) were used as negative controls.

As reported before, most overexpressed EpoR does not reach the cell surface (50); JAK2 is required for EpoR cell surface expression. Therefore, cell surface expression of FLAG-tagged PrlR/EpoR chimera and the HA-EpoR in SH-SY5Y cells was measured by FACS analysis (Fig. 2B). Because the receptors are tagged on the extracellular domain, in the absence of cell permeabilization primary antibodies only detect receptors on the cell surface. As shown in Fig. 2B, overexpressed PrlR/EpoR chimeras reach the cell surface, as infected cells have a higher level of APC fluorescence than control cells infected with the empty vector (SH-SY5Y-GFP). Surface expression of the PrlR/EpoR-F8 was highest, and the PrlR/EpoR-WT lowest. For the HA-EpoR, surface expression of the murine EpoR was slightly higher than that of the human EpoR (Fig. 2B).

As differentiated SH-SY5Y neuronal cells were to be used for the apoptosis experiments, we confirmed that overexpression of these receptors does not interfere with neuronal differentiation. The panels in Fig. 3 show representative displays of normal differentiation of SH-SY5Y cells overexpressing PrlR/EpoR chimeras or the HA-EpoR. Panels I in Fig. 3 show that transfected cells developed neurites in response to retinoic acid, which were comparable in length to those in the parental SH-SY5Y cells (top row). As shown in panels II in Fig. 3, GFP-positive neurites were observed, confirming normal differentiation of SH-SY5Y cells overexpressing PrlR/EpoR chimeras or the HA-EpoR. We also verified expression of a neuronal marker p-NFH in these differentiated cells (Fig. 3, panels III). Differentiated SH-SY5Y cells overexpressing PrlR/EpoR chimeras or the HA-EpoR express p-NFH at levels similar to the parental SH-SY5Y cells. None of the cells express the astrocyte marker GFAP (data not shown). The same pattern was observed for SH-SY5Y cells expressing other PrlR/EpoR chimeras not shown here. Taken together, these data confirmed that overexpression of PrlR/EpoR chimeras or the HA-EpoR does not interfere with neuronal differentiation of SH-SY5Y cells.

Prolactin Exerts Antiapoptotic Effects in SH-SY5Y Cells Overexpressing Wild-type PrlR/EpoR Chimeras but Not in Cells Overexpressing Mutant PrlR/EpoR Chimeras—To examine whether the downstream signaling pathways activated by the EpoR intracellular domain can exert

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4 M. Um, A. W. Gross, and H. F. Lodish, submitted for publication.
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As shown in Fig. 4A, upon induction of apoptosis by staurosporine, Prl supported survival of the SH-SY5Y cells overexpressing the wild-type PrlR/EpoR chimera but not of the cells overexpressing mutant PrlR/EpoR chimeras. In cells overexpressing the PrlR/EpoR-WT chimera, apoptosis was reduced by ~2-fold by 30 pm Prl treatment (Fig. 4A), similar to the effects of Epo on parental SH-SY5Y cells (4) or SH-SY5Y cells overexpressing either the murine or human HA-EpoR (Fig. 4B). In contrast, cells overexpressing mutant PrlR/EpoR chimeras showed no response or even slightly increased apoptosis following Prl treatment. As expected, the negative control cells where only GFP was expressed (Fig. 4A, SH-SY5Y-GFP) exhibited no effects by Prl treatment upon apoptosis. Therefore, some downstream signaling pathways activated through the wild-type EpoR intracellular domain can exert antiapoptotic effects in differentiated SH-SY5Y cells.

**Activation of Both the STAT5 and AKT Signaling Pathways Is Required for the Antiapoptotic Effects Mediated by EpoR Signaling**—We performed biochemical studies to ascertain the activation of individual signaling pathways by mutant PrlR/EpoR chimeras. As shown in Fig. 5, SH-SY5Y cells overexpressing the PrlR/EpoR-WT chimera show full activation of both STAT5 (Fig. 5A, lanes 1 and 2) and AKT (Fig. 5B, lanes 1 and 2) following Prl treatment. On the contrary, other mutant PrlR/EpoR chimera-expressing cells show impaired or absent activation of either STAT5 or AKT or both (Fig. 5, A and B, lanes 3–12). As expected, cells overexpressing the PrlR/EpoR-F8, lacking all cytoplasmic tyrosine residues, exhibited activation of neither STAT5 nor AKT following Prl treatment (Fig. 5, A, lanes 3 and 4, and B, lanes 3 and 4), confirming the significant role of the eight cytoplasmic tyrosine residues of the EpoR in signaling in SH-SY5Y cells. As expected from studies in hematopoietic cells (25, 26, 47, 48), in cells overexpressing the PrlR/EpoR F7Y343, Prl induced phosphorylation of STAT5 (Fig. 5A, lanes 5 and 6), albeit weaker than did the PrlR/EpoR-WT chimera, but did not activate AKT (Fig. 5B, lanes 5 and 6). The converse mutation, in which only tyrosine 343 was mutated to phenylalanine (PrlR/EpoR Y343F), caused a loss of STAT5 activation (Fig. 5A, lanes 9 and 10) but no impairment of AKT activation by Prl (Fig. 5B, lanes 9 and 10). In cells overexpressing the PrlR/EpoR-F7Y479 chimera, AKT, but not STAT5, was phosphorylated following Prl treatment (Fig. 5, A, lanes 7 and 8, and B, lanes 7 and 8). Together these results confirm the roles of tyrosine residues in the EpoR cytoplasmic domain in activation of two downstream signaling pathways STAT5 and AKT in differentiated SH-SY5Y cells.

On the other hand, ERK 1/2 was activated following Prl treatment of all of the PrlR/EpoR chimeras-expressing cells (Fig. 5C). Even the PrlR/EpoR-F8 chimera (Fig. 5C, lanes 3 and 4) activated ERK 1/2 to an extent comparable with the PrlR/EpoR-WT chimera (Fig. 5C, lanes 1 and 2). This result is consistent with a previous report showing that in erythroid-type cells, ERK 1/2 is activated by a mutant EpoR in which all tyrosine residues were deleted or mutated to phenylalanine (42). Because JAK2 can associate with this EpoR mutant and become activated by Epo, ERK 1/2 is thought to be activated by JAK2 without phosphorylation of the EpoR.

As controls, HA-EpoR-overexpressing cells exhibited phosphorylation of all STAT5, AKT, and ERK 1/2 following Epo treatment (Fig. 5, A–C, lanes 13–16). Total expression of STAT5 (Fig. 5A, lower panels), AKT (Fig. 5B, lower panels), and ERK 1/2 (Fig. 5C, lower panels) was not changed following Prl or Epo treatment in any cell type.

We also examined activation of NF-kB by Prl in the PrlR/EpoR chimera-expressing cell lines. Similar to the parental SH-SY5Y cells treated with Epo, we observed modest accumulation of NF-kB p65 in the nuclear fraction of the cells overexpressing the PrlR/EpoR-WT 30 min.

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FIGURE 3. Normal differentiation of SH-SY5Y cells overexpressing PrlR/EpoR chimeras and the HA-EpoR. Cells were differentiated with 5 μM all-trans-retinoic acid in Neurobasal-A medium for 7 days. Panels I represent the phase contrast images of differentiated cells. Panels II, stained for GFP, show normal neurite outgrowth of GFP-positive cells overexpressing either PrlR/EpoR chimeras or the HA-EpoR. Panels III display expression of the neuronal marker p-NFH. The arrowheads indicate examples of neurites where expression of p-NFH and GFP overlaps.
after Prl addition (Fig. 5D, upper panels), which was much lower than that generated by TNF-α treatment (Fig. 5D) (53, 54). None of the mutant PrlR/EpoR chimeras showed accumulation of NF-κB p65 in nuclear fractions at any time point, except for the PrlR/EpoR-Y343F chimera that exhibited slight accumulation only at 60 min after Prl treatment. Epo treatment of EpoR-overexpressing SH-SY5Y cells similarly induced modest accumulation of NF-κB p65 in the nuclear fraction at 30 min after Epo treatment. Electrophoretic mobility shift assay using consensus NF-κB binding oligonucleotides as a DNA probe also showed the same pattern of modest activation of NF-κB following Prl treatment (Fig. 5D, lower panels).

The contribution of each signaling pathway downstream of the EpoR in antiapoptosis is summarized in Table 1. Activation of MAPK alone (PrlR/EpoR-F8) cannot inhibit apoptosis in differentiated SH-SY5Y cells. Activation of STAT5 and MAPK together (PrlR/EpoR-F7Y343) or AKT and MAPK together (PrlR/EpoR-F7Y479) or AKT, MAPK, and NF-κB together (PrlR/EpoR-Y343F) are insufficient for antiapoptotic effects. Only when both STAT5 and AKT are activated together, staurosporine-induced apoptosis prevented in differentiated SH-SY5Y cells (PrlR/EpoR-WT).

To confirm the requirement of activation of AKT for the antiapoptotic effects mediated by EpoR signaling, we examined effects of LY294002 and wortmannin on the antiapoptotic activity of Prl for SH-SY5Y cells overexpressing the wild-type PrlR/EpoR chimera. These two reagents block PI 3-kinase activity and hence prevent AKT activation. LY294002 and wortmannin were added to the cells at the same time as Prl. As shown in Fig. 6, in the presence of LY294002 or wortmannin, Prl treatment could not protect the cells overexpressing the wild-type PrlR/EpoR chimera from apoptosis induced by staurosporine. These results confirmed that the PI 3-kinase/AKT signaling pathway is critical for the antiapoptotic activity of EpoR signaling.

**STAT5 and AKT Are Phosphorylated following Prl Treatment Even in the Presence of 0.1 μM Staurosporine**—We used staurosporine because it is known to induce apoptosis in many cell types, including SH-SY5Y cells (57, 58), and Epo was shown to reduce apoptosis induced by staurosporine in several cell types (62–64). However, staurosporine is a potent protein kinase inhibitor. Thus, phosphorylation of certain signaling molecules might not occur in the presence of staurosporine, and this could be related to the antiapoptotic effects noted in the PrlR/EpoR-WT chimera- or EpoR-overexpressing SH-SY5Y cells. To address these questions, we performed biochemical studies on cells treated with 0.1 μM staurosporine. In these studies staurosporine was present during 5 h of growth factor starvation, harvesting, and Prl treatment and was removed only when the cells were lysed. As shown in Fig. 7A, STAT5 was phosphorylated following Prl treatment of PrlR/EpoR-WT-expressing cells in the presence of 0.1 μM staurosporine. Activation of AKT required phosphorylation of both a threonine residue on the kinase domain and a serine residue on the hydrophobic regulatory domain (see Introduction). A previous report showed that phosphorylation of the threonine residue, which is essential for AKT activation, is especially susceptible to staurosporine (65). Therefore, we examined phosphorylation of the threonine residue on the kinase domain of AKT by Prl treatment of the PrlR/EpoR-WT-expressing cells in the presence of 0.1 μM staurosporine. Fig. 7B shows that, similar to STAT5, 0.1 μM staurosporine treatment did not interfere with phosphorylation of the activating threonine residue in the AKT kinase domain. We also confirmed that Prl-triggered phosphorylation of the serine residue on the AKT hydrophobic domain also occurred in the presence of 0.1 μM staurosporine treatment (data not shown).

**DISCUSSION**

**Signaling Pathways Downstream of the EpoR**—Using the PrlR/EpoR chimera system, in which a single component of the downstream signaling pathways is selectively retained or inactivated by mutating cytoplasmic tyrosine residues of the EpoR, we demonstrate that activation of multiple signaling pathways is required for the antiapoptotic activity of Epo in differentiated SH-SY5Y neuroblastoma cells. In hematopoietic cells, activation of one pathway, such as STAT5 or AKT, can partially support cell survival (47, 48, 61, 66). In contrast, the antiapoptotic effects of Epo in neuronal cells require activation of multiple pathways, suggesting that their functions are likely less redundant than in hematopoietic cells. The STAT5 and AKT signaling pathways seem to be especially critical, as impairment of either of these pathways completely blocks the antiapoptotic activity of EpoR signaling in differentiated SH-SY5Y cells.
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In addition to the STAT5, AKT, and MAPK signaling pathways, other signaling pathways are activated by the EpoR in hematopoietic cells (24, 25). For several reasons differentiated human neuroblastoma SH-SY5Y cells are an appropriate system for studying apoptosis in neurons. First, cultured SH-SY5Y cells can be efficiently differentiated to neuron-like cells by treatment with retinoic acid. These cells had long neurites and efficiently expressed the neuronal marker protein phospho-neurofilament H. Second, primary neuronal cultures are a mixture of heterogeneous types of neurons and contaminated glia, but SH-SY5Y cells are homogeneous. Third, the growth rate of undifferentiated SH-SY5Y cells is much faster, a doubling time of 35–40 h, than that of primary neuronal cells. Fourth, by transducing the cells to express the murine ecotropic retrovirus, we were able to use retroviral infection to overexpress proteins in hematopoietic cells (24, 35–40). Tyrosine 343 of the EpoR (Fig. 1, F7Y343) is respon-

**TABLE 1**

Role of the cytoplasmic tyrosine residues of the EpoR in signaling and antia apoptotic activity

| EpoR intracellular domain | STAT5 | AKT | ERK 1/2 | NF-κB | Antiapoptotic activity |
|---------------------------|-------|-----|---------|-------|-----------------------|
| Wild-type EpoR            | +     | +   | +       | +     | +                     |
| F8                        | −     | −   | −       | −     | −                     |
| F7Y343                    | +     | +   | −       | −     | −                     |
| F7Y479                    | +     | +   | −       | −     | −                     |
| Y343F                     | −     | +   | +       | −     | −                     |

**FIGURE 6.** Activation of AKT is required for the antia apoptotic effects of Prl for SH-SY5Y cells overexpressing the wild-type PrlR/EpoR chimera. 10 μM LY294002 or 100 nM wortmannin was added at the same time as 30 pm Prl to cells overexpressing the wild-type PrlR/EpoR chimeras; Prl was added either 24 h before (pretreatment) or upon (concomitant treatment) induction of apoptosis by 0.1 μM staurosporine. As in Fig. 4, apoptotic cell population was identified by FACS analysis using annexin V binding and 7-AAD staining. The graph displays the Relative Apoptosis Proportion. About 40–90% of cells underwent apoptosis by staurosporine treatment. The indicated values are the averages of four independent experiments performed in duplicate. Standard deviations are depicted as error bars. ***, p < 0.0001 by t test.

**FIGURE 7.** STAT5 and AKT are phosphorylated by Prl treatment in the presence of 0.1 μM staurosporine. SH-SY5Y cells overexpressing the PrlR/EpoR-WT chimera were starved of growth factors for 5 h in the absence (−) or presence (+) of 0.1 μM staurosporine and then were either untreated (−) or treated with Prl for 10 min (+) in the continued absence (−) or presence (+) of 0.1 μM staurosporine. Lysates were immunoprecipitated with anti-STAT5 (A) or anti-AKT (B) antibodies. Western blot analysis was performed with anti-p-STAT5 (A, upper panels) or p-Thr-AKT (B, upper panels) antibodies. For quantification of protein loading, the membranes were stripped and reprobed with anti-STAT5 (A, lower panels) or anti-AKT (B, lower panels) antibodies.

For several reasons differentiated human neuroblastoma SH-SY5Y cells are an appropriate system for studying apoptosis in neurons. First, cultured SH-SY5Y cells can be efficiently differentiated to neuron-like cells by treatment with retinoic acid. These cells had long neurites and efficiently expressed the neuronal marker protein phospho-neurofilament H. Second, primary neuronal cultures are a mixture of heterogeneous types of neurons and contaminated glia, but SH-SY5Y cells are homogeneous. Third, the growth rate of undifferentiated SH-SY5Y cells is much faster, a doubling time of ~55 h, than that of primary neuronal cells. Fourth, by transducing the cells to express the murine ecotropic receptor, we were able to use retroviral infection to overexpress proteins of interest, here PrlR/EpoR chimeras and the EpoR. In contrast, transfection or infection of primary neurons is always problematic because of their post-mitotic status.

In addition to the STAT5, AKT, and MAPK signaling pathways, other signaling pathways are activated by the EpoR in hematopoietic cells (24, 35–40). Tyrosine 343 of the EpoR (Fig. 1, Y343F and F7Y343) is respon-
sible for activation of both STAT5 and AP-1 transcription factors (48, 67). Thus, loss of antiapoptotic effects by mutation of tyrosine 343 of the EpoR to phenylalanine (Fig. 1, Y343F) could be explained by disruption of other downstream signaling pathways in addition to STAT5. The converse mutation, where tyrosine 343 remains intact yet seven other tyrosine residues are mutated to phenylalanine (Fig. 1, F7Y343), exhibits normal EpoR-mediated activation of STAT5 and AP1 (48, 67). This mutant receptor, however, was also unable to provide antiapoptosis in SH-SY5Y cells. These results suggest that the antiapoptotic effects mediated by EpoR signaling in these cells require multiple downstream pathways, including STAT5 and possibly AP-1. However, an additional signaling pathway is also necessary.

This additional pathway is likely downstream of the AKT kinase. Tyrosine 479 of the EpoR (Fig. 1, F7Y479) is the primary recruitment site for PI 3-kinase and thus crucial for AKT activation (47, 60, 61). Mutation of all tyrosine residues in the cytoplasmic domain of the EpoR, except tyrosine 479, to phenylalanine (Fig. 1, F7Y479) results in impairment of STAT5 activation but preserves AKT activation. This mutation causes loss of the antiapoptotic effects by EpoR signaling in SH-SY5Y cells, confirming that the AKT signaling pathway is necessary but not sufficient for antiapoptotic signaling by the EpoR in SH-SY5Y neuronal cells.

Digicaylioglu and Lipton (12) suggest that activation of the EpoR in neurons triggers cross-talk between the signaling pathways of JAK2 and NF-κB. They show that activation of JAK2 by the EpoR in neuronal cells leads to phosphorylation of IκB, which results in the nuclear translocation of NF-κB and subsequent transcriptional activation of NF-κB-dependent neuroprotective genes. In their studies, phosphorylation of IκB following Epo treatment in neuronal cells was very slow; phosphorylation of both serine and tyrosine residues of IκB was observed at 1 h and maintained at 3 h after Epo treatment. Our study in SH-SY5Y cells shows that NF-κB p65 accumulates in nuclear fraction only modestly at 30 min after EpoR activation. In contrast, as in erythroid cells, activation of STAT5, AKT, and ERK 1/2 in SH-SY5Y cells is very rapid, occurring within 10 min after EpoR activation. This short time frame suggests that activation of STAT5 and ERK 1/2 in SH-SY5Y cells is mediated directly by phosphorylation by JAK2, as in hematopoietic cells (25–28). In the case of AKT, recruitment and resultant activation of PI 3-kinase by JAK2 is responsible for phosphorylation of AKT in hematopoietic cells (29–31). Therefore, activation of NF-κB is likely to be an indirect activation by these immediate signaling pathways, rather than via direct activation by JAK2. Interestingly, the PrlR/EpoR-Y343F chimera was able to activate the NF-κB signaling pathway, albeit more weakly than the PrlR/EpoR-WT chimera. This result is consistent with previous studies showing that AKT is the upstream kinase that regulates the NF-κB signaling pathway (68, 69). In specific types of cells, STAT5 also plays a role in regulating the NF-κB signaling pathway (70). We speculate that in SH-SY5Y cells both AKT and STAT5 contribute to activation of the NF-κB signaling pathway by inducing expression of a protein that, in turn, triggers NF-κB activation.

Other reports have suggested that activation of NF-κB plays an important role in the effects of Epo on various types of cells, including mitogenic and antiapoptotic effects in hematopoietic cell lines (71, 72), neurogenesis of neuronal stem cells (73), and differentiation of neuronal stem cells into astrocytes (74). These results imply that activation of NF-κB by Epo is not restricted to neuronal cells. Kolonitsas et al. (75), however, reported that Epo does not activate NF-κB in myeloid/erythroid TF-1 cells. In certain cancer cell lines, Epo treatment reduced rather than enhanced NF-κB activation (76, 77). Taken together, these results suggest that the importance of the NF-κB signaling pathway in Epo varied in different cell types.

Role of the EpoR in Antiapoptotic Activity of Epo in Neuronal Cells—Recent reports suggested that the cytoprotective effects of Epo may be separated from its hematopoietic effects through signaling by an alternative receptor (59). Instead of the “classical” homodimeric EpoR complex, a heterotrimer complex consisting of an EpoR monomer and a dimer of the common β chain was suggested to be responsible for Epo cytoprotective effects.

By using the PrlR/EpoR chimera system, we show that activation of signaling pathways downstream of the classical EpoR blocks apoptosis in SH-SY5Y cells. These PrlR/EpoR chimeras contain the extracellular domain of the PrlR and provide antiapoptotic effects that are identical to those induced by the EpoR. Our results suggest that the EpoR cytoplasmic domain per se is sufficient to generate antiapoptotic signals. Consistent with this suggestion, in another paper4 we show that the classical homodimeric EpoR complex is responsible for the antiapoptotic effects of Epo in differentiated SH-SY5Y cells. We speculate that the receptor mediating the cytoprotective effects of Epo might be different depending on the type of cells. It would be interesting to determine whether other forms of the EpoR complexes exist in SH-SY5Y cells, such as a heteromer with the common β chain and, if so, whether this complex contributes to the antiapoptotic effects of Epo.

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