A Novel, Nerve Growth Factor-activated Pathway Involving Nitric Oxide, p53, and p21WAF1 Regulates Neuronal Differentiation of PC12 Cells*

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During development, neuronal differentiation is closely coupled with cessation of proliferation. We use nerve growth factor (NGF)-induced differentiation of PC12 pheochromocytoma cells as a model and find a novel signal transduction pathway that blocks cell proliferation. Treatment of PC12 cells with NGF leads to induction of nitric oxide synthase (NOS) (Peunova, N., and Enikolopov, G. (1995) Nature 375, 68–73). The resulting nitric oxide (NO) acts as a second messenger, activating the p21WAF1 promoter and inducing expression of p21WAF1. Cyclin-dependent kinase inhibitor NO activates the p21WAF1 promoter by p53-dependent and p53-independent mechanisms. Blocking production of NO with an inhibitor of NOS reduces accumulation of p53, activation of the p21WAF1 promoter, expression of neuronal markers, and neurite extension. To determine whether p21WAF1 is required for neurite extension, we prepared a PC12 line with an inducible p21WAF1 expression vector. Blocking NOS with an inhibitor decreases neurite extension, but induction of p21WAF1 with isopropyl-1-thio-β-β-galactopyranoside restored this response. Levels of p21WAF1 induced by isopropyl-1-thio-β-β-galactopyranoside were similar to those induced by NGF. Therefore, we have identified a signal transduction pathway that is activated by NGF; proceeds through NOS, p53, and p21WAF1 to block cell proliferation; and is required for neuronal differentiation by PC12 cells.

Neuronal differentiation is closely linked to cessation of cell proliferation, but how these states are connected remains a major unanswered question. Nerve growth factor (NGF) induces both cell cycle arrest and differentiation for PC12 pheochromocytoma cells (1). These effects are associated with induction of nitric oxide synthase (NOS) (2, 3), the p53 tumor suppressor (4), and the p21WAF1 cyclin-dependent kinase inhibitor (5–7). However, the relationship among these signaling events, arrest, and differentiation is poorly understood.

Nitric oxide (NO) is a regulatory molecule that influences many processes, perhaps including neuronal proliferation and differentiation. NO is synthesized from arginine by a family of three NOS proteins: neuronal NOS (nNOS), endothelial NOS (eNOS), and inducible NOS (iNOS) (8). In mature mammals, NO acts as a neurotransmitter, a regulator of blood pressure, and a toxin for killing pathogens. A role for NO in the developing nervous system is plausible because nNOS is absent from proliferating neuroblasts but is coexpressed with early markers of neuronal differentiation (9, 10). In Drosophila, NOS is expressed in developing imaginal discs (11). Inhibition of NOS in larvae results in enhanced cell proliferation, and elevated NOS expression stunts development. In rats, NGF enhances expression of nNOS by cholinergic neurons of the basal forebrain (12). NGF treatment of PC12 cells induces expression of all three isoforms of NOS (2, 3), and inhibitors of NOS block NGF-induced cessation of proliferation and neurite extension for PC12 cells (3). Hence, NO acts as a regulator of cell proliferation which, in turn, influences process outgrowth.

The tumor suppressor p53 may play a role in neural development, in addition to its well-established function as an inducer of apoptosis or cell cycle arrest after certain types of cell stress, including DNA damage (13). Mice lacking functional p53 genes have an unusually large number of birth defects, such as failure of neural tube closure (14). In some mouse embryos, the absence of p53 leads to exencephaly in which overgrowth of neural tissue in the fore- and midbrain leads to abnormalities in cranial development (15). This overgrowth probably results from excessive cell proliferation rather than decreased cell death. Consistent with this role, a recent study found that p53 is required for NGF-induced neurite extension by PC12 cells (4).

p21WAF1 binds to and inhibits cyclin-dependent kinases and induces cell cycle arrest at G1/S (16). p53 is a potent transcriptional activator for p21WAF1 (16, 17), but expression of p21WAF1 also can be induced by p53-independent mechanisms (18–22). In vivo, expression of p21WAF1 is enhanced by myoblasts differentiate into muscle cells (23–25). p21WAF1 is not expressed in the mitotic germinal layer of the olfactory epithelium but is expressed by olfactory neurons (25). We (5) and others (6, 7, 26) have reported up-regulation of p21WAF1 expression in NGF-treated cell lines. We have demonstrated that p21WAF1 is required for survival of differentiating neuroblastoma cells (5), but van Grunsven et al. (6) have suggested that after differentiation of PC12 cells, continued expression of p21WAF1 is not required to maintain their differentiated phenotype. Hence, it may be that p21WAF1 plays its most important role during differentiation.
In this report, we provide the first evidence for a connection among these NGF-induced events. We find that NO activates the p21WAF1 promoter, resulting in expression of p21WAF1 protein. This link is partially dependent on p53, a potent transcriptional activator for p21WAF1 (16). NO also is required for NGF-induced expression of two markers associated with neuronal differentiation. Using a PC12 line bearing an inducible expression vector for p21WAF1, we find that induction of recombiant p21WAF1 restores NGF-induced differentiation for cells treated with a NOS inhibitor. These data demonstrate a signal transduction pathway that is activated by NGF; proceeds through NOS, p53, and p21WAF1 to block cell proliferation; and is required for NGF-induced neuronal differentiation by PC12 cells.

EXPERIMENTAL PROCEDURES

Cell Culture—PC12 cells from Dr. David Kaplan (Montreal Neurological Institute) were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated horse serum, 5% heat-inactivated fetal bovine serum, and 100 μg/ml gentamycin at 37 °C under 5% CO2. The cells were found to be mycoplasma-free by a direct culture method (MYCOTRIM, Irvine Scientific, Santa Ana, CA). For PC12 cell differentiation, plastic dishes were treated overnight at 4 °C with 15 μg/ml rat tail collagen (Sigma) and 15 μg/ml poly-D-lysine (>300,000 Da, Sigma). The dishes were rinsed with distilled water, and then PC12 cells were plated in defined medium, as described (27), and treated with 100 ng/ml NGF (2.5 S, Bioproducts for Science, Indianapolis). After 3–4 days of treatment, cells with neurites at least five cell diameters long were scored as positive.

NOS Inhibitors—N-Nitro-L-arginine methyl ester (L-NAME) competes with arginine for binding to all three isoforms of NOS and blocks enzymatic activity (8). In these studies, we used 20 μm L-NAME and the same concentration of the inactive enantiomer, D-NAME, as a control. L-NAME used at this concentration is thought to be specific for NOS and with little effect on PC12 metabolism, growth rates, and NGF induction of immediate-early genes (3).

Preparation of a p21WAF1 Inducible Cell Line—We prepared a PC12 line bearing an inducible expression vector (LacSwitch, Stratagene, La Jolla, CA) for p21WAF1. In this system, mammalian cells are transfected with both a Lac-repressor-expressing vector and a lac-operator-containing p21WAF1 vector. Expression of p21WAF1 occurs within 48 h after the addition of 25 mM (S)-isopropyl-1-thio-β-D-galactopyranoside-β-1′,2′-p-iodophenyl-3′-D-galactopyranoside (IPTG). The p21WAF1 plasmid for these experiments was prepared by J. Earheart and R. Pittman (University of Pennsylvania School of Medicine). In brief, the protein-encoding portion of the p21WAF1 cDNA was inserted into the pOPRSV1 plasmid (Stratagene) which includes a neomycin resistance gene. Expression of p21WAF1 is driven by a Rous sarcoma virus promoter, but, in the absence of IPTG, is inducible by adding lac repressor operator sites. Plasmid p3S-SS, which has the lac repressor gene under control of a cytomegalovirus early promoter, also was from Stratagene and was used without modification.

PC12 cells were transfected with the p3S Lac repressor plasmid, using the cationic detergent Lipofectin (Life Technologies, Inc.). Plasmid DNA (6–7 μg/25-mm2 flask) was diluted in 100 μl of Dulbecco’s modified Eagle’s medium (serum-free). At the same time in a second tube, 20 μl of 1 mg/ml Lipofectin was diluted with 100 μl of Dulbecco’s modified Eagle’s medium. After a 30-min incubation at room temperature, the two solutions were combined and incubated for an additional 15 min at room temperature. This mix was then diluted with 2.5 ml of complete medium including serum, added to a 25-mm2 tissue culture flask containing about 3 × 105 cells, and incubated overnight at 37 °C. The resulting cells were selected for 14 days with medium containing 125 μg/ml hygromycin. This mass culture was derived from about 30 different G418-resistant colonies. The morphology and rates of proliferation of these cells were not obviously affected by the transfections.

Western Blotting—After treatment with NGF and NO drugs, cells were extracted as described (17). These samples (~40 μg of protein/lane) were boiled under reducing conditions, subjected to electrophoresis on a 12.5% polyacrylamide gel, and electrotransferred to an Immobilon-P membrane (Millipore, Bedford, MA). Residual proteins in the gel were stained with Coomassie Blue to confirm even loading of the gel. The membrane was blocked for 1 h with 10% powdered milk in 0.2% Tween 20, Tris-buffered saline, and then incubated with 2 μg/ml anti-p53 monoclonal antibody Pab-1801 (Oncogene Science, Cambridge, MA), 1 μg/ml rabbit anti-p21WAF1 antibody C-19 (Santa Cruz Biotechnology, Santa Cruz, CA), or 35 μg/ml anti-neuronal nitric oxide repressor (nNOS) monoclonal antibody mab35 (25). Immunoreactive proteins were detected with horseradish peroxidase-conjugated secondary antibodies (Amersham, Arlington Heights, IL) and a chemiluminescence reagent (29). The films were scanned with a Hewlett Packard ScanJet 3c. Brightness and contrast were adjusted with Photoshop, making the same adjustments for each band. Montages were assembled with CorelDraw, using only images from the same experiment and film.

Diaphorse and Immunostaining—Cells were fixed for 10 min at room temperature with 4% paraformaldehyde in PBS and immunostained with 1 μg/ml of anti-p21WAF1 rabbit antibody C-19 and then with a rhodamine-conjugated secondary antibody. The cells were then incubated with 1.0 mg/ml of NAPDH, 0.25 mg/ml of nitro blue tetrazolium and 0.1% Triton HCl for 4 h at 37 °C (30). Samples were mounted in Citifluor (Ted Polla Inc., Reading, PA) and were viewed with a Zeiss Axiolab microscope and a 25× oil immersion objective lens, using bright field and fluorescence optics to detect diaphorase and p21WAF1, respectively.

For detection of neuronal markers, cells were fixed for 10 min with 35% methanol, 5% acetic acid at −20 °C. The samples were stained with 35 μg/ml monoclonal antimonos and anti-mAbs35 against the α subunit of the nicotinic acetylcholine receptor (nACHR) (28) or ascites diluted 1:200 from hybridoma MAP1B-4 directed against microtubule-associated protein 1B (MAP1B) (31, 32). These antibodies were diluted with 0.3% bovine serum albumin, 0.2% Tween 20 in Tris-buffered saline. Both of these markers are expressed during development, although the time of initial onset is earlier for MAP1B (33).

Micrographs were recorded with Kodak T-MAX 400 film and digitized with a Nikon Coolscan. Brightness and contrast were adjusted with Adobe Photoshop, and montages were assembled with CorelDraw. The figures were printed using a Kodak Colorsear PS printer on Kodak Extathrowal XLS paper.

p21WAF1 Promoter Activity—PC12 cells were treated for 60 h with or without NGF, L-NAME, or D-NAME. The pLacOP-1 and pLacIFG, described above, were cotransfected with promoter constructs WWP-Luc or DM-Luc (16) (6 μg/25-mm2 flask) and β-galactosidase plasmid pCH110 (6 μg/25-mm2 flask). After 16–20 h, cells were extracted for 15 min at room temperature with cell culture lysis reagent (Promega; 25 mM Tris phosphate, pH 7.8, 2 mM dithiothreitol, 2 mM EDTA, 10% glycerol, and 1% Triton X-100). These extracts were clarified by centrifugation and mixed with 30 μl of NADPH, 0.25 mg/ml of nitroblue tetrazolium, and 0.1 mM EDTA, 33.3 mM dithiothreitol, 270 μg/ml of coenzyme A, 470 μl of luciferase assay reagent (20 μl Tricine, 1.07 mM Mg(OH)2, 1.33 mM Mg(OAc)2, 30 mM H2O, 0.2 mM MgSO4, 0.1 mM EDTA, 33.3 mM dithiothreitol, 270 μg/ml of coenzyme A, 470 μl of luciferase assay reagent, pH 7.8) were immediately placed in a model N Luminometer analyzer (Packard), and luciferase activities were measured. β-Galactosidase activities were measured using a kit from Promega. Assay 2% buffer (120 mM Na2HPO4, 80 mM NaH2PO4, 2 mM MgCl2, 100 mM β-mercaptoethanol, 1.33 mg/ml o-nitrophenyl-β-D-galactopyranoside) was mixed with an equal volume (100 μl) of cell extract. Samples were incubated overnight at 37 °C, and the reaction was stopped by the addition of 50 μl of 1 M Na2CO3. Optical densities at 420 nm were measured. Luciferase activities were normalized with β-galactosidase activities to eliminate any differences in transfection efficiencies.

RESULTS

Using NGF-treated PC12 cells as a model system, we tested the relationship among several signaling events that occur during neuronal differentiation. We found that the NOS inhibitor, L-NAME, but not the inactive enantiomer, D-NAME, inhibits NGF-induced neurite extension by 73 ± 15% (mean ± S.D., n = 6), in agreement with Penanova and Enikolopolov (3).

We assayed expression of two markers associated with neuronal differentiation. As judged by immunofluorescence microscopy, MAP1B (34, 35) is up-regulated by NGF (Fig. 1). For this experiment, untreated PC12 cultures showed 12 ± 1% of cells positive for MAP1B, but NGF-treated cultures showed 87 ± 3% cells positive. L-NAME, but not D-NAME, reduced this NGF-induced increase in MAP1B expression (L-NAME + NGF-treated cells, 88 ± 4% positive; L-NAME + NGF-treated cells,
14 ± 2% positive). Averaging three experiments, l-NAME inhibited NGF induction of MAP1B-positive cells by 85 ± 10%. The pattern of expression for nAChR closely resembled that for MAP1B (36) (micrographs not shown). By Western blotting, we observed the same pattern of expression for nAChR (Fig. 2). Averaging three experiments, the relative intensities of the nAChR bands for control, NGF, NGF + d-NAME, NGF + L-NAME, and l-NAME were 1.0, 2.6 ± 0.2, 3.7 ± 1.6, 0.8 ± 0.4, and 0.8 ± 0.3, respectively. The anti-MAP1B antibody does not detect MAP1B protein by Western blotting. Hence, NO, like cell cycle arrest, is required for NGF-induced differentiation.

We then analyzed the role of NO in regulating expression of p53 and p21WAF1 (4–7). As judged by scanning densitometry, NGF induced accumulation of p53 and p21WAF1 by 71.3 ± 4.3-fold and 4.7 ± 1.1-fold (n = 3), respectively, compared with levels in untreated cells (Fig. 3). l-NAME, but not d-NAME, inhibited NGF-induced accumulation of p53 by 87 ± 7% and p21WAF1 by 61 ± 7%. This is the first report that NO, at physiological levels, acts as a second messenger to enhance expression of p53 and p21WAF1, although it was reported that treatment of cells with NO donors induces accumulation of p53 (37–44).

To evaluate further the relationship between NO and p21WAF1 expression, we transfected PC12 cells with a p21WAF1 promoter-luciferase reporter construct (WWP-Luc (16)) and found that treatment with NGF significantly enhanced the activity of the p21WAF1 promoter (p = 0.0001; Student’s t test) (Fig. 4). This response was inhibited by l-NAME but not by d-NAME. The parallel regulation of p21WAF1 promoter activity and p21WAF1 protein levels (Figs. 3 and 4) suggests that NGF increases p21WAF1 levels by an NO-dependent increase in p21WAF1 promoter activity.

To determine whether NGF regulates p21WAF1 promoter activity via p53, a potent transcriptional activator of p21WAF1 (16), we utilized a truncated promoter lacking the p53 binding site (DM-Luc (16)). Cells transfected with this shorter construct gave substantially reduced responses to NGF (Fig. 4). Therefore, transcriptional activation by p53 is a major mechanism by which NGF induces p21WAF1 expression. However, there was significant activation (p = 0.0001) of the shorter promoter construct, indicating a second mechanism independent of the p53 binding site.

We also assessed the relationship between NOS, p21WAF1, and neurite extension for individual cells. We double stained PC12 cells for diaphorase activity and p21WAF1. Diaphorase staining allows visualization of NOS enzymatic activity, resulting in colored cells that can be detected by bright-field light microscopy (45, 46). This method is specific for NOS because other diaphorases are inactivated by fixation with parafomaldehyde. p21WAF1 was detected by immunofluorescence microscopy, using an anti-p21WAF1 antibody. For untreated cultures, few PC12 cells displayed diaphorase staining, p21WAF1 immunoreactivity, or neurites (Fig. 5, A, C, and E, respectively). NGF treatment increased the percentages of cells with diaphorase activity (92%), p21WAF1 immunoreactivity (85%), and neurites (77%) (Fig. 5, B, D, and E, respectively). The staining for p21WAF1 was almost exclusively nuclear, in agreement with other studies (47, 48).

To test the role of p21WAF1 in NGF-induced differentiation, we prepared a PC12 line bearing an inducible p21WAF1 expression vector (Fig. 6). This line differentiated in response to NGF, in the same manner as the parental PC12 line, and l-NAME reduced neurite extension (Fig. 6, A, D, and E). However, induction of p21WAF1 expression with IPTG restored NGF-induced differentiation of l-NAME-treated cells (Fig. 6B). Addition of IPTG alone had no effect on neurite extension (not shown), but IPTG did reduce proliferation, as judged by bromodeoxyuridine labeling, by 58 ± 5%. IPTG did not enhance the effect of NGF (Fig. 6, C and E). We also noted that p21WAF1 levels induced by IPTG (320 ± 30% relative to untreated cells) were similar to those induced by NGF (250 ± 20%) (Fig. 7).
Hence, p21 WAF1 is the functionally important target of the NGF-NOS pathway, but p21WAF1, like NO, is not sufficient by itself for PC12 differentiation.

DISCUSSION

The major finding of this study is a novel, NGF-activated signal transduction pathway that regulates neurite extension. NGF binds to its receptors and induces expression of NOS, perhaps by activating mitogen-activated protein kinases (49) and the transcription factor, nuclear factor-κB (50, 51), which are associated with NOS expression (52–54). In ongoing studies2 we have found that TrkA can induce NOS expression, but we have not yet assessed the role of the other NGF receptor, gp75. NO, in turn, raises levels of p53, a protein required for NGF-induced differentiation of PC12 cells (4). p53 protein activates transcription of p21WAF1 by binding to a p53 binding site in the p21WAF1 promoter, but transcription of p21WAF1 also is activated by a second mechanism that is not dependent on the p53 binding site and might involve the AP2 transcriptional activator protein (55) or the p300 transcriptional coactivator protein (56). The p21WAF1 protein is known to block the cell cycle at the G1/S transition. As judged by neurite extension and expression of neuronal markers, neuronal differentiation occurs when the NGF-NOS-p21WAF1-cytostasis pathway is activated in conjunction with the mitogen-activated protein kinase SNT and, perhaps, other NGF-regulated pathways (57).

For this pathway, the molecular targets of NO are not known, but we can suggest several possibilities. The best characterized target for NO is guanylate cyclase (9). NO activates guanylate cyclase, raising levels of cGMP and activating the cGMP-dependent kinase. The relevance of guanylate cyclase to NGF-induced differentiation is suggested by the increase in cGMP levels in PC12 cells after treatment with NGF (58). NO also reacts with ribonucleotide reductase, an enzyme required for nucleotide biosynthesis and cell proliferation (59). In addition, high levels of NO damage DNA and, thereby, induce expression of p53 (37). It is unlikely that NGF induces sufficient levels of NO to damage DNA, but we cannot exclude it from consideration.

The effects of NO include elevated levels of p53 (37–44), a protein required for NGF-induced neuritogenesis of PC12 cells (4). It is possible that p53 activity is additionally modified by other mechanisms. p53 is regulated by association with a variety of other proteins such as mdm-2 (13). Since p53 is subject to redox regulation and has free cysteines that might react with NO (60, 61), p53 itself might be an NO target. p53 activity is regulated by phosphorylation by casein kinase II, protein kinase C, and cyclin-dependent kinases (13). In addition, subcellular localization plays an important role in p53 function. NGF treatment of PC12 cells induces translocation of p53 from the cytoplasm to the nucleus (4).

In a recent study, it was reported that NGF-induced expression of p21WAF1 is p53-independent (56). In contrast, we find that activation of the p21WAF1 promoter occurs by two mechanisms. The first is initiated by binding of p53 to a site in the p21WAF1 promoter. The second is independent of this p53 binding site. These contradictory findings may be related to differ-
ences in culture conditions which affect both the extent of differentiation and the expression of cell cycle-associated proteins. For measurements of p21WAF1 promoter activity, we treated the PC12 cells for 3 days in defined medium, which considerably speeds both NGF-induced differentiation (27) and induction of p21WAF1 (62). The other group treated their cells for only 2 days in serum-containing medium. Hence, at the time of the promoter assay, the cells in our experiments were probably more differentiated than those in the other study. In addition to the degree of differentiation, the culture medium may affect considerably the expression of cell cycle-associated proteins. NGF induces expression of p21WAF1 in defined medium (this study and Ref. 62) or in the presence of serum (6, 7, 26) but not in Dulbecco’s modified Eagle’s medium lacking both serum and growth factors (6). Further studies are required to resolve these differences, but ultimately we feel that p53 will be shown to play a role in this pathway because p53 is required for NGF-induced neurite extension by PC12 cells (4).

NO leads to p21WAF1 induction but also regulates the activities of other enzymes and the expression of additional gene products (9). Which of these events is relevant to cell cycle arrest and neurite extension? To answer this question, we prepared a PC12 line with an inducible p21WAF1 expression vector. Blocking NOS with l-NAME inhibits neurite extension, but induction of p21WAF1 with IPTG restores the response. Furthermore, the levels of p21WAF1 induced by NGF and by IPTG are similar, so it is unlikely that our conclusion is based on an artifact of overexpression. Hence, the lesion in the pathway caused by l-NAME is complemented by IPTG-induced p21WAF1, and we conclude that p21WAF1 is a biologically relevant downstream product.

These studies have been carried out using PC12 cells, which are the most commonly used model for NGF-activated signal transduction and differentiation. However, these findings are relevant to other neuronal cell types, both in vitro and in vivo. For example, neuroblastoma, a pediatric tumor that probably results from excessive cell proliferation during gestation, has a high rate of spontaneous regression in which the tumor decreases in size and, in some cases, disappears. This favorable outcome is thought to be caused by differentiation of tumor cells and is correlated with expression of TrkA (63–65). Activation of JNK kinases is associated with cell death resulting from neurotrophin deprivation (78). Moreover, these JNK kinases, p21WAF1 might play a role in injury-induced responses. Hence, this model defines a key pathway required for NGF-induced differentiation of PC12 cells and serves as a prototype for the analysis of additional pathways that regulate cell proliferation during differentiation, the course of neural tumors, and responses to injury and other stimuli in mature neurons.

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