

A Dissertation Presented

By

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THE REGULATION OF nNOS DURING NEURONAL DIFFERENTIATION AND
THE EFFECT OF NITRIC OXIDE ON Hm2-p53 BINDING

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I also want to thank my parents and my wife, Amy. Your unwavering support kept me going for these six long years.

ABSTRACT

Nitric oxide is a ubiquitous signaling molecule with both physiological and pathological functions in biological systems. Formed by the enzymatic conversion of arginine to citrulline, NO, has known roles in circulatory, immune and nervous tissues. In the nervous system nitric oxide has been implicated in long-term potentiation, neurotransmitter release, channel function, neuronal protection and neuronal degeneration. Much of our work has focused on yet another role for nitric oxide in cells, namely, neuronal differentiation.

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). The resulting nitric oxide (NO) acts as a second messenger, activating the p21(WAF1) promoter and inducing expression of p21(WAF1) cyclin-dependent kinase inhibitor. NO activates the p21(WAF1) promoter by p53-dependent and p53-independent mechanisms. Blocking production of NO with an inhibitor of NOS reduces accumulation of p53, activation of the p21(WAF1) promoter, expression of neuronal markers, and neurite extension. To determine whether p21(WAF1) is required for neurite extension, we prepared a PC12 line with an inducible p21(WAF1) expression vector. Blocking NOS with an inhibitor decreases neurite extension, but induction of p21(WAF1) with isopropyl-1-thio-beta-D-galactopyranoside restored this response. Levels of p21(WAF1) induced by isopropyl-1-thio-beta-D-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 p21(WAF1) to block cell proliferation; and is required for neuronal differentiation by PC12 cells.

In further studies of this pathway, we have examined the role of MAP kinase pathways in neuronal nitric oxide synthase (nNOS) induction during the differentiation of PC12 cells. In NGF-treated PC12 cells, we find that nNOS is induced at RNA and protein levels, resulting in increased NOS activity. We note that neither nNOS mRNA, nNOS protein nor NOS activity is induced by NGF treatment in cells that have been infected with a dominant negative Ras adenovirus. We have also used drugs that block MAP kinase pathways and assessed their ability to inhibit nNOS induction. Even though U0126 and PD98059 are both MEK inhibitors, we find that U0126, but not PD98059, blocks nNOS induction and NOS activity in NGF-treated PC12 cells. Also, the p38 kinase inhibitor, SB 203580, does not block nNOS induction in our clone of PC12 cells. Since the JNK pathway is not activated in NGF-treated PC12 cells, we determine that the Ras-ERK pathway and not the p38 or JNK pathway is required for nNOS induction in NGF-treated PC12 cells. We find that U0126 is much more effective than PD98059 in blocking the Ras-ERK pathway, thereby explaining the discrepancy in nNOS inhibition. We conclude that the Ras-ERK pathway is required for nNOS induction.

The activation of soluble guanylate cyclase and the production of cyclic GMP is one of the best characterized modes of NO action. Having shown that inhibition of NOS blocks PC12 cell differentiation we tested whether nitric oxide acts through soluble guanylate cyclase to lead to cell cycle arrest and neuronal differentiation. Unlike NOS

inhibition, the inhibition of soluble guanylate cyclase does not block the induction of neuronal markers. Moreover, treatment of NGF-treated, NOS-inhibited PC12 cells with a soluble analog of cyclic GMP was unable to restore differentiation of those cells. Hence, cGMP is not a component of this pathway and we had to consider other mechanisms of NO action.

It has become increasingly evident that another manner by which NO may exert its effects is by S-nitrosylation of cysteine residues. We tested, *in vitro* whether nitric oxide may control p53 by S-nitrosylation and inactivation of the p53 negative regulator, Hdm2. Treatment of Hdm2 with a nitric oxide donor inhibits Hdm2-p53 binding, the first step in Hdm2 regulation of p53. The presence of cysteine or DTT blocks this inhibition of binding. Moreover, nitric oxide inhibition of Hdm2-p53 binding was found to be reversible. Sulfhydryl-sensitivity and reversibility are consistent with nitrosylation. Finally, we have identified a critical cysteine residue that nitric oxide modifies in order to disrupt Hdm2-p53 binding. Mutation of this residue from a cysteine to an alanine does not interfere with binding but rather eliminates the sensitivity of Hdm2 to nitric oxide inactivation.

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CHAPTER I

INTRODUCTION

Chemistry of nitric oxide

Nitric oxide is a unique and ubiquitous signaling molecule. While the chemistry of NO is complex and has been reviewed elsewhere, several aspects of this chemistry bear mentioning here. NO is a free radical that is highly diffusible because of its low molecular weight and high solubility. Limits on this diffusion exist, however, due to its many chemical interactions. The three most important of these are interactions with superoxide anion, transition metals, and thiol groups in proteins (1-5) (Table 1).

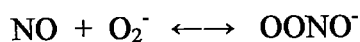
Nitric oxide reacts with the superoxide anion, O_2^- , to form the potent oxidizer peroxynitrite (Table 1). Peroxynitrite is a chemically complex molecule that can react with sulfhydryls and zinc thiolate moieties. Peroxynitrite can also nitrate and hydroxylate aromatic amino acid residues. Finally, it can oxidize lipids, DNA and proteins and is thought to be the key mediator of NO-induced cytotoxicity and neurodegeneration (1-3).

Nitric oxide can react with transition metals like the iron in heme groups (Table 1). Probably the best characterized NO modification of a heme-containing protein is soluble guanylate cyclase. Nitric oxide binds the heme moiety of soluble guanylate cyclase producing a conformational change that activates the enzyme, resulting in the

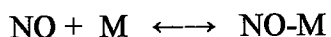
increased production of cyclic GMP (1). The NO-heme modification is reversible, thus serving as a means of regulating the enzyme. Targets of cGMP include cGMP-dependent protein kinase, cGMP-gated ion channels and cyclic nucleotide phosphodiesterases (1,4). Additional heme-containing proteins that are targets for NO include hemoglobin, and cyclooxygenase (3,6).

TABLE 1
MAJOR REACTIONS OF NO

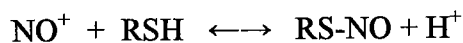
1. Reaction of NO with superoxide (O_2^-)



2. Reaction of NO with transition metals (M)



3. Reaction of NO with thiols (RSH)



Recently, it has become apparent that nitrosylation of cysteine residues by nitric oxide is a major regulatory reaction (Table 1). While nitrosylated cysteine residues are labile and difficult to detect, several examples are now known. S-nitrosoalbumin, and S-

nitrosoglutathione (GSNO) and S-nitrosocysteine all can serve as intracellular pools of NO (5). Meanwhile nitrosylated hemoglobin serves as a sensor for blood flow depending on the availability of oxygen (7). In addition there are now examples of proteases, transcription factors, and receptors that are S-nitrosylated (Table 2).

The specificity of S-nitrosylation can be achieved by subcellular localization of nitric oxide synthases, enzymes responsible for the production of NO. Similar to NO modification of heme-containing proteins, S-nitrosylation is reversible. This reversibility distinguishes nitrosylation from some other types of oxidation which are irreversible and can be toxic (2). NO itself is regulated by oxidation/reduction reactions. NO^\bullet , NO^- and NO^+ are all of potential biological significance but in the case of S-nitrosylation it is nitrosium ion (NO^+) that reacts efficiently with sulfhydryls (4,8,9).

A family of three nitric oxide synthase proteins; neuronal NOS (nNOS), endothelial NOS (eNOS) and inducible NOS (iNOS) (Figure 1-1) synthesize nitric oxide from arginine (Figure 1-1). Both eNOS and nNOS are Ca^{2+} -dependent while iNOS is Ca^{2+} -independent. nNOS and iNOS exist in both soluble and membrane-bound forms while eNOS is exclusively membrane-bound (3). All three NOS genes have been deleted in mice. nNOS null mice have changes in their resistance to stroke and in their behavior but no changes were observed in hippocampal long term potentiation (10). iNOS null mice have increased susceptibility to bacterial and viral infections. These mice also exhibit an increased vulnerability to tumor development (11,12). Finally, eNOS null

Table 2
S-Nitrosylated Proteins

Protein	Reference
Ras	(13)
Src kinase	(14)
GAPDH	(15,16)
Transcription Factors	
Acl	(17)
Jun	(18)
Receptors	
EGF-R	(19)
Ryanodine receptor	(20,21)
NMDA receptor	(22)
Glucocorticoid receptor	(23)
NO pools	
Hemoglobin	(7)
Albumin	(8)
Proteases	
Caspase-3	(24-26)
Papain	(27)
HIV-protease	(28)

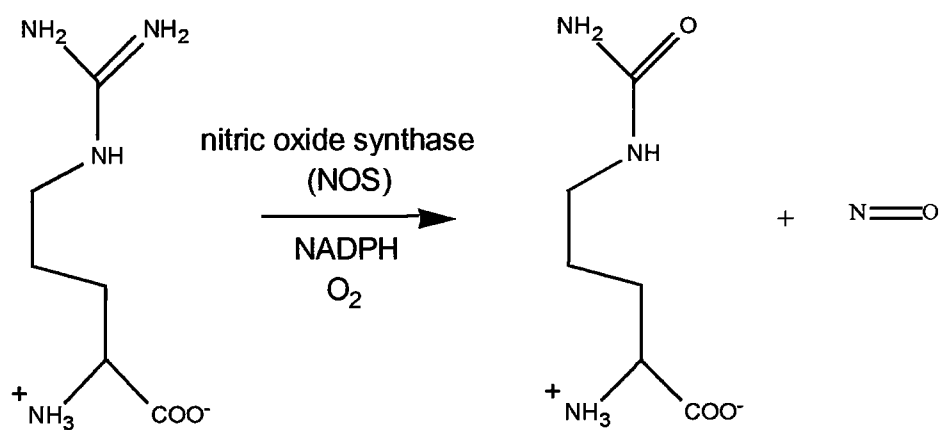


Figure 1-1. Biosynthesis of nitric oxide by nitric oxide synthase

mice are deficient for acetylcholine-induced vasodilation and have elevated blood pressure. These mice also demonstrate an increased susceptibility to stroke (3).

The study of nitric oxide has been enhanced considerably by the existence of drugs that can either release nitric oxide (NO donors) or inhibit nitric oxide production (NOS inhibitors). While there are numerous NO donors and NOS inhibitors and the chemistry of these drugs has been studied thoroughly, we will highlight just one donor and one NOS inhibitor that we employed extensively. L-NAME (*N*^ω-nitro-L-arginine) is one of the more commonly used inhibitors (Figure 1-2). This arginine analog is a competitive inhibitor of NOS and does not show NOS isoform specificity. One advantage of this drug is the ability to use the inactive enantiomer, D-NAME, as a negative control. DETA NONOate belongs to the class of NONOate nitric oxide donors (Fig 1-3). NONOates are adducts of NO with nucleophiles in which a nitric oxide dimer is formally bound to the nucleophilic residue via a nitrogen atom. NONOates release NO spontaneously, and the rate of release is unaffected by biological reactants (9). DETA/NONOate is also very useful in cell-based assays because of its long half-life (9).

Neuronal differentiation and cessation of proliferation

Neuronal differentiation refers to the physiological, biochemical and morphological changes that cells undergo in order to become mature neurons. The neurotrophins are a family of proteins that serve as differentiation factors. One of these

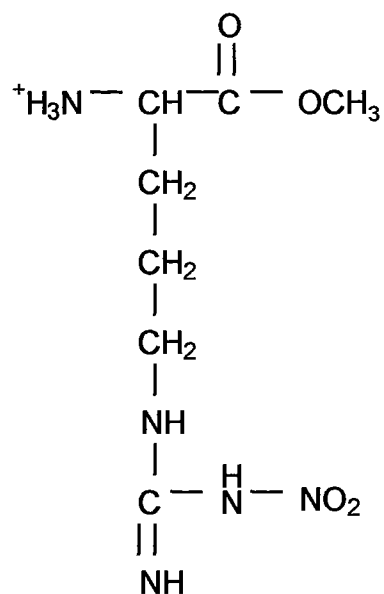


Figure 1-2. NOS inhibitor *N*^ω-Nitro-L-arginine methyl ester

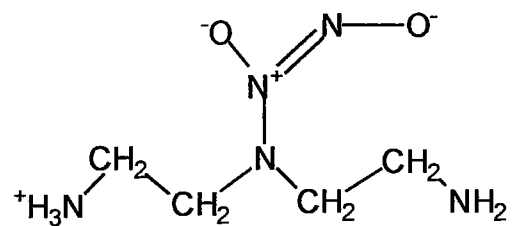


Figure 1-3. Structure of DETA NONOate

neurotrophins, NGF, is a survival and differentiation factor for sympathetic and some types of sensory neurons (29,30). The discovery of the NGF receptor TrkA, which belongs to a family of transmembrane receptors with tyrosine kinase activity has resulted in an improved understanding of NGF-mediated signal transduction (31-34). PC12 cells, a transformed rat pheochromocytoma cell line, have been an important tool in the NGF field. Upon stimulation with NGF, these cells extend neurites and differentiate into sympathetic-like neurons (35).

Cessation of proliferation is a key aspect of neuronal differentiation. Two key proteins involved in cessation of proliferation during neuronal differentiation are p53 and p21WAF1. p21WAF1 is a cyclin-dependent-kinase inhibitor that binds cdc2, cdk2 and cdk4 (36,37) and is important in blocking the cell cycle at the G₁-S transition (38,39). Mice lacking p21WAF1 undergo normal development suggesting there is significant redundancy in its function. However, these mice also demonstrate some deficiencies in G₁ checkpoint control (40). p21WAF1 is required for survival of differentiating neuroblastoma cells and differentiated PC12 cells express elevated levels of p21WAF1 (41,42).

The p53 tumor suppressor protein is involved in growth arrest and apoptosis (reviewed in (43-45)). One way that p53 functions to block cell cycle progression is by inducing transcription of p21WAF1 (36,46). Accordingly, p53 blocks the cell cycle, like p21WAF1, at the G₁-S transition (38,39,46). Work with p53 knockouts has revealed a role for p53 in neuronal development. p53 knockout mice have a large number of birth

defects such as failure of neural tube closure (47). As with p21 WAF1, p53 is induced in NGF-treated PC12 cells (48).

A key player in the regulation of p53 is the Mdm2 protein. The *mdm2* gene was first detected in a screen for oncogenes present on a mouse *double minute* chromosome (49). Mdm2 can regulate p53 in two ways. Mdm2 binds p53 and can block its ability to activate transcription (50). Mdm2 also regulates p53 levels by promoting its degradation (51,52). Conversely, p53 stimulates the transcription of the *mdm2* gene thus forming a negative feedback loop for the tight control of p53 levels (53,54) (Figure 1-4).

MAP kinase pathways during neuronal differentiation

Mitogen-activated protein (MAP) kinases, found in all eukaryotes, are common participants in signal transduction pathways from the membrane to the nucleus. The core unit of MAP kinase pathways is a three-member protein kinase cascade. MAPKs are phosphorylated and activated by MAPK kinases (MKKs) which are in turn activated and phosphorylated by MKK kinases (MKKKs) (55,56). In mammals, the MAP kinase pathways can be organized into three main groups (57). The Ras-Raf-MEK-ERK pathway is important for mitogenic, differentiation and survival responses (58-60). ERK1 and ERK2 are the best studied MAP kinases of this pathway family. The other two MAP kinase pathways are, in general, more involved in stress and apoptotic responses and are referred to as the JNK pathway and the p38 kinase pathway. JNKs, of which there are three, were isolated and characterized in part by their ability to

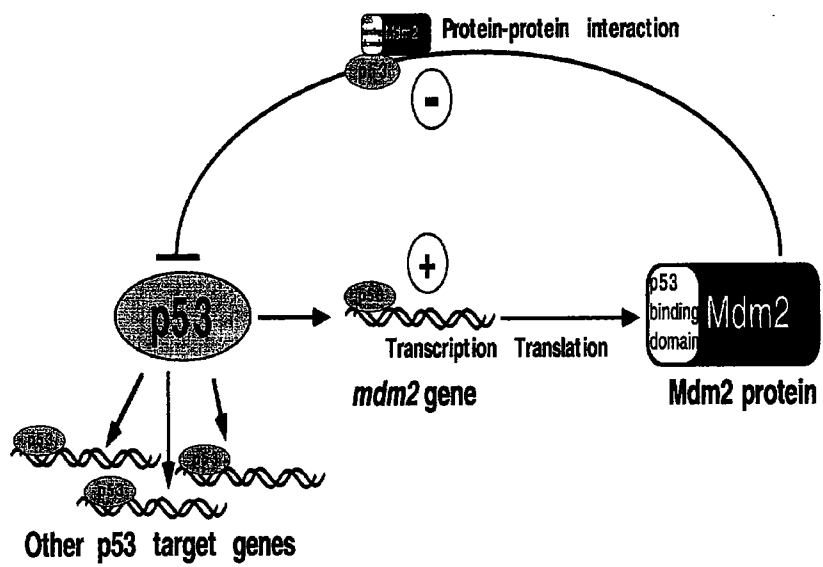


Figure 1-4. The p53-Mdm2 autoregulatory loop (45).

phosphorylate the transcription factor c-Jun and are strongly activated by stress-inducing stimuli (61-63). The third MAP kinase, p38, is a homolog of the yeast HOG1. In mammalian cells, the p38 kinase pathway is induced by lipopolysaccharide and high osmolarity. Currently, four isoforms of p38 MAP kinase are known (59,61,62). Hence, the MAP kinase pathways act as conduits, conveying information from the cell surface to the cytoplasm and nucleus.

In PC12 cells, the Ras-ERK pathway plays a critical role in transmitting signals from NGF. Upon binding of NGF to its receptor TrkA, adaptor molecules Shc, Grb2, and Sos recruit and activate the small G protein Ras (29). The activation of Ras initiates a kinase cascade that proceeds from Raf to MEK 1/2 to ERK 1/2. In PC12 cells, NGF elicits a sustained activation of this pathway, whereas epidermal growth factor, EGF, causes only a transient activation of this pathway (29,30). Recently, it has been shown that the protein Rap1 mediates the sustained activation of the pathway (64). It was initially thought that sustained activation of the Ras-ERK pathway was sufficient to differentiate PC12 cells (65). However, this theory relied heavily on overexpression studies. In addition, the discovery of NGF-stimulated Ras-independent signaling events in PC12 cells has modified these earlier interpretations. It is now thought that the Ras-ERK pathway is necessary but not sufficient for NGF-induced differentiation of PC12 cells (29,30,66).

The role of the p38 and JNK pathways in PC12 differentiation also has been analyzed. Initially, it was reported that p38 was not activated in NGF-treated PC12 cells but rather was activated after NGF withdrawal (67). However, two more recent reports

show that p38 is activated by NGF in PC12 cells (68,69). Moreover, p38 may play an essential role in the differentiation of PC12 cells (68). Also, expression of a constitutively active MEK results in the activation of p38, demonstrating cross talk between the Ras-ERK pathway and the p38 kinase pathway (68). The p38 kinase pathway may also synergize with the Ras-ERK pathway in order to transmit signals to the nucleus. This is the case in PC12 cells with the activation of the transcription factor CREB (69). Finally, the JNK pathway does not appear to be induced by NGF in PC12 cells and therefore is not thought to play a role in NGF-induced differentiation of PC12 cells (67,69).

The study of these pathways has inevitably led to the discovery of various inhibitors that block these pathways. For the Ras-ERK pathway, the inhibitor PD98059 has been used extensively to elucidate the involvement of this MAP kinase cascade in various cellular processes (70,71). Recently, another inhibitor of the Ras-ERK pathway, U0126, has been introduced (72). PD98059 inhibits activation of MEK. U0126 inhibits MEK activation and activity. PD98059 is more potent towards MEK1 than MEK2 while U0126 is equally potent towards both MEK isoforms and is more potent than PD98059 (70,72). A potent and specific inhibitor of the p38 kinase pathway, SB203580, has also been developed. SB203580 is specific for p38- α/β and does not inhibit p38- γ/δ (71,73,74). All of these drugs have been useful in elucidating the physiological substrates of the respective kinases, as well as defining cellular processes dependent on the MAP kinase pathways (71,75) (Figure 1-5).

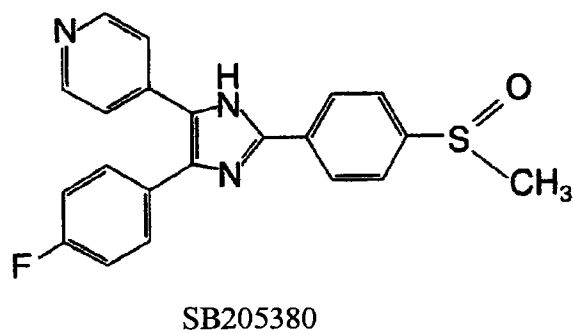
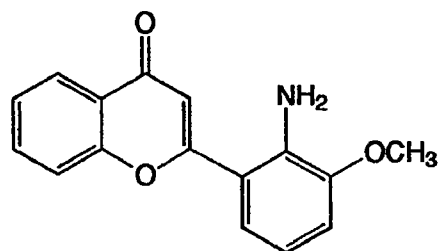
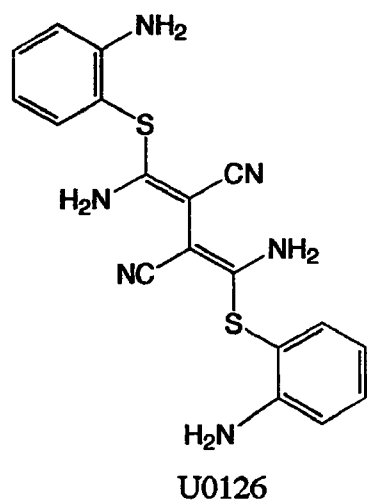


Figure 1-5. Structure of MAP kinase pathway inhibitors

Nitric oxide as a cytostatic signal during neuronal differentiation

A large volume of work has been done to show that under various cellular conditions nitric oxide can induce p53 levels (76-78). In addition, it has been shown that NO regulates proliferation in the imaginal discs of developing *Drosophila* through the Rb pathway (79,80). It has also been demonstrated that NOS levels are induced during differentiation of PC12 cells (81,82) and that this production of NO serves as a signal for growth arrest (83). Moreover, blocking NO production prevents cell cycle arrest and, thereby, prevents differentiation of PC12 cells. Conversely, treating PC12 cells with nitric oxide donors can inhibit thymidine incorporation (83).

Thesis Goals

Knowing that nitric oxide is a factor in activating p53 and can serve as a cytostatic agent during neuronal differentiation we set out to do three things:

1. Link NO to the induction of p53 and p21WAF1 during neuronal differentiation.
2. Investigate the regulation of nNOS during neuronal differentiation.
3. Provide a mechanism by which NO can elevate p53 levels.

CONTRIBUTIONS

During the initial stages of my time in the Ross lab, I worked very closely with a postdoc in the lab, Wojciech Poluha. Chapter II of this thesis is a manuscript on which he is the first author and I am second (84). I include this chapter for two reasons. First, the results from this paper serve as a framework for the rest of my work in the lab. Second, I had a very active role in performing the experiments and preparation of the manuscript. Wojciech and I worked together to obtain the results in figures 2-1, 2-2, 2-3, 2-4 and 2-7. In figure 2-1 and 2-2, I did all of the cell culture and treatments and then Wojciech took the cells from me and did staining or western blots. For figure 2-3, I performed repetitions of Wojciech's initial results. I also assisted Wojciech in his initial luciferase experiments in figure 2-4. Finally, I did the western blot for figure 2-7.

The data presented in chapters III, IV and V of this thesis was performed by myself with occasional assistance and advice from members of the Ross lab.

CHAPTER II

A NOVEL, NGF-ACTIVATED PATHWAY INVOLVING NITRIC OXIDE, p53 and p21WAF1 REGULATES NEURONAL DIFFERENTIATION OF 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 (35). These effects are associated with induction of nitric oxide synthase (NOS) (81,83), the p53 tumor suppressor (48), and the p21WAF1 cyclin-dependent kinase inhibitor (41,42,85). However, the relationship between 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) (86). 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 since nNOS is absent from proliferating neuroblasts but is coexpressed with early markers of neuronal differentiation (87,88). In *Drosophila*, NOS is expressed in developing imaginal discs (79). 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 (89). NGF treatment of PC12 cells induces expression of all three

isoforms of NOS (81,83), and inhibitors of NOS block NGF-induced cessation of proliferation and neurite extension for PC12 cells (83). 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, following certain types of cell stress, including DNA damage (43). Mice lacking functional p53 genes have an unusually large number of birth defects, such as failure of neural tube closure (47). 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 (90). 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 (48).

p21WAF1 binds to and inhibits cyclin-dependent kinases and induces cell cycle arrest at G₁/S (36). p53 is a potent transcriptional activator for p21WAF1 (36,46), but expression of p21WAF1 also can be induced by p53-independent mechanisms (91-95). *In vivo*, expression of p21WAF1 is enhanced as myoblasts differentiate into muscle cells (96). p21WAF1 is not expressed in the mitotic germinal layer of the olfactory epithelium but is expressed by olfactory neurons (96). We (41) and others (42,85,97) 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 (41), but van Grunsven et al. (85) have suggested that following 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 between these NGF-induced events. We find that nitric oxide (NO) activates the p21WAF1 promoter, resulting in expression of p21WAF1 protein. This link is partially dependent on p53, a potent, transcriptional activator for p21WAF1 (36). 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 recombinant 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.

Materials and Methods

Cell Culture

PC12 cells from Dr. David Kaplan (Montreal Neurological Institute) were maintained in DMEM supplemented with 10% heat-inactivated horse serum, 5% heat-inactivated fetal bovine serum, and 100 µg/ml of gentamycin at 37°C under 5% CO₂. These cells were found to be mycoplasma-free by the 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 of rat tail collagen (Sigma Chemical

Co., St. Louis, MO) and 15 μ g/ml of 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 (98), and treated with 100 ng/ml of NGF (2.5 S, Bioproducts for Science, Indianapolis, IN). 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 (86). In these studies, we used 20 mM 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 (83).

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 hrs following addition of 25 mM isopropyl thio β -galactoside (IPTG).

J. Earheart and R. Pittman (University of Pennsylvania School of Medicine) prepared the p21WAF1 plasmid for these experiments. 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 suppressed by adjoining *lac* operator sites. Plasmid p3'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 p3'SS Lac repressor plasmid, using the cationic detergent Lipofectin (Gibco). Plasmid DNA (6–7 μg per 25-cm² flask) was diluted in 100 μl of DMEM medium (serum-free). At the same time in a second tube, 20 μl of 1 mg/ml Lipofectin was diluted with 100 μl of DMEM. 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-cm² tissue culture flask containing about 3×10^6 cells and incubated overnight at 37°C. The resulting cells were selected for 14 days with medium containing 125 $\mu\text{g}/\text{ml}$ of hygromycin. This mass culture was derived from about 20 different hygromycin-resistant colonies. These cells were then transfected by the same procedure with the pOPRSV1 p21WAF1 plasmid bearing the p21WAF1 cDNA and selected for 14 days with 100 $\mu\text{g}/\text{ml}$ of G418. The mass culture from the second transfection 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

Following treatment with NGF and NO drugs, cells were extracted as described (46). 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 hr with 10% powdered milk in 0.2% Tween-20, Tris-buffered saline and then incubated with 2 $\mu\text{g}/\text{ml}$ of anti-p53 monoclonal antibody Pab-1801 (Oncogene Science, Cambridge, MA), 1 $\mu\text{g}/\text{ml}$ of rabbit anti-p21WAF1 antibody C-19 (Santa Cruz Biotechnology, Santa Cruz, CA) or 35 $\mu\text{g}/\text{ml}$ of anti-nicotinic acetylcholine receptor (nAChR) monoclonal antibody mab35 (99). Immunoreactive proteins were detected with horseradish peroxidase-conjugated secondary antibodies (Amersham, Arlington Heights, IL) and a chemiluminescence reagent (100). 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.

Diaphorase and Immunostaining

Cells were fixed for 10 min at room temperature with 4% paraformaldehyde in PBS and immunostained with 1 $\mu\text{g}/\text{ml}$ of anti-p21WAF1 rabbit antibody C-19 and then a rhodamine-conjugated secondary antibody. The cells were then incubated with 1.0 mg/ml of NADPH, 0.25 mg/ml of nitro blue tetrazolium and 0.1 M Tris HCl for 4 hr at

37°C (101). Samples were mounted in Citifluor (Ted Pella Inc., Reading, PA) and were viewed with a Zeiss Axioskop microscope and a 25X 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 95% methanol, 5% acetic acid at -20°C. The samples were stained with 35 µg/ml of monoclonal antibody mab35 against the α subunit of the nicotinic acetylcholine receptor (nAChR) (99) or ascites diluted 1:200 from hybridoma MAP1B-4 directed against microtubule-associated protein 1B (MAP1B) (102,103). These antibodies were diluted with 0.3% BSA, 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 (104).

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 Colorease PS printer on Kodak Extatherm XLS paper.

p21WAF1 Promoter Activity

PC12 cells were treated for 60 hours with or without NGF, L-NAME, or D-NAME. Then, using Lipofectin as described above, they were cotransfected with promoter constructs WWP-Luc or DM-Luc (36) (6 µg/25-cm² flask) and β -galactosidase plasmid pCH110 (6 µg/25-cm² flask). The 2.4 kb WAF1 promoter region was obtained by PCR and cloned into pBluescript KS(+) to yield plasmid WWP-Luc. DM-Luc which

lacks the p53-binding element was obtained by digesting WWP-Luc with SacI and recircularization. After 16-20 hrs, 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 1, 2-diaminocyclohexane N, N, N', N'-tetraacetic acid, 10% glycerol and 1% Triton X-100). These extracts were clarified by centrifugation, stored at -70°C and then used immediately after thawing. Mixtures of 20 µl of cell extract and 100 µl of Luciferase Assay Reagent (20 mM tricine, 1.07 mM (MgCO₃)₄Mg(OH)₂·3H₂O, 2 mM MgSO₄, 0.1 mM EDTA, 33.3 mM dithiothreitol, 270 µM Coenzyme A, 470 µM luciferin, 530 µM ATP, pH 7.8) were immediately placed in Model N Luminometer Analyzer (Packard) and luciferase activities were measured.

β-galactosidase activities were measured using a kit from Promega. Assay 2x buffer (120 mM Na₂HPO₄, 80 mM NaH₂PO₄, 2 mM MgCl₂, 100 mM β-mercaptoethanol, 1.33 mg/ml of *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 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 $73 \pm 15\%$ (mean \pm S.D., $n = 6$), in agreement with Peunova and Enkolopov (83).

We assayed expression of two markers associated with neuronal differentiation. As judged by immunofluorescence microscopy, MAP1B (105,106) is upregulated by NGF (Fig. 2-1). For this experiment, untreated PC12 cultures showed $12 \pm 1\%$ of cells positive for MAP1B, but NGF-treated cultures showed $87 \pm 3\%$ cells positive. L-NAME, but not D-NAME, reduced this NGF-induced increase in MAP1B expression (D-NAME+NGF-treated cells, $88 \pm 4\%$ positive; L-NAME+NGF-treated cells, $14 \pm 2\%$ positive). Averaging 3 experiments, L-NAME inhibited NGF induction of MAP1B-positive cells by $85 \pm 10\%$. The pattern of expression for nAChR closely resembled that for MAP1B (107) (micrographs not shown).

By Western blotting, we observed the same pattern of expression for nAChR (Fig. 2-2). Averaging 3 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 (41,42,48,85). As judged by scanning densitometry, NGF induced accumulation of p53 and p21WAF1 by 7.1 ± 4.3 fold and 4.7 ± 1.1 fold ($n=3$), respectively, compared to levels in untreated cells (Fig. 2-3). L-NAME, but not D-NAME, inhibited NGF-induced accumulation of p53 by $87 \pm 7\%$ and p21WAF1 by $61 \pm 7\%$. This is the first report that

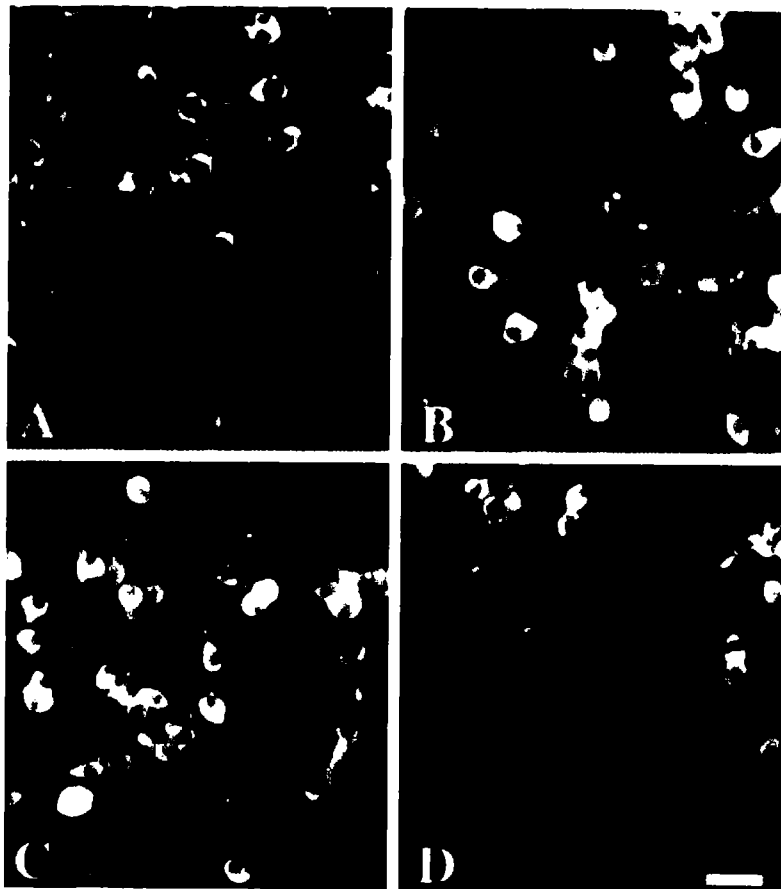


Figure 2-1. NGF-induced expression of MAP1B is dependent on NO. Cells were immunostained for MAP1B and examined by immunofluorescence microscopy. Panel A, no addition; panel B, +NGF (100 ng/ml); panel C, +NGF + D-NAME (20 mM); panel D, +NGF + L-NAME (20 mM). Bar = 25 μ m.

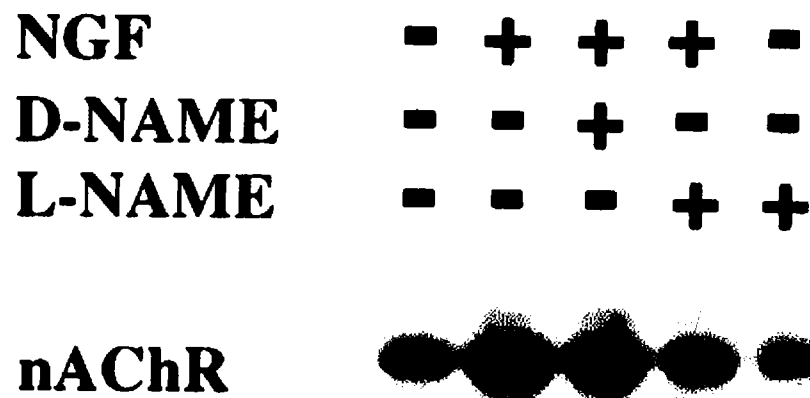


Figure 2-2. NGF-induced expression of nAChR is dependent on NO. PC12 cells were treated as in Fig. 1 and extracted for Western blotting.

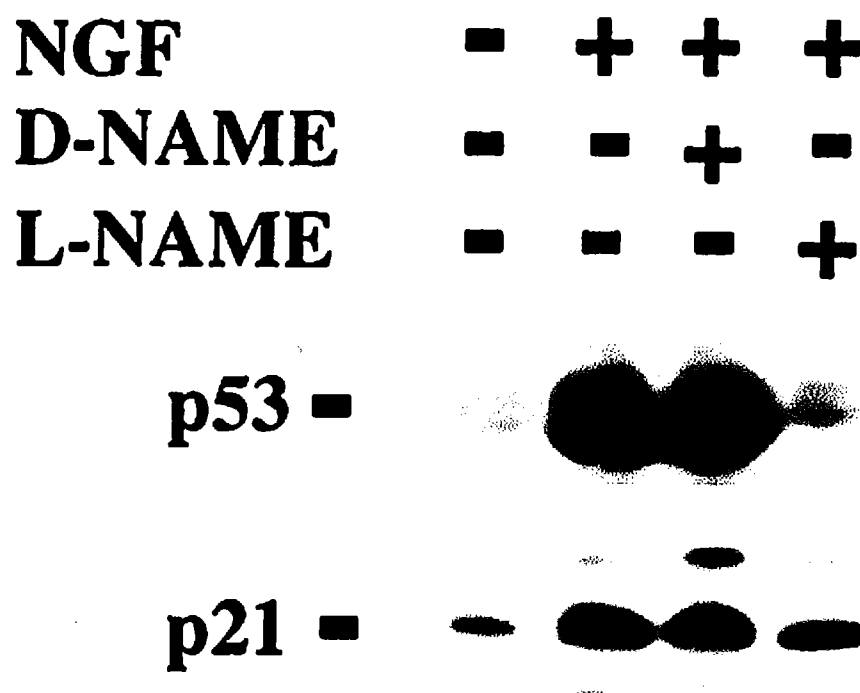


Figure 2-3. NGF and NO enhance expression of p53 and p21WAF1. Cells were treated as in Fig. 1 and then extracted. p53 and p21WAF1 were assayed by Western blotting.

NO, at physiological levels, acts as a second messenger to enhance expression of p53 and p21, although it was reported that treatment of cells with NO donors induces accumulation of p53 (77,108-113).

To evaluate further the relationship between NO and p21WAF1 expression, we transfected PC12 cells with a p21WAF1 promoter-luciferase reporter construct (WWP-Luc (36)) and found that treatment with NGF significantly enhanced the activity of the p21WAF1 promoter ($p \leq 0.0001$, Students-*t* test)(Fig. 2-4). This response was inhibited by L-NAME, but not by D-NAME. The parallel regulation of p21WAF1 promoter activity and p21WAF1 protein levels (Figs. 2-3,4) 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 (36), we utilized a truncated promoter lacking the p53 binding site (DM-Luc (36)). Cells transfected with this shorter construct gave substantially reduced responses to NGF (Fig. 2-4). Therefore, transcriptional activation by p53 is a major mechanism by which NGF induces p21WAF1 expression. However, there was significant activation ($p \leq 0.0001$) 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 (114,115). This method is specific for NOS because other diaphorases are inactivated by fixation with

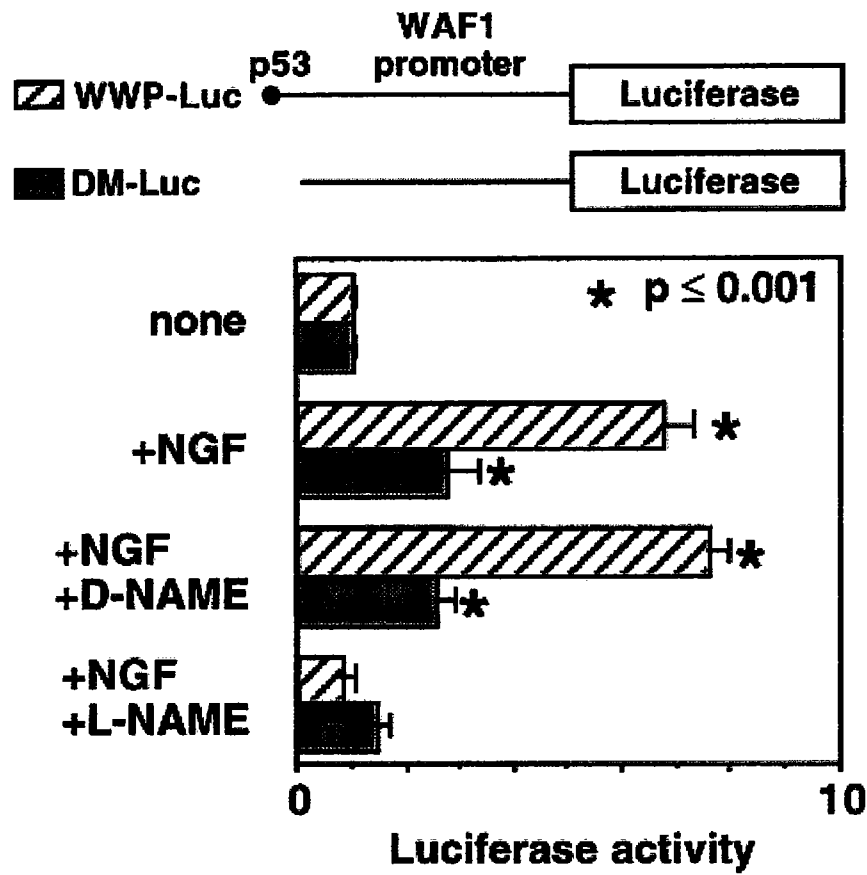


Figure 2-4. Activation of p21WAF1 promoter by NGF and NO. The diagram shows the promoter-reporter constructs WWP-Luc and DM-Luc. The p53 binding site present in WWP-Luc, but not in DM-Luc, is shown as a filled circle. Cells were treated with drugs, cotransfected with the promoter-reporter plasmids and a β -galactosidase plasmid, and then luciferase and β -galactosidase activities were measured. Luciferase activities were normalized with β -galactosidase activities to eliminate small differences in transfection efficiencies. Six experiments were averaged and reported as the ratio of treated cells relative to untreated WWP-Luc and DM-Luc transfected cells, respectively. Errors were propagated as described (116).

paraformaldehyde. 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. 2-5A, C, E). NGF treatment increased the percentages of cells with diaphorase activity (92%), p21WAF1 immunoreactivity (85%) and neurites (77%)(Fig. 2-5B, D, E). The staining for p21WAF1 was almost exclusively nuclear, in agreement with other studies (46,117).

To test the role of p21WAF1 in NGF-induced differentiation, we prepared a PC12 line bearing an inducible p21WAF1 expression vector (Fig. 2-6). This line differentiated in response to NGF, in the same manner as the parental PC12 line, and L-NAME reduced neurite extension (Fig. 2-6A, D, E). However, induction of p21WAF1 expression with IPTG restored NGF-induced differentiation of L-NAME-treated cells (Fig. 2-6B). Addition of IPTG alone had no effect on neurite extension (not shown), but IPTG did reduce proliferation, as judged by BrdU labeling, by $58 \pm 5\%$. IPTG did not enhance the effect of NGF (Fig. 2-6C, E). We also noted that p21WAF1 levels induced by IPTG ($320 \pm 30\%$ relative to untreated cells) were similar to those induced by NGF ($250 \pm 20\%$)(Fig. 2-7). Hence, p21WAF1 is the functionally important target of the NGF-NOS pathway, but p21WAF1, like NO, is not sufficient by itself for PC12 differentiation.

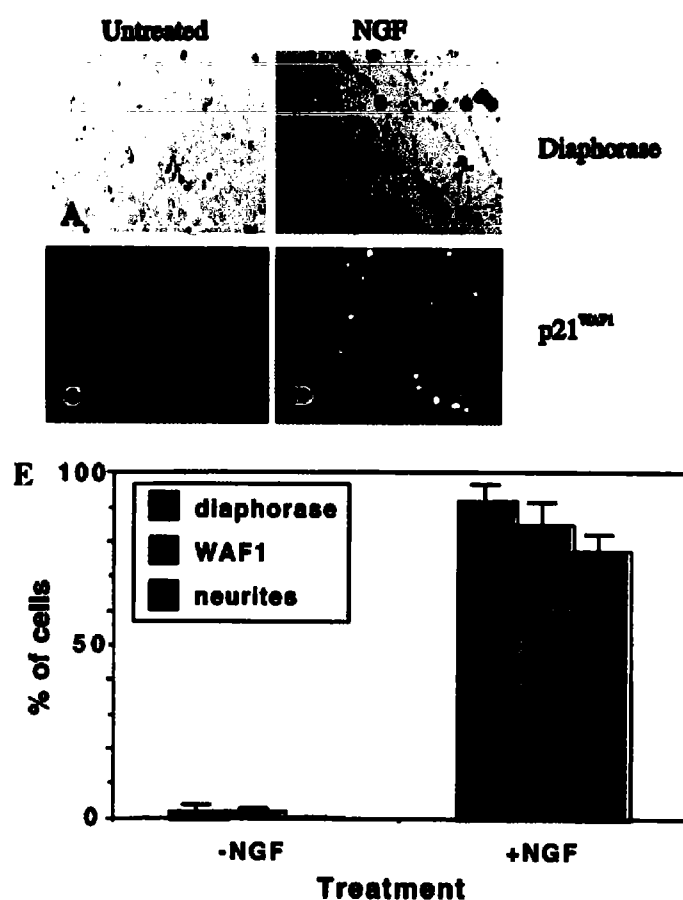


Figure 2-5. Differentiated PC12 cells express NOS and p21 WAF1. PC12 cells in defined medium were treated for 3 days without (panels A and C) or with 100 ng/ml NGF (panels B and D). The cells were double stained for diaphorase activity (panels A and B) and p21 WAF1 (panels C and D). Averages and S.D. values from three independent experiments are shown in panel E. Bar = 10 μ m.

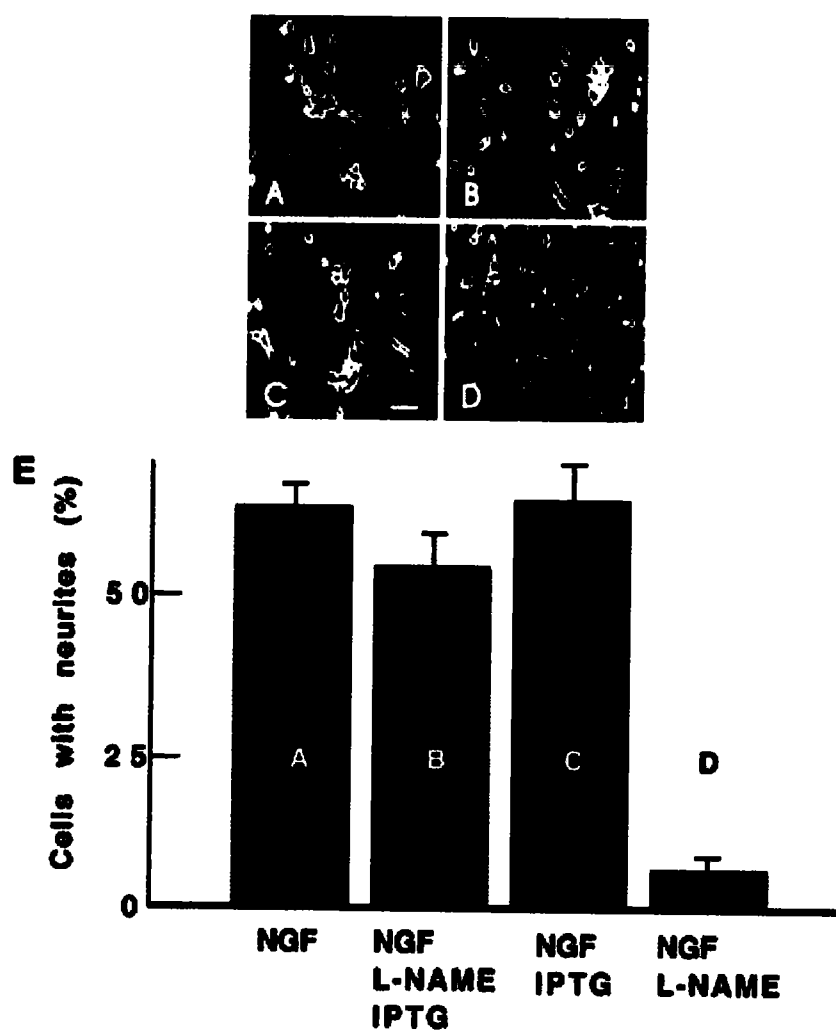


Figure 2-6. Role of p21WAF1 in NGF-induced differentiation. PC12 cells bearing an inducible p21WAF1 expression vector were treated for 3 days with 100 ng/ml NGF (panel A), NGF + L-NAME (20 mM) + IPTG (25 mM) (panel B), NGF + IPTG (panel C), or NGF + L-NAME (panel D). Panel E, quantification of neurite extension (average \pm S.D.). Bar = 30 μ m.

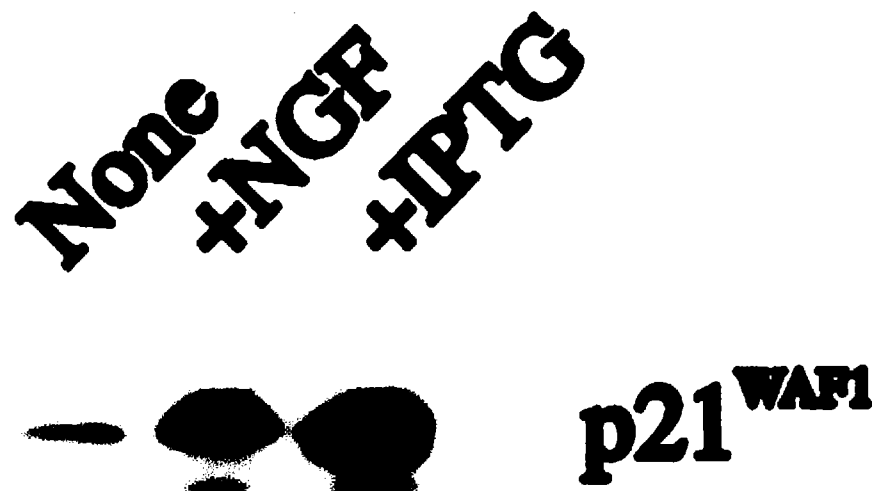


Fig. 2-7. Expression of p21WAF1 induced by NGF and IPTG. PC12 cells bearing an inducible p21WAF1 expression vector were treated for 3 days with no addition, 100 ng/ml NGF, or 25 mM IPTG. Expression of p21WAF1 was assayed by Western blotting.

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 MAP kinases (58) and the transcription factor, NF- κ B (118,119), which are associated with NOS expression (120-122). In ongoing studies (Bulsecq and Ross, not shown), we have found that TrkA can induce NOS expression but 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 (48). 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 which is not dependent on the p53 binding site and might involve the AP2 transcriptional activator protein (123) or the p300 transcriptional co-activator protein (124). 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 MAP kinase, SNT and, perhaps, other NGF-regulated pathways (29).

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 (87). 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 following treatment with NGF (125). NO

also reacts with ribonucleotide reductase, an enzyme required for nucleotide biosynthesis and cell proliferation (126). In addition, high levels of NO damage DNA and, thereby, induce expression of p53 (77). It is unlikely 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 (77,108-113), a protein required for NGF-induced neuritogenesis of PC12 cells (48). 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 (43). Since p53 is subject to redox regulation and has free cysteines which might react with NO (127,128), p53 itself might be an NO target. p53 activity is regulated by phosphorylation by casein kinase II, protein kinase C and cyclin-dependent kinases (43). 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 (48).

In a recent study, it was reported that NGF-induced expression of p21WAF1 is p53-independent (124). 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 differences 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 three days in defined medium which considerably speeds both NGF-induced differentiation (98) and induction of p21WAF1 (129). The other group treated their cells for only two 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 considerably affect expression of cell cycle-associated proteins. NGF induces expression of p21 WAF1 in defined medium (this study and (129)) or in the presence of serum (42,85,97) but not in DMEM lacking both serum and growth factors (85). Further studies are required to resolve these differences, but ultimately we feel that p53 will be shown to play a role in this pathway, since p53 is required for NGF-induced neurite extension by PC12 cells (48).

NO leads to p21 WAF1 induction but also regulates the activities of other enzymes and the expression of additional gene products (87). 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 p21 WAF1 expression vector. Blocking NOS with L-NAME inhibits neurite extension, but induction of p21 WAF1 with IPTG restores the response. Furthermore, the levels of p21 WAF1 induced by NGF and by IPTG are similar, so it is unlikely that our conclusion is based an artifact of overexpression. Hence, the lesion in the pathway caused by L-NAME is complemented by IPTG-induced p21 WAF1, and we conclude that p21 WAF1 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 which probably results from blocked differentiation 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 due to differentiation of tumor cells and is correlated with expression of TrkA (130-132). In addition, nearly all neuroblastomas have functional p53 (133-135) and p21WAF1 (136) genes. Hence, neuroblastomas have the molecular components required for the NGF-NOS-p21WAF1 pathway. We have found that NOS is required for both expression of p21WAF1 and survival of differentiating neuroblastoma SH-SY5Y cells (41) (not shown). This cell culture model suggests that the NGF-NOS-p21WAF1 pathway may play a role in spontaneous regression of neuroblastoma.

This signal transduction pathway discovered in PC12 cells probably also plays a role in normal development of the nervous system. Neurotrophins, in particular neurotrophin-3, may be signals that terminate neuroblastic proliferation (137,138). Likewise, a role for NOS in controlling neuroblastic proliferation is suggested by the expression of NOS during neuronal differentiation (88,139). Disruption of the NGF-NOS-p21WAF1 pathway may have adverse consequences for development. Mice lacking p53 genes have an unusually large number of birth defects, including failure of neural tube closure (47) and exencephaly in which overgrowth of neural tissue in the fore- and midbrain precludes normal formation of the cranium (90). This overgrowth probably results from excessive cell proliferation rather than decreased cell death. In *Drosophila*, inhibition of NOS results in excessive proliferation during development (79). The NGF-NOS-p21WAF1 pathway also may play a role in the regulating phenotype of postmitotic neurons. NGF induces expression of both NOS and choline acetyltransferase in developing basal forebrain neurons (89).

Finally, the pathway may be relevant to neuronal responses to injury. Following injury of CNS neurons, both NOS and p53 are induced (140-142). Although speculative, it would be interesting to determine if p21WAF1 also is induced. In addition to cyclin-dependent kinases, p21WAF1 inhibits casein kinase-2 (143) and stress-activated JNK kinases (144). Activation of JNK kinases is associated with cell death resulting from neurotrophin deprivation (67). By modulating these 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.

CHAPTER III

THE RAS-MAP KINASE PATHWAY IS REQUIRED FOR THE INDUCTION OF NEURONAL NITRIC OXIDE SYNTHASE IN DIFFERENTIATING PC12 CELLS

Introduction

MAP kinase pathways are key regulators of many cellular processes. In mammals, there are three well-characterized MAP kinase pathways (57). The Ras-Raf-MEK pathway is important for mitogenic, differentiation and survival responses (58-60). The other two MAP kinase pathways are involved in stress and apoptotic responses and are referred to as the JNK pathway and the p38 kinase pathway. JNKs were isolated and characterized in part by their ability to phosphorylate the transcription factor c-Jun and are strongly activated by stress-inducing stimuli (61,62). The third MAP kinase, p38, is a homolog of the yeast HOG1. In mammalian cells, the p38 kinase pathway is induced by LPS and high osmolarity (59,61,62). Hence, the MAP kinase pathways act as conduits, conveying information from the cell surface to the cytoplasm and nucleus.

In PC12 cells, the Ras-ERK pathway plays a critical role in transmitting signals from nerve growth factor (NGF). Upon binding of NGF to its receptor TrkA, adaptor molecules Shc, Grb2, and Sos recruit and activate the small G protein Ras (29). The activation of Ras initiates a kinase cascade that proceeds from Raf to MEK to ERK. In PC12 cells, NGF elicits a sustained activation of this pathway, whereas epidermal growth factor causes only a transient activation of this pathway (29,30). Recently, it has been shown that the protein Rap1 mediates the sustained activation of the pathway (64). It was

initially thought that sustained activation of the Ras-ERK pathway was sufficient to differentiate PC12 cells (65). However, this theory relied heavily on overexpression studies. In addition, the discovery of NGF-stimulated Ras-independent signaling events in PC12 cells has modified these earlier interpretations. It is now thought that the Ras-ERK pathway is necessary but not sufficient for NGF-induced differentiation of PC12 cells (29,30,66).

The role of the p38 and JNK pathways in PC12 differentiation also has been analyzed. Initially, it was reported that p38 was not activated in NGF-treated PC12 cells but rather was activated after NGF withdrawal (67). However, two more recent reports show that p38 is activated by NGF in PC12 cells (68,69). Moreover, p38 may play an essential role in the differentiation of PC12 cells (68). Also, expression of a constitutively active MEK results in the activation of p38, demonstrating cross talk between the Ras-ERK pathway and the p38 kinase pathway (68). The p38 kinase pathway may also synergize with the Ras-ERK pathway in order to transmit signals to the nucleus. This is the case in PC12 cells with the activation of the transcription factor CREB (69). Finally, the JNK pathway does not appear to be induced by NGF in PC12 cells and therefore is not thought to play a role in NGF-induced differentiation of PC12 cells (67,69).

The study of these pathways has inevitably led to the discovery of various inhibitors that block these pathways. For the Ras-ERK pathway, the inhibitor PD98059 has been used extensively to elucidate the involvement of this MAP kinase cascade in various cellular processes (70,71). Recently, another inhibitor of the Ras-ERK pathway, U0126,

has been introduced (72). PD98059 inhibits activation of MEK whereas U0126 inhibits both MEK activation and activity. PD98059 is more potent towards MEK1 than MEK2 while U0126 is equally potent towards both MEK isoforms and is more potent than PD98059 (70,72). A potent and specific inhibitor of the p38 kinase pathway, SB203580, has also been developed. SB203580 is specific for the ubiquitously expressed α and β isoforms of p38. (71,73,74,145). These drugs have been useful in elucidating the physiological substrates of the respective kinases, as well as defining cellular processes dependent on the MAP kinase pathways (71,75).

Nitric oxide (NO) performs a multitude of physiological and pathological functions in the nervous system. NO in cells is produced by nitric oxide synthase (NOS) of which there are three isotypes; nNOS or bNOS (neuronal or brain NOS), eNOS (endothelial NOS) and iNOS (inducible NOS). In the nervous system, NO is involved in learning and memory, neurotransmitter release, development and neuronal damage or degeneration (3). It has also been postulated that NOS levels are induced during differentiation of PC12 cells by NGF. The nNOS gene includes two promoters, 5'1 and 5'2. NGF induction of nNOS is due to activation of the 5'2 promoter (146). Although there is one report of nNOS induction by the mitogen, epidermal growth factor (EGF) (82), we (data not shown) and others (83,146) have not detected nNOS induction by EGF. The production of NO in response to NGF serves as a signal for growth arrest (83). Blocking NO production prevents cell cycle arrest and, thereby, prevents differentiation of PC12 cells. This cytostatic signal of NO appears to act through p53 and the cyclin-dependent kinase inhibitor p21WAF1 (84). In addition, it has been shown that NO regulates

proliferation in the imaginal discs of developing *Drosophila* through the Rb pathway (79,80).

We set out to investigate which MAP kinase pathways are necessary for the induction of nNOS in NGF-treated PC12 cells. We find that the Ras-ERK pathway is necessary for the induction of nNOS protein and enzymatic activity. The p38 pathway and the JNK pathway do not appear to be required for this induction. Finally, we resolve conflicting results obtained with the MEK inhibitors, U0126 and PD98059. We find that U0126 inhibits NOS induction much more effectively than PD98059 because in PC12 cells, U0126 inhibits the Ras-ERK pathway much more effectively than PD98059.

Materials and Methods

Cell culture

Rat pheochromocytoma (PC12) cells were a gift from David Kaplan. Cells were grown at 37°C and 5% CO₂ in DMEM supplemented with 10% FBS and 5% donor horse serum (35). Treatments with NGF (100 ng/ml) were performed in flasks coated with collagen (15 µg/ml) and poly-D-lysine (15 µg/ml). For experiments with various kinase inhibitors, cells were pretreated with the drugs for 30-45 minutes before the addition of NGF. For differentiation of PC12 cells NGF and drugs were replenished every 48 hours.

Adenoviruses and Infection Protocol

Virus-containing cell lysates (DNRas; Ser 17 to Asn, and XRaf; truncated at amino acid 257 and Cys 168 to Ser) were prepared as described (60). Cells were infected with virus-containing lysates in a minimal volume of media for 1 hour at 37°C. Growth media was then added to increase the volume. Twenty-four hours after infection, the media containing the adenovirus was removed and replaced with fresh growth media. Infection of cells with a MOI of 150 did not produce significant toxicity.

Materials

NGF (2.5 S) was purchased from Bioproducts for Science. PD98059 was obtained from New England Biolabs. U0126 was purchased from Promega. SB203580 and the NOS assay kit were purchased from Calbiochem. TRI reagent for total RNA preparation was from Molecular Research Center, Inc. RT PCR kit was purchased from Perkin Elmer. Monoclonal Anti-NOS-Brain (nNOS) antibody was purchased from Sigma and Phospho-p44/42 MAP Kinase antibody was from New England Biolabs. Anti-MAP kinase antibody (C14) was from Santa Cruz. Arginine, L-{2,3-³H} was purchased from New England Nuclear.

Western blots

To prepare total cellular extracts cells were removed from the flasks with HBSS + EDTA and collected by centrifugation. After the supernatant was removed cells were resuspended in lysis buffer (10 mM Tris pH 7.4, 1% SDS, 1 mM PMSF and 5 µg/ml

aprotinin). An equal volume of 2X sample buffer (250 mM Tris-HCl pH 6.8, 4% SDS, 10% glycerol, 0.006% bromophenol blue and 2 % β -mercaptoethanol) was added to 25 μ g of total protein, the samples were boiled and then separated by SDS-PAGE. Proteins were then electrophoretically transferred to PVDF and the membranes were blocked with 10% milk in TTBS (0.2% Tween-20) for one hour at room temperature. Primary antibody incubations were for one hour at room temperature using the appropriate dilutions (monoclonal nNOS 1:3000, monoclonal phospho-p44/42 MAP kinase 1:1000, and anti-p44/42 MAP kinase 1:100). Membranes were washed for thirty minutes with three changes, and the secondary antibody incubation (anti-mouse horseradish peroxidase-conjugated from Amersham 1:1500) was for one hour. After washing expression was detected using enhanced chemiluminescence (100).

Neurite extension

Cells were plated on collagen and poly-D-lysine coated 25-cm² flasks at 1.3×10^5 cells per flask and pictures were taken after 2, 4 and 6 days. Cells with extensions longer than two cell diameters were scored as positive for neurites.

NOS assay

NOS assays were performed as detailed in the Nitric Oxide Synthase Assay Kit from Calbiochem. Cells were collected, frozen in liquid nitrogen and then resuspended in 1X homogenization buffer (25 mM Tris-HCl, pH 7.4, 1 mM EDTA and 1 mM EGTA). Extracts were tested in the presence of 0.6 mM Ca^{++} , using 100 μ g of protein per sample

and 1 μCi of $\{^3\text{H}\}$ -arginine per assay. Arginine concentration was 25 μM . Reactions were carried out for thirty minutes at 37°C. Assays were linear for both time and PC12 cell extract concentration (data not shown). Citrulline was then separated from arginine with equilibrated ion-exchange resin and the radioactivity in the flow-through was determined by scintillation counting. Counts for negative control samples, no lysate or lysate treated with the NOS inhibitor *N*^o-nitro-L-arginine methyl ester (L-NAME) (\approx 30,000 cpm/ experiment), were subtracted and results were presented as cpm/ 100 μg of lysate.

RT PCR

For RT PCR experiments, total RNA was prepared using TRI reagent. 1 μg of total RNA was used to prepare cDNAs, using random hexamer primers. For the PCR the nNOS primers were 5' AAG CAG TCA GAT GGC TCT A 3' (forward primer) and 5' TCT GTA GCG GTA TTC ATT CT 3' (reverse primer), which generates a 551 base pair product. The PCR conditions were 92°C for 1 minute (denaturing step), 50°C for one minute (annealing step), and 72°C for one minute (extension step) for 30 cycles followed by a final 10 minute 72°C extension step. For G3PDH, primers were 5' GTC AAC GGA TTT GGT CGT ATT 3' (forward primer) and 5' AGT CTT CTG GGT GGC AGT GAT 3' (reverse primer), which generates a 540 base pair product. PCR conditions were 4 minutes at 95°C followed by 25 cycles of 45 seconds at 95 °C, 45 seconds at 56°C and one minute at 72°C. A final 10 minute 72°C extension step was included. PCR products were resolved on 1.2 % agarose gels.

Results

Ras is required for induction of nNOS RNA, protein and NOS activity

In PC12 cells, NGF induced nNOS at the RNA level, as judged by RT PCR (Fig 3-1A). We also found that nNOS protein (Fig 3-1B) and NOS activity (Fig. 3-1C) were induced in PC12 cells treated with NGF. By four days the induction of NOS activity was statistically significant ($p < .001$). The induction of nNOS mRNA, protein and NOS activity are in agreement with earlier experiments (82,83,146). We failed to see an induction of iNOS or eNOS by western blots (data not shown), which is in agreement with earlier findings (82).

To test the possibility that the Ras-ERK pathway is necessary for nNOS induction, we compared PC12 cells with cells infected with an adenovirus that expresses a dominant negative Ras (60). The dominant negative Ras17N functions by binding more tightly to Ras-specific GEFs than does normal Ras and thereby, preventing activation of endogenous Ras. Ras17N, in turn, cannot bind to downstream targets even when bound to GTP (147). As previously reported (60), we found that this dominant negative adenovirus blocks differentiation of PC12 cells but has no effect on survival or proliferation (data not shown). At a MOI of 140, PC12 cells infected with the DNRas virus showed greatly reduced induction of nNOS RNA, protein or activity when treated with NGF for six days (Fig. 3-2A, B and C). The residual NOS activity in Fig. 3-2C may be due to incomplete infection of these cultures. We estimate that 80-90% of cells are

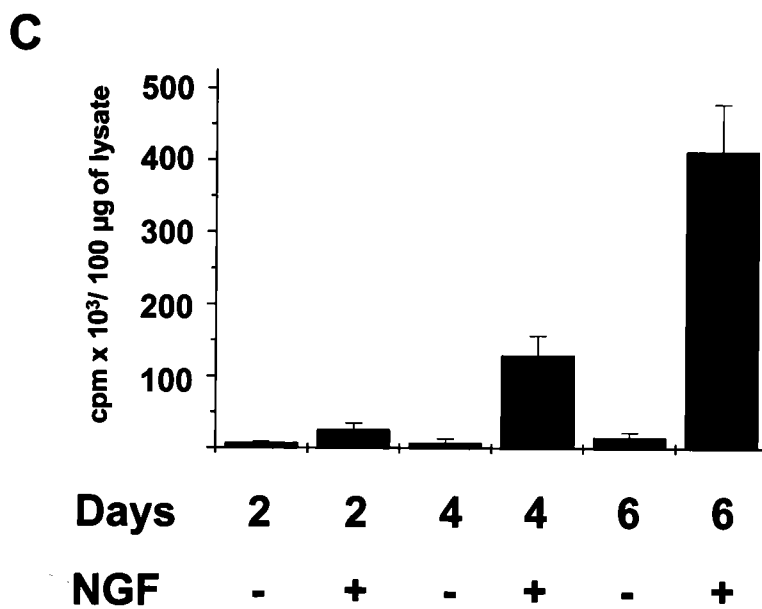
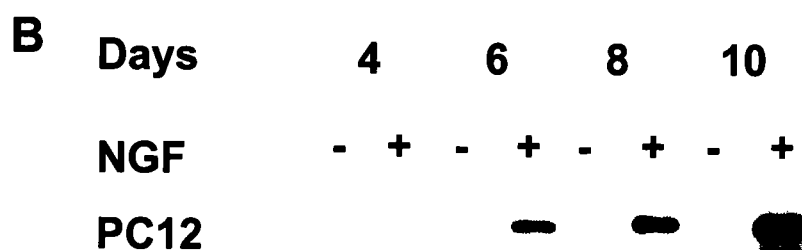
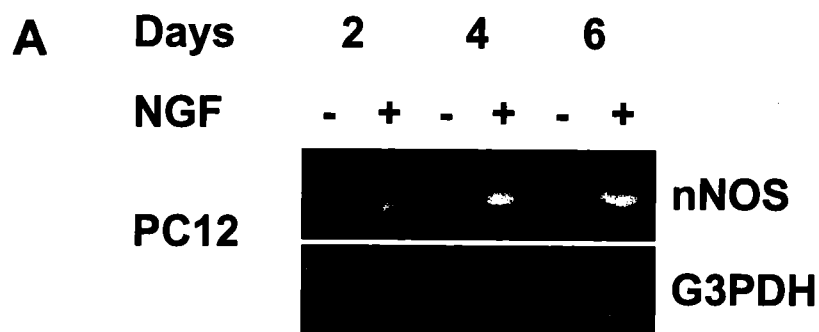


Figure 3-1. nNOS is induced at the RNA, protein and activity levels. Cells were treated with or without 100 ng/ml of NGF (- or +) for the indicated number of days. (A) RT PCR was performed on PC12 cells. The media and NGF were replenished every 48 hours. At the indicated times total RNA was prepared and used for RT PCR (1 μ g of total RNA per reaction). PCR was carried out using primers specific for nNOS or the housekeeping gene G3PDH. (B) Western blots were performed using total cellular extracts of PC12 cells. This Western blot is representative of at least three experiments. (C) NOS assays performed after 2, 4 and 6 days of NGF treatment are presented as cpm $\times 10^3$ /100 μ g of lysate and are measured as a mean \pm SEM (n = 9). Samples with no extract or extracts treated with 1 mM L-NAME were used as negative controls. Extracts from untreated cells were always within 5-10 % of these two negative controls.

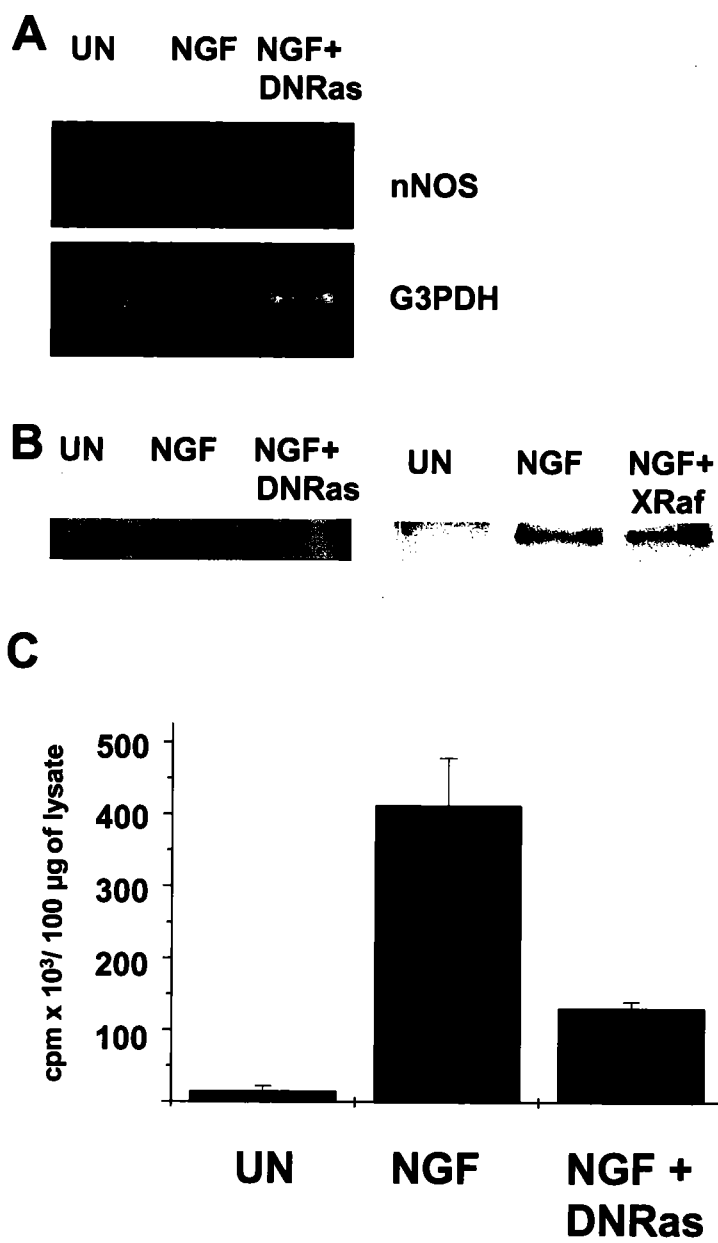


Figure 3-2. Ras is required for the induction of NOS. (A) After six days of treatment RT PCR was performed on untreated (UN), NGF-treated and NGF-treated DNRas (MOI 140) infected PC12 cells. RT PCR was performed for nNOS and the housekeeping gene G3PDH. (B) Western blots for nNOS were performed on untreated, NGF-treated, NGF-treated DNRas infected and NGF-treated control virus Xraf infected PC12 cells after 6 days of treatment. These Western blots are representative of three independent experiments. (C) NOS assays comparing NGF and NGF-DNRas infected PC12 cells after 6 days of treatment are presented as least three experiments. (C) NOS assays performed after 6 days of NGF treatment are presented as $\text{cpm} \times 10^3 / 100 \mu\text{g}$ of lysate and are measured as a mean \pm SEM ($n = 6$).

infected (60)(data not shown). Thus, we have demonstrated that a functional Ras is required for the production of nitric oxide in differentiating PC12 cells.

To control against adenoviral specific effects, we performed infections using an adenovirus that expresses an inactivated Raf (XRaf). XRaf has no kinase domain and exhibits decreased Ras binding. It neither activates the pathway nor acts as a dominant negative, making it an effective negative control (148). Neurite extension by NGF-treated XRaf-infected cells was indistinguishable from NGF-treated, uninfected cells (data not shown). Additionally, infection with XRaf did not block nNOS induction by NGF (Fig. 3-2B) or induce nNOS in the absence of NGF (data not shown).

U0126, not PD98059, blocks nNOS induction and NOS activity

Having shown that Ras is required for nNOS induction, we tested whether downstream effectors of the MAP kinase pathway were necessary for this induction. Previous work has shown the MEK inhibitor PD98059 is able to block the differentiation of PC12 cells (149-151). Recently, another MEK inhibitor, U0126, has also been used to block the Ras-ERK pathway (72). We tested each of these drugs ability to block nNOS induction. The PD98059 drug did not inhibit NGF-induced nNOS levels, even at concentrations of 50 μ M (Fig. 3-3A). It is impractical to use this drug at higher concentrations because it precipitates out of solution. U0126, however, was very effective in blocking this induction at concentrations of 10 μ M to 50 μ M (Fig. 3-3A). We also checked whether blocking the MAP kinase pathway effected NOS activity. 50 μ M

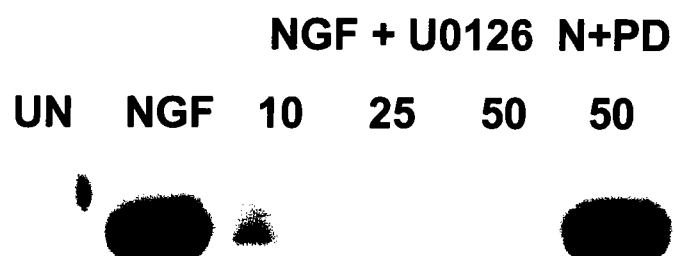
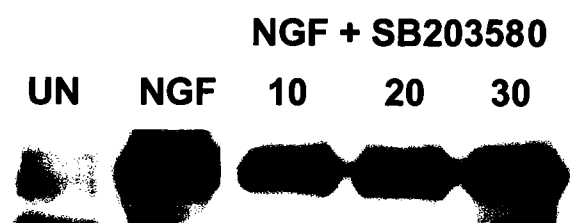
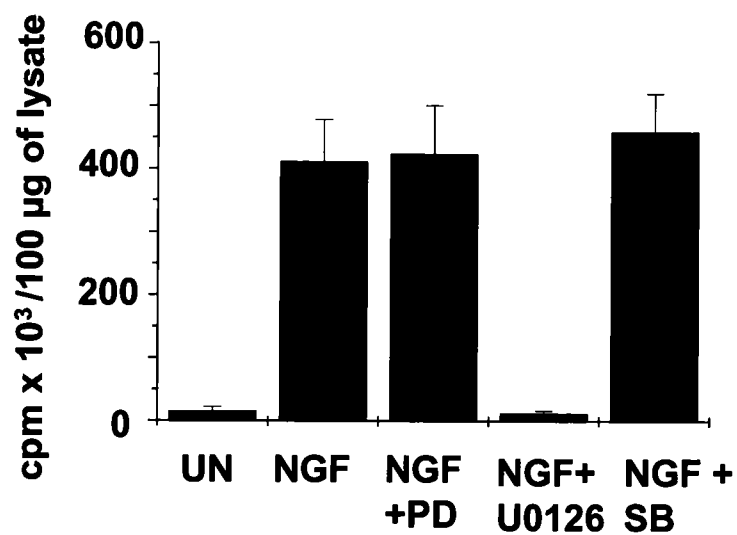
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Figure 3-3. (A) Effect of MEK inhibitors on nNOS induction. Cells were pretreated with carrier alone, U0126 (10, 25 and 50 μ M) or 50 μ M PD98059 for 30 minutes before addition of 100 ng/ml of NGF. NGF, media and drugs were replenished every 48 hours. (B) Effect of SB203580 on nNOS induction. PC12 cells were pre-treated with increasing concentrations of SB203580 (10, 20 and 30 μ M) for 30 minutes and then NGF (100 ng/ml) was added. SB203580 and NGF were replenished every 48 hours. After 6 days extracts were prepared and Westerns performed. This blot is representative of four independent experiments. (C) Effect of MEK and p38 inhibitors on NGF-induced NOS activity. Cells were pretreated with 50 μ M of PD98059, 50 μ M of U0126 or 30 μ M SB203580 for 30 minutes before addition of 100 ng/ml of NGF. NGF, media and drugs were replenished every 48 hours. After 6 days homogenates were prepared and citrulline assays using 100 μ g of protein per sample were performed. Data are presented as cpm \times 10^3 /100 μ g of lysate and are measured as a mean \pm SEM (n = 6).

of PD98059 was ineffective in blocking NGF-induced NOS activity six days ($p = 0.450$) (Fig. 3-3C). U0126 at 50 μM completely blocked NGF-induced NOS activity after six days (Fig. 3-3C). These results were statistically significant ($p < .001$). Similar results were obtained after four days (data not shown). The nNOS Western blots and the NOS activity assays gave very similar results: PD98059 has negligible effects on nNOS induction while U0126 is a potent inhibitor of nNOS induction.

p38 kinase does not regulate nNOS induction

Recently, it was reported that the p38 kinase pathway is involved in the differentiation of PC12 cells (68). We investigated whether the p38 inhibitor, SB203580, would have any effect on neurite extension and induction of nNOS. Treatment with 30 μM SB203580 inhibited neurite extension by 38% ($p < 0.01$). In contrast, Fig. 3-3B shows that SB203580 (10-30 μM) did not inhibit the induction of nNOS protein levels. Similarly, 30 μM SB203580 did not inhibit NGF-induced NOS activity after 6 days ($p = 0.668$) (Fig. 3-3C).

Effectiveness of MEK inhibitors, U0126 and PD98059

We wanted to investigate the discrepancy between the PD98059 and U0126 results. Surprisingly, we found that PD98059 only partially blocked neurite extension in our clone of PC12 cells (Fig 3-4A and B). In contrast, U0126 was very effective in

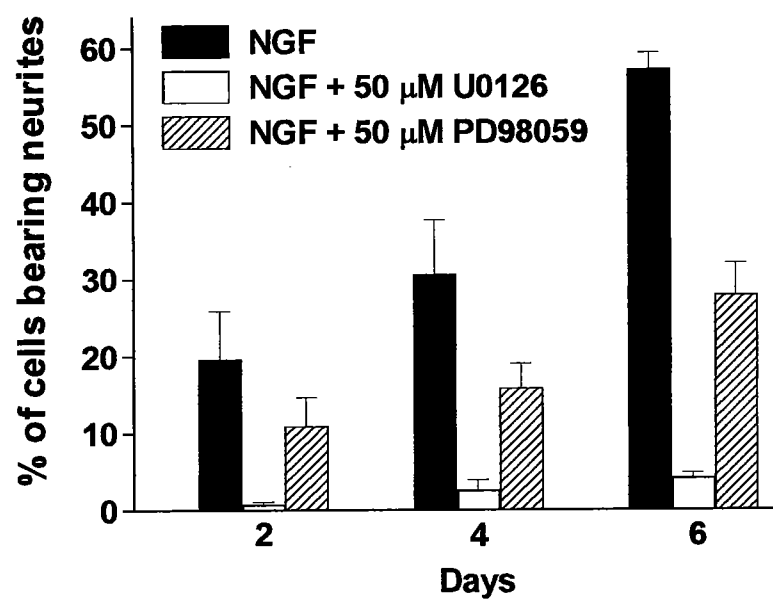
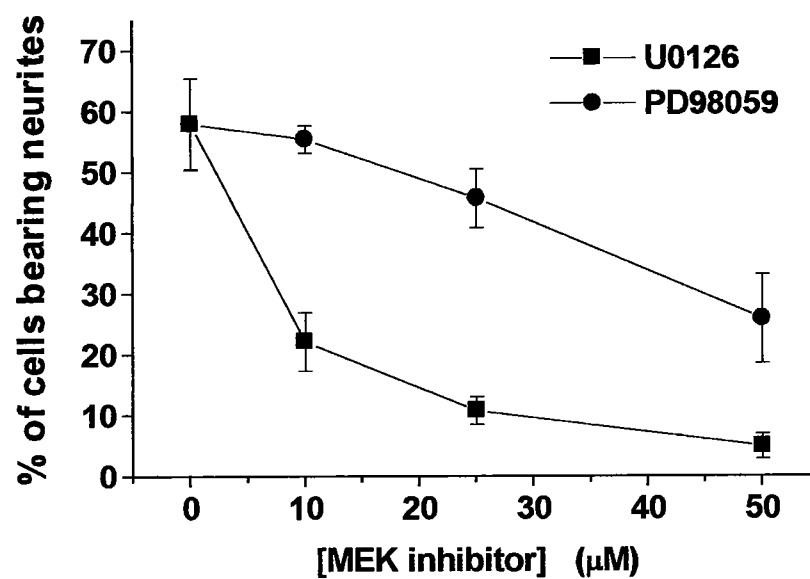
A**B**

Figure 3-4. Effect of MEK inhibitors on neurite extension. Drugs were dissolved in DMSO. Carrier alone controls showed no adverse effects. Cells were pretreated with drug for 30 minutes before addition of 100 ng/ml NGF. Media, drugs and NGF were replenished every 48 hours. Cells were scored positive for neurites if they had at least one neurite two cell diameters long. (A) For the time course five separate fields of the same flask were photographed and scored for neurites. The experiment was performed in triplicate and data is presented as a mean \pm SEM (B) For the dose response five fields were photographed and scored for neurites after six days of treatment with either MEK inhibitor. The experiment is presented as a mean \pm SEM.

blocking neurite extension. After 6 days of NGF treatment, $57 \% \pm 2 \%$ (SEM) of NGF-treated cells had neurites. Only $4.3 \% \pm 1 \%$ (SEM) of NGF plus 50 μ M U0126-treated cells while $28 \% \pm 4 \%$ (SEM) of NGF plus 50 μ M PD98059-treated cells scored positive for neurites (Fig. 3-4A). Similarly, increasing doses of U0126 were much more effective than PD98059 in blocking neurite extension after six days of NGF treatment (Fig. 3-4B).

Because PD98059 was only partially effective in blocking neurite extension and unable to block nNOS induction, we compared its ability to block Ras-ERK signaling with that of U0126. In PC12 cells, ERK becomes phosphorylated within ten minutes of NGF treatment. (29,30,58). Since ERKs are the physiological substrates of MEKs, we were able to address the efficacy of both MEK inhibitors with an antibody that recognizes phosphorylated ERK1 and ERK2. Fig. 3-5 shows that U0126 is much more effective in blocking ERK phosphorylation than PD98059. Blots were then reprobed with an anti-ERK antibody to verify that the samples contained equal levels of ERK (Fig. 3-5). We also confirmed that PD98059 was only partially effective in blocking ERK activity in a MAP kinase assay (data not shown). We conclude that U0126 is a more effective MEK inhibitor than PD98059 in our cells, and this explains why U0126 blocks NOS induction much more effectively than PD98059.

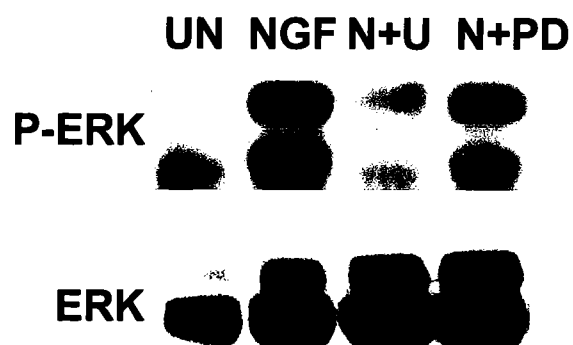


Figure 3-5. Effect of MEK inhibitors on ERK phosphorylation. Cells were pre-incubated with serum free media for one hour in order to lessen basal levels of ERK phosphorylation and pre-treated with either 50 μ M U0126, 50 μ M PD98059 or carrier alone for 30 minutes. NGF was added for 15 minutes and cellular extracts were then prepared. Western blots were performed with an antibodies specific for ERK and phosphorylated ERKs. This experiment is representative of three independent results.

Discussion

We have used NGF-treated PC12 cells as a model to study the upregulation of nNOS mRNA and protein levels. The Ras-ERK pathway has been extensively studied in this system and is required for many of the morphological and biochemical changes induced by NGF (29,30,58). By two different means we have demonstrated that the Ras-ERK pathway is required for the induction of nNOS. First, we have shown in wild type PC12 cells that nNOS is induced at the RNA level as seen using RT PCR and at the protein level by Western blotting. In PC12 cells infected with an adenovirus that expresses a dominant negative Ras, nNOS is not induced at either the RNA level or the protein level (Fig. 3-2A and 3-2B). We have also shown by citrulline assay that a competent Ras is necessary for the induction of NOS activity (Fig. 3-2C). Although we did not examine the transcriptional regulation of nNOS, Rife et al. have suggested that MAP kinase might influence expression through transcription factors associated with the 5'2 nNOS promoter (146). It is interesting to note that the induction of nNOS mRNA and NOS activity apparently precedes that of nNOS protein. This finding may be due to the relative low sensitivity of the Western blot analysis as compared with the NOS activity assay. Second, we discovered that U0126, but surprisingly not PD98059, blocked the induction of nNOS protein and activity (Fig. 3-3A, and 3-3C). Finally, we have observed that the p38 kinase inhibitor SB203580 did not block NGF-induced nNOS (Fig. 3-3B and 3-3C). Since the JNK kinase pathway is not activated in NGF-treated

PC12 cells (69), we conclude the Ras-ERK pathway, not the p38 or JNK kinase pathways, is required for nNOS induction.

The different potencies for U0126 and PD98059 were puzzling at first. PD98059 has been used extensively to investigate MAP kinase signaling. In particular, PD98059 has been reported to inhibit neurite extension in PC12 cells (149-151). Yet in our clone of PC12 cells, PD98059 did not block nNOS induction and only partially blocked ERK phosphorylation and neurite extension (Fig. 3-3A, 3-3C, 3-4 and 3-5). A possible explanation for this difference is that various clones of PC12 cells contain different numbers of TrkA receptors so that signaling varies from clone to clone. Alternatively, the potency of PD98059 as a MAP kinase inhibitor might vary from clone to clone. Indeed, the developers of PD98059 noted that this drug did not block neurite extension in some clones (70). Thus it seems that even the residual activation of MAP kinase signaling as seen in NGF plus PD98059 treated PC12 cells (Fig. 3-5) is sufficient to induce nNOS (Fig. 3-3A and 3-C). In contrast, the drug U0126 was very effective in blocking MEK activity, neurite extension and nNOS induction (Fig. 3-3A, 3-3C, 3-4 and 3-5). Most likely, this is because U0126 is significantly more potent than PD98059 (72). Moreover, U0126 is equally potent towards MEK1 and MEK2 while PD98059 is more potent towards MEK1 (72,152). This is important because both isoforms of MEK are activated with similar kinetics in NGF-treated PC12 cells (153). In conclusion, U0126 is a much more effective drug and should be used in future studies of PC12 cells.

This report provides the first evidence of nNOS regulation by the Ras-ERK pathway. It is already known that the expression of the other two NOS isotypes can be

regulated by MAP kinases. Estrogen has been shown to induce eNOS in pulmonary artery endothelial cells through the Ras-ERK pathway (154). Similarly, fibroblast growth factor and epidermal growth factor stimulate expression of eNOS through Ras-ERK in ovine fetoplacental artery endothelial cells (155). In addition, all three MAP kinase pathways have been implicated in iNOS regulation. The Ras-ERK pathway has been associated with cytokine-induced iNOS in cardiac myocytes and microvascular endothelial cells (121,156). The JNK pathway has been reported to be important for iNOS expression during costimulation of mouse macrophages with IFN-gamma and TNF-alpha (157). Likewise, the p38 pathway has been implicated in iNOS induction in mouse astrocytes, bovine cartilage-derived chondrocytes and in an oligodendrocyte cell line (CG4 cells) (158-160). In addition, p38 may downregulate iNOS in glomerular mesangial cells (161). The p38 pathway can synergize with the Ras-ERK pathway to regulate iNOS (162-164). Thus consistent with their pervasive role in signaling, the MAP kinase pathways participate in the expression of all three NOS isotypes.

While it is now clear that the MAP kinase pathways regulate the expression of nitric oxide synthases, it is becoming evident that NO itself or NO-related species are important regulators of MAP kinase signaling. Ras is a target of NO and the site of the molecular interaction between Ras and nitric oxide has been determined (13,165). It has been reported that NO-activation of Ras in PC12 cells is important for survival responses (166). These observations have been extended to primary cortical cultures in rats where it has been shown that NO controls NMDA receptor-induced activation of Ras (167). In addition to Ras, all three MAP kinase pathways also appear to be susceptible to NO-

induced activation (168,169). Hence, it is becoming apparent that nitric oxide can modify components of the MAP kinase pathways. It remains to be determined whether NO modification of MAP kinase pathways creates a feedback loop to modulate signaling from cell surface receptors and to induce crosstalk between the three MAP kinase pathways.

CHAPTER IV

NITRIC OXIDE DOES NOT ACT THROUGH GUANYLATE CYCLASE DURING DIFFERENTIATION OF PC12 CELLS

Introduction

We have described a novel signaling pathway during the differentiation of PC12 cells involving NGF, nitric oxide, p53 and p21 WAF1 (84). It is unclear the mechanism by which NO acts to induce differentiation and cell cycle arrest in this system. One of the best characterized modes of NO action is through the activation of guanylate cyclase and the production of cGMP (1). Cyclic GMP, in turn can activate cyclic GMP-dependent protein kinase (PKG) and these signaling molecules can control several neuronal functions, including neurotransmitter release, ion channels, and calcium signaling (170).

A role for cGMP in the differentiation of several different cell types has been reported. An increase in the intracellular cGMP concentration induced granulocytic differentiation of the human promyelocytic cell line HL-60 (171). Similarly, the differentiation of rat osteoblast-like cells, human melanocytes, Sertoli cells, and normal human bone marrow cells all involve cyclic GMP (172-175).

In PC12 cells, cGMP has multiple, varied roles. Cyclic-GMP acts in opposition to tetanus toxin blockade of acetylcholine release (176). Nitric oxide can amplify

calcium-induced gene transcription in PC12 cells and this effect is cGMP-dependent (177). Conversely, NO selectively inhibits voltage-dependent calcium influx in neuronal cells through a cGMP-dependent mechanism (178). Others have shown NO, through cGMP, to modulate agonist-evoked intracellular Ca^{2+} (179,180) which in turn can activate the ryanodine receptor (181). Stimulation of the cGMP pathway by NO induces expression of the immediate early genes c-fos and junB in PC12 cells (182). In trophic factor-deprived PC12 cells, NO can delay death by a cGMP-mediated mechanism (183).

We examined the possibility that NGF-induced differentiation of PC12 cells through NO requires the activation of guanylate cyclase. We find that unlike the inhibition of NOS which inhibits differentiation, inhibition of guanylate cyclase does not inhibit differentiation as measured by the induction of the neuronal marker β -tubulin III. Moreover, 8Br-cGMP, a cell-permeable analog of cGMP failed to induce differentiation of NGF-treated, NOS-inhibited PC12 cells.

Materials and Methods

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 $\mu\text{g}/\text{ml}$ gentamycin at 37 °C under 5% CO_2 . These cells were found to be mycoplasma-free by the 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 (98), 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.

Drugs

N-Nitro-L-arginine methyl ester (L-NAME) competes with arginine for binding to all three isoforms of NOS and blocks enzymatic activity (9). In these studies, we used 20 mM 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 (83). LY83583 (Calbiochem) is a competitive inhibitor of soluble guanylate cyclase. 8 Br-cGMP (Sigma) is a soluble analog of cyclic GMP.

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 monoclonal anti- β -tubulin isotype III (Sigma) 1:800 in TTBS for 1 h. Immunoreactive proteins were detected with horseradish peroxidase-conjugated secondary antibodies (Amersham, Arlington Heights, IL) and a chemiluminescence reagent (100).

Results

Previously we have shown that inhibition of NOS during NGF-induced neuronal differentiation blocks differentiation as measured by neurite extension or expression of neuronal markers (84). We tested whether blocking cGMP production with the guanylate cyclase inhibitor LY83583 could also block the expression of NGF-induced differentiation. NGF induces the neuronal marker β -tubulin III after three days in defined media (Figure 4-1). LY83583 does not inhibit NGF-induced levels of β -tubulin III (Figure 4-1) or neuronal differentiation (data not shown). It should be noted that this concentration of LY83583 has been shown to inhibit guanylate cyclase activity in NGF-treated PC12 cells (184). We attempted to restore differentiation of NGF-treated, NOS-inhibited PC12 cells with a soluble analog of cGMP. 8 Br-cGMP was not able to restore neurite extension in L-NAME-treated PC12 cells (Figure 4-2).

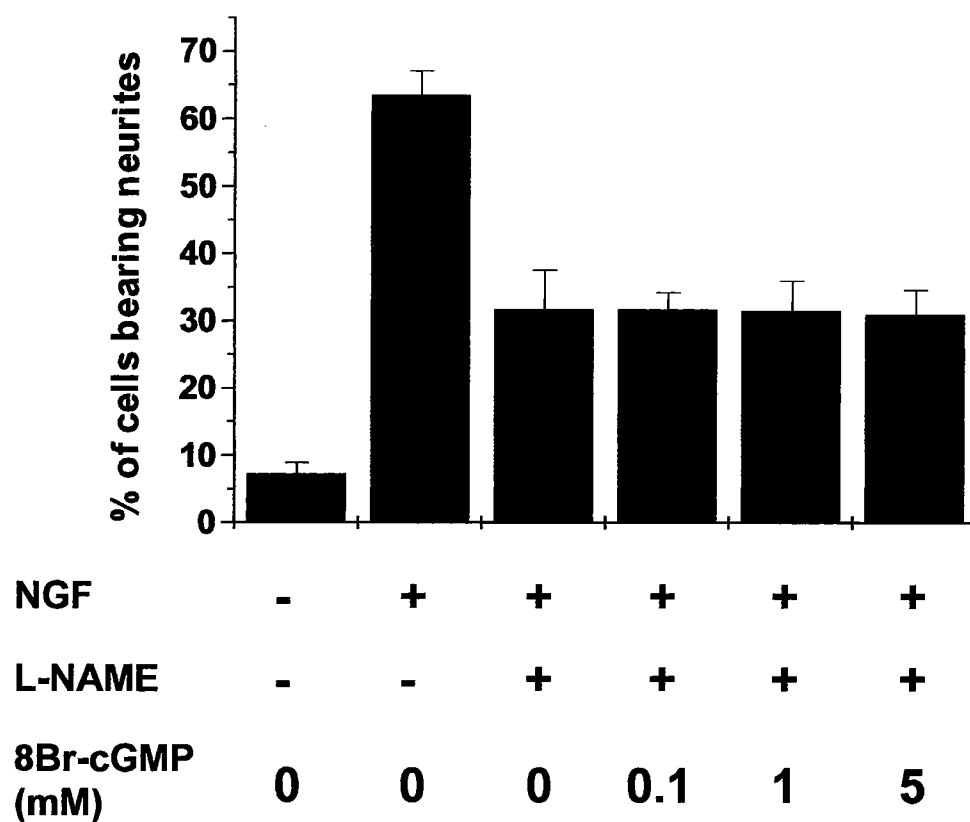


Figure 4-2. 8 Bromo-cyclic GMP cannot rescue L-NAME inhibited differentiation. Cells were cultured for three days in defined media and either untreated, treated with NGF (100 ng/ml), treated with L-NAME (20 mM) and/or 8Br-cGMP (0.1, 1 and 5 mM). Data is presented as a mean \pm SEM of three independent experiments.

Discussion

Our evidence indicates that NO does not act through guanylate cyclase during the differentiation of PC12 cells. Conflicting data exists over the importance of cyclic GMP during PC12 differentiation. One group reports that nerve growth factor increases the cyclic GMP level and activates the cyclic GMP phosphodiesterase in PC 12 cells (125). Moreover, another group has reported that NO donors enhance neurotrophin-induced neurite outgrowth through a cGMP-dependent mechanism (185). Conversely, a recent report shows evidence that NGF decreases soluble guanylate cyclase levels in PC12 cells (186). While the evidence for a role for cyclic GMP in NGF-treated PC12 cells is conflicting our results along with another group (184) argue against a role for cGMP in PC12 neuronal differentiation.

It has become increasingly evident that nitric oxide does not act exclusively through cGMP. Nitric oxide is involved in neuronal process outgrowth and remodeling in dorsal root ganglion neurons in a cGMP independent manner (187). In PC12 cells, nitric oxide attenuates potassium-stimulated dopamine release in a cyclic GMP independent fashion (188). NO can regulate the activity of caspases independent of cGMP (24,25). It is now known that nitrosylation of cysteine residues by NO is a major mechanism for NO action. We next investigated whether NO functions through nitrosylation to regulate p53 levels. Earlier we demonstrated that NO participates in p53 induction during PC12 differentiation (Chapter 2).

CHAPTER V
NITRIC OXIDE INHIBITS HDM2-p53 BINDING
THROUGH S-NITROSYLATION OF HDM2

Introduction

The tumor suppressor p53 is an important regulatory molecule involved in cell cycle arrest during development and as a mediator of apoptosis in response to stress (43-45). Mutation of p53 is one of the most common genetic lesions in human cancer. Although p53 is dispensable for normal cell growth and development, cells and mice lacking functional p53 show a predisposition to cell proliferation, chromosomal abnormalities, and tumorigenesis, suggesting that p53 plays a critical role in maintenance of genome integrity (43). p53 functions as a transcription factor that activates transcription of genes involved in cell cycle arrest (p21WAF1 and cyclin G), DNA damage (GADD45) and apoptosis (Bax) (43). Mdm2 is another transcriptional target of p53 and its interactions with p53 are the focus of this study (53,54).

The mdm2 gene was first detected in a screen for oncogenes present on a *mouse double minute* chromosome (49). The human homolog of the gene, hdm2, has been shown to be overexpressed in a number of human tumors (189). Hdm2 can regulate p53 in two ways. Hdm2 binds p53 and inactivates its transcriptional activity (50). Hdm2 also regulates p53 levels by promoting its degradation (51,52). Hdm2 is an E3 ligase that

ubiquitinates p53 (190). Thus Hdm2 participates in a negative feedback loop that keeps p53 levels in tight check.

Nitric oxide is a ubiquitous signaling molecule with diverse and varied functions. NO has been shown to be a signal that increases p53 levels in physiological and pathological situations. During neuronal differentiation of PC12 cells NO acts as a cytostatic agent by inducing p53 (84). Similarly, in vascular smooth muscle cells NO activates p21WAF1 through p53 (191). During apoptosis, NO plays a critical role in p53 induction but the molecular mechanism of this induction is not known (76,192).

One way that nitric oxide exerts its actions in cells is through S-nitrosylation of cysteine residues. In the case of Ras, S-nitrosylation enhances its activity (13). For most proteins, though, S-nitrosylation is an inactivating modification. Caspase-3 is inactivated by S-nitrosylation *in vivo* (25). Several other proteins including receptors, proteases, and transcription factors are reportedly S-nitrosylated (5) (Table 2).

It has recently been shown that Hdm2-p53 binding is sensitive to oxidation and that the sulfhydryl-modifying agent N-ethylmaleimide destroys the binding capacity of Hdm2 to p53 (193). In this study we test whether nitric oxide can effect the ability of Hdm2 to bind p53. We find that NO reacts with Hdm2 and thereby inhibits Hdm2-p53 binding. Moreover, we demonstrate that this inhibition is reversible, consistent with nitrosylation of cysteine. Finally, we identify a cysteine residue proximal to the Hdm2-p53 binding domain that is responsible for the NO inhibition of binding.

Materials and Methods

Reagents

Dithiothreitol, N-ethylmaleimide, cysteine, reduced glutathione and tetramethylbenzidine (TMB) dihydrochloride tablets were all purchased from Sigma. DETA/NO (NOC-18) was purchased from Alexis.

GST-Hdm2

The plasmid GST-Hdm2 (a.a. 1-188) was a gift from David Lane (194). Bacteria cultures were grown to OD 0.8. They were cooled to room temperature, induced with 1 mM IPTG and then grown for 4 h at 27°C. Cells were harvested and pellets were flash frozen in liquid nitrogen. Pellets were resuspended in ice cold buffer A (0.5 M NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, 1mM PMSF, 1 mM EDTA, 10 mM 2-mercaptoethanol, pH 7.3) and lysed twice in a French press. After centrifugation the soluble fraction was incubated with 1 ml of Glutathione Sepharose 4B beads for 1 hour at room temperature and then overnight at 4°C. The entire mixture was then spun and washed 6 times with PBS and then the beads were packed in a column. The protein was then eluted with buffer B (50 mM Tris/HCl, 10 mM reduced glutathione, 0.5 M NaCl, 1 mM EDTA, 1 mM PMSF, 10 mM 2-mercaptoethanol pH 8.0). Fractions containing GST-Hdm2 were pooled, aliquotted and stored at -20°C.

Preparation of p53 protein in SF9 cells

His-tagged, full-length p53 baculovirus was a gift from Steve Jones. Sf9 cells were infected and cells were harvested 48 hours after infection. The cells were extracted at 4°C with 1% Triton X-100, 150 mM NaCl, 50 mM Tris/HCl (pH 7.5) and 1 mM PMSF for 30 minutes. After centrifugation extract was passed through a 0.45µm filter. The p53 protein was purified on a His-Bind Quick 900 Cartridge (Novagen). Protein expression and purity were checked by western blotting with a monoclonal anti-6xHis antibody (Clontech). Purified p53 was aliquotted and stored at -20°C.

ELISA Assay

The Hdm2-p53 interaction was assayed by ELISA using a similar assay to one described previously (194). EIA assay plates (Costar) were coated overnight at 4°C with 1 µg/ml of p53 in PBS. Plates were blocked with PBST-M (PBS, containing 5% milk powder and 0.1% Tween 20) for one hour at room temperature. GST-Hdm2 (2.5 µg/ml) in PBS was incubated with NO donor and with or without cysteine or DTT for one-half hour at room temperature. An equal volume of 2X PBST-M was then added to the sample and then applied to the ELISA plate for one hour at room temperature. After three washes wells were probed with a polyclonal anti-GST antibody (1:10,000) from Sigma in PBST-M and then a HRP-linked polyclonal anti-rabbit antibody (1:1,500) from Amersham in PBST-M. Substrate development was performed with TMB tablets (0.1 mg/ml) in 0.05 M phosphate-citrate buffer pH 5.0 containing 0.006% hydrogen peroxide. After 15 minutes reactions were stopped with an equal volume of 1M phosphoric acid and read at 450 nm. All washing between steps was done with PBS.

Site directed mutagenesis

Site directed mutagenesis and generation of the mutant plasmid GST-Hdm2C77A was performed with the QuickChange™ Site-Directed Mutagenesis Kit from Stratagene following standard protocols. The mutant sequence was confirmed by automated sequencing.

Results

We tested whether treating Hdm2 with a nitric oxide donor would affect its binding to p53. We employed a GST-Hdm2 fusion protein that includes the first 188 amino acids of Hdm2. This fragment contains the p53-binding domain. In ELISA assays we confirmed earlier results that the thiol-modifying agent N-ethylmaleimide (NEM) inhibits Hdm2-p53 binding (Figure 5-1). Using the nitric oxide donor DETA/NO (1, 5 and 10 mM) we demonstrated that nitric oxide inhibits this binding as well (Figure 5-1). This inhibition is blocked by the addition of an excess of reducing agent. When excess DTT (10 mM) is present DETA/NO was unable to inhibit binding (Figure 5-2). Presumably, the excess DTT competes with Hdm2 for the nitric oxide. Moreover, addition of excess amounts of cysteine also blocked nitric oxide inhibition of Hdm2-p53 binding (Figure 5-3). These results are consistent with nitric oxide effecting binding by nitrosylating sulfhydryl residues.

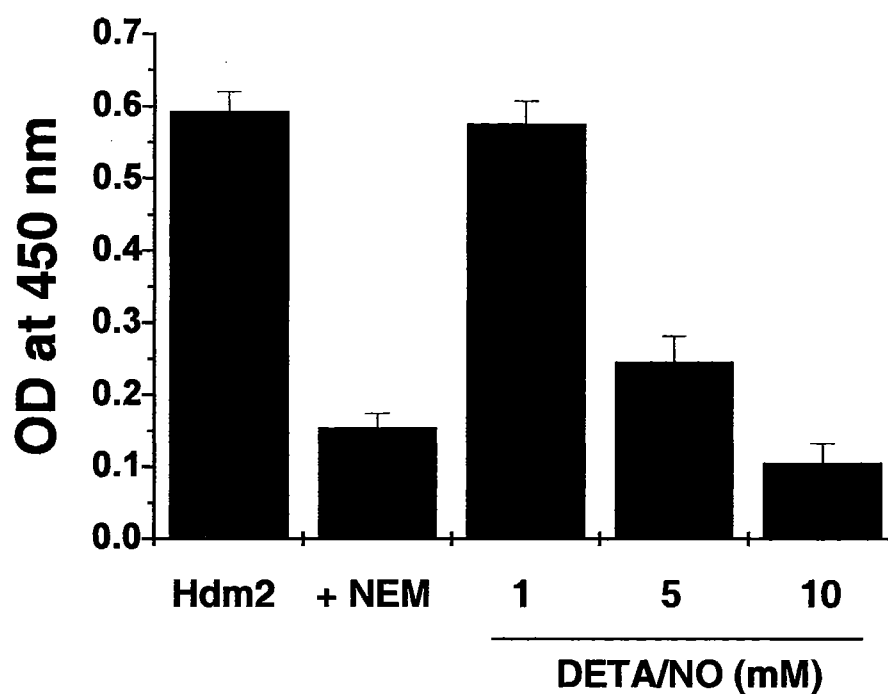


Figure 5-1. DETA/NO inhibits Hdm2-p53 binding. ELISA assays were performed on p53-coated plates treated with Hdm2 (2.5 $\mu\text{g/ml}$) alone, Hdm2 + 10 mM NEM or Hdm2 + increasing amounts of DETA/NO (mM). Data is presented as a mean \pm SEM (n=15).

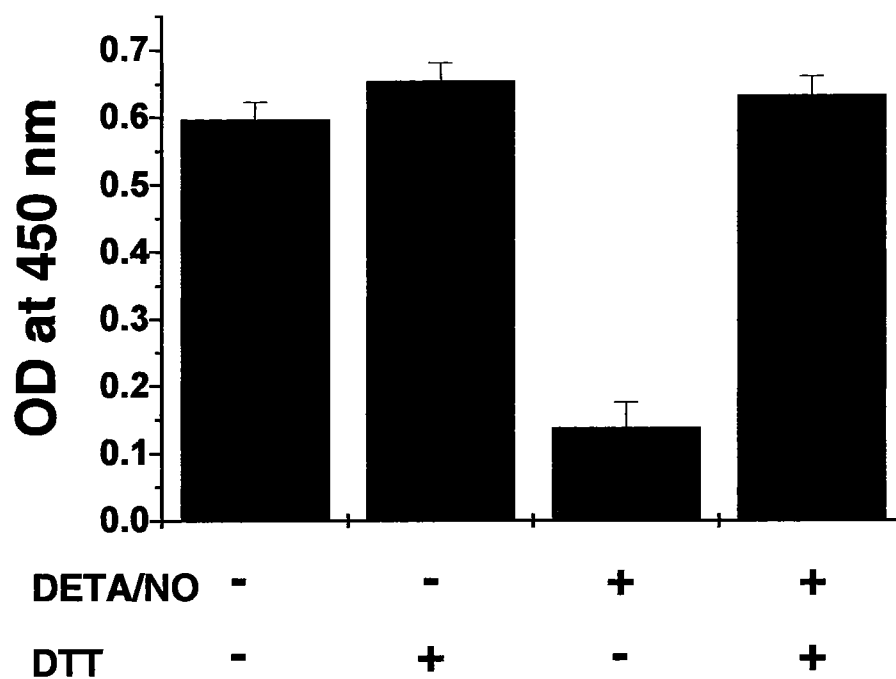


Figure 5-2. DTT blocks DETA/NO inhibition of Hdm2-p53 binding. ELISA assays were performed on p53-coated plates that were presented with Hdm2 (2.5 $\mu\text{g/ml}$) alone, Hdm2 + 10 mM DTT, Hdm2 + 10 mM DETA/NO or Hdm2 + 10 mM DTT and 10 mM DETA/NO. Data is presented as a mean \pm SEM ($n = 9$).

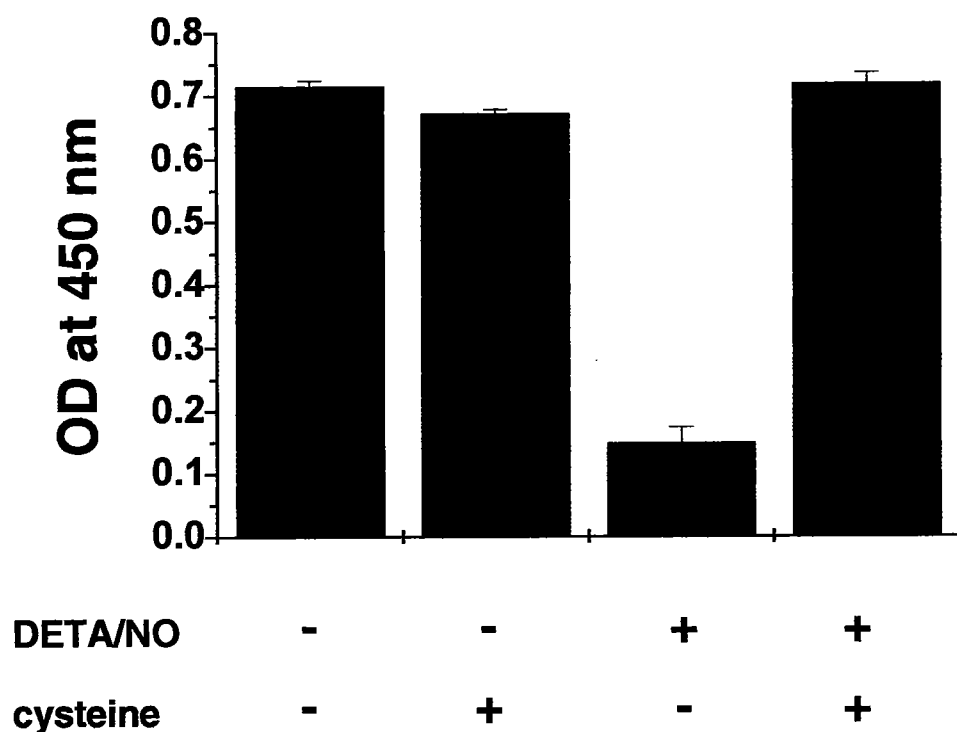


Figure 5-3. Cysteine blocks NO inhibition of Hdm2-p53 binding. ELISA assays were performed on p53-coated plates treated with Hdm2 (2.5 μ g/ml) alone, Hdm2 + 10 mM cysteine, Hdm2 + 10 mM DETA/NO or Hdm2 + 10 mM cysteine and 10 mM DETA/NO. Data is presented as a mean \pm SEM (n = 9).

In order to ensure that the effect of nitric oxide was specific for Hdm2 we performed several controls. We coated ELISA plates with GST-Hdm2 instead of p53 and tested whether the presence of nitric oxide effected the binding of the anti-GST antibody to GST-Hdm2. In these experiments, nitric oxide had no effect (data not shown). We also tested whether nitric oxide was modifying p53 instead of Hdm2. Plates coated with p53 were treated with DETA/NO and tested for their ability to bind Hdm2. In these experiments DETA/NO had no effect on Hdm2-p53 binding (data not shown). From these experiments we conclude the effect of nitric oxide on Hdm2-p53 binding is due to a reaction of NO with Hdm2.

We tested whether the effect of nitric oxide was reversible. DETA/NO was reacted with Hdm2 for one-half hour and then the mixture was split in two. Half of the sample was then incubated with DTT for an additional one-half hour while the other half of the sample was untreated. The results from these experiments clearly indicate the inhibition by nitric oxide to be reversible, again consistent with nitrosylation of a sulfhydryl group (Fig 5-4).

Previously, it had been shown that the Hdm2-p53 reaction was susceptible to oxidation (194). This fact coupled with our results with NO led us to examine which cysteine residue is modified by NO and thereby inhibits binding. In our Hdm2 fragment there are three cysteine residues; C2, C77 and C127. C77 seemed to be the most likely residue to be modified due to its proximity to the Hdm2 and p53 binding interface (193,195) (Figure 5-5). In addition we found that this C77 is highly conserved in human, mouse, chicken, frog and zebrafish (Fig 5-5). We mutated C77 to A in Hdm2 and tested

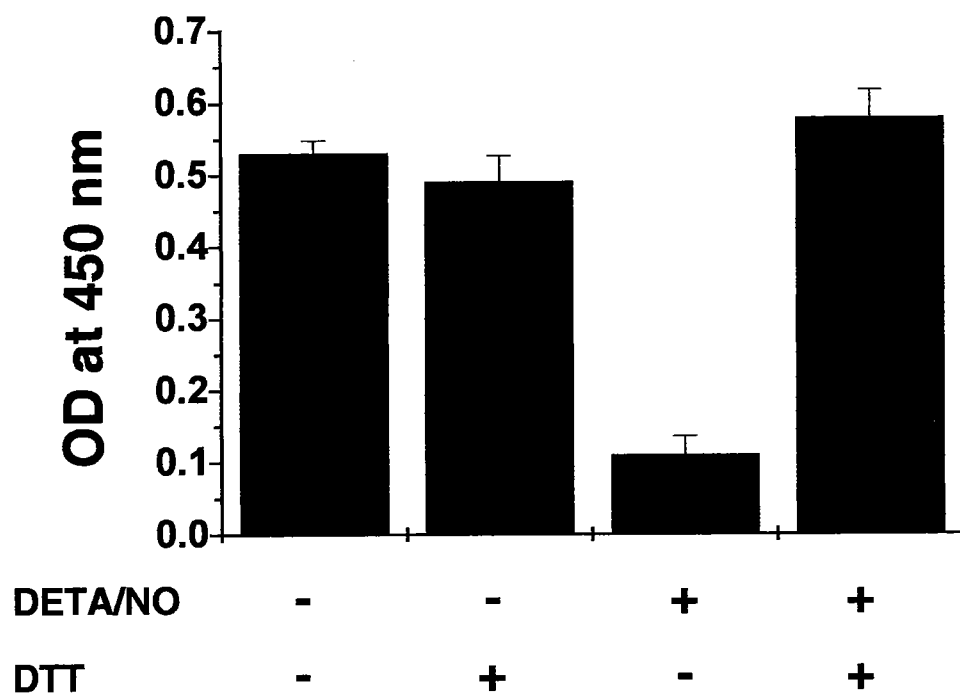


Figure 5-4. DETA/NO inhibition of Hdm2-p53 binding is reversible. ELISA assays were performed on p53-coated plates. Hdm2 (2.5 $\mu\text{g/ml}$) alone or Hdm2 + 10 mM DETA/NO were incubated for one-half hour then half of each sample was then incubated with 10 mM DTT for one-half hour. Data is presented as a mean \pm SEM (n=9).

Figure 5-5. The N-terminus of Hdm2 contains three cysteines (C2, C77 and C127). C77 is highly conserved and is located within the p53-binding domain (aa 50-100). Sequences were aligned using the Clustal Method in MegAlign.

the ability of this mutated protein to bind p53 and the effect of nitric oxide on this binding. The C77A mutation did not affect normal binding when compared to the wild type protein (Fig. 5-6). However, when the mutant protein was treated with DETA/NO there was no inhibition of Hdm2-p53 binding (Fig. 5-7). Thus, the C77 to A mutation renders Hdm2 insensitive to NO inhibition and provides strong evidence that NO exerts its action through this particular cysteine residue.

Discussion

We have presented a potential mechanism by which NO increases p53 levels. Nitric oxide inhibits binding of Hdm2, a negative regulator of p53 levels, to p53 (Fig. 5-1). This inhibition of binding would allow p53 levels to go unchecked and remain high or increase. This inhibition can be blocked by the reducing agent DTT or the NO scavenger, cysteine (Fig. 5-2 and 5-3). Importantly, the inhibition of Hdm2-p53 binding is reversible suggesting this may be a relevant form of regulation *in vivo* (Fig. 5-4). Additionally, we have identified C77 in our Hdm2 fragment as the target of NO. Mutation of this residue to alanine has no effect on normal binding (Fig. 5-6). Nitric oxide has no effect on Hdm2C77A-p53 binding unlike wild-type Hdm2-p53 binding, which is inhibited by nitric oxide (Fig. 5-7).

In this study we focus on the N-terminus of Hdm2 that contains the p53 binding domain. However, Hdm2 regulates p53 by two mechanisms. In addition to its ability to bind and inactivate p53, Hdm2 also regulates p53 stability by targeting the p53 protein

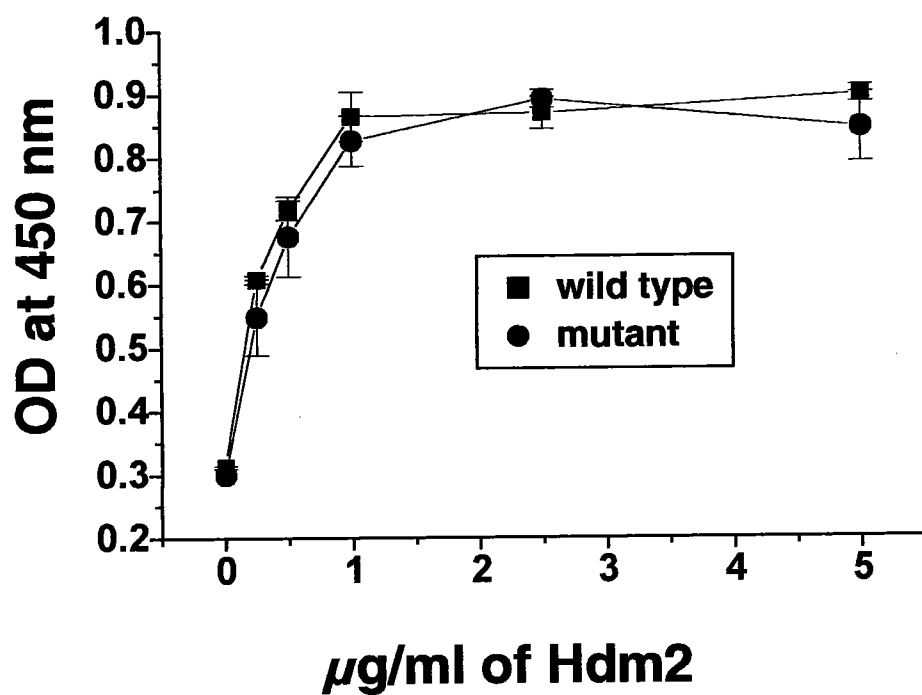


Figure 5-6. Wild type and mutant Hdm2 bind to p53 similarly. ELISA assays were performed on p53-coated plates using increased concentrations of wild type and mutant (C77A) Hdm2.

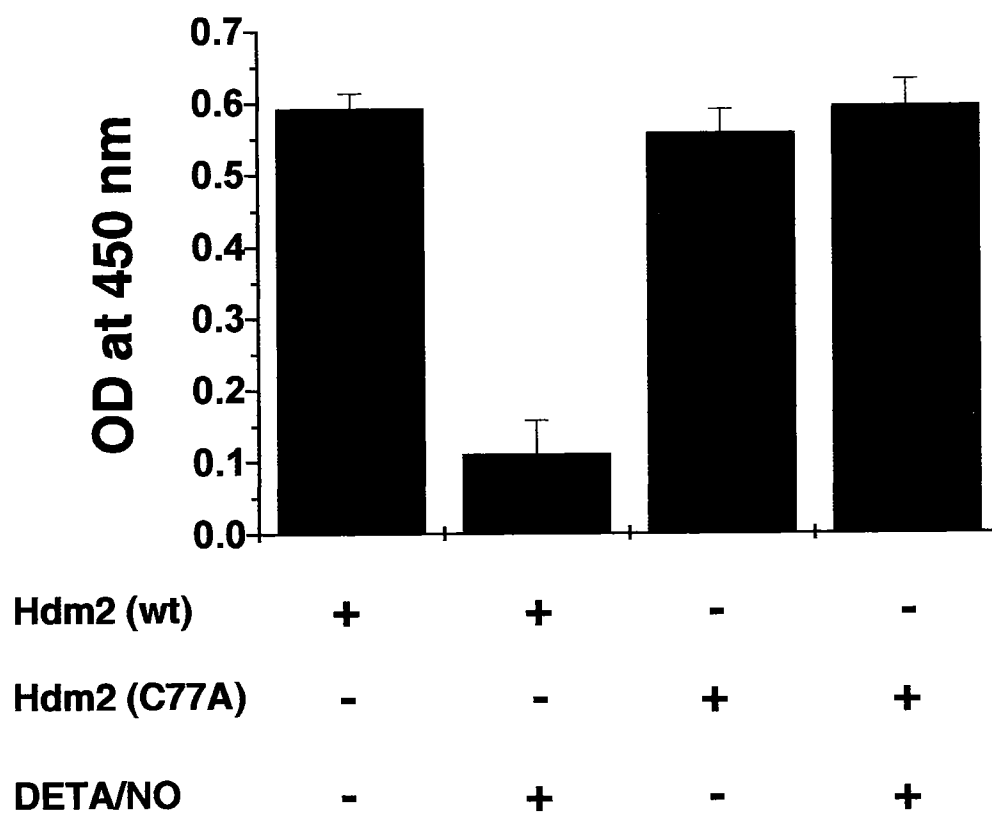


Figure 5-7. Mutant Hdm2 lacks responsiveness to DETA/NO. ELISA assays were performed on p53-coated plates with either Hdm2 wild type (2.5 $\mu\text{g/ml}$) or Hdm2 (C77A) (2.5 $\mu\text{g/ml}$) with or without 10 mM DETA/NO. Data is represented as a mean \pm SEM (n=9).

for proteasomal degradation (51,52). A recent report suggests that Mdm2, the murine version of Hdm2, is a RING finger-dependent ubiquitin protein ligase for itself and p53. This study identifies several cysteine residues in the Mdm2 RING finger that are critical for its ability to degrade p53 (190). Thus, it is possible that in full length Hdm2, these cysteines are additional targets of NO.

The importance of C77 of Hdm2 is not immediately apparent as it does not participate directly in p53 binding. However, the crystal structure does show that C77 is proximal to the p53-binding pocket (Figure 5-8 A). Specifically, C77 lies in close proximity to two residues, V75 and P91, that participate in Van der Waal's interactions with p53 (195,196). C77 is buried in a hydrophobic pocket directly behind the p53-interacting residues (P91, V75, V88 and L82) (Fig. 5-8 B). Reaction of NO with C77 will make this sidechain both larger and more polar. One can propose two possible models. The nitrosylated C77 sidechain might pop out of the hydrophobic pocket, which would distort the p53-interaction domain. Alternatively, the nitrosylated sidechain might remain in the pocket, distorting the layer of p53-interacting residues, which make up much of the hydrophobic pocket. Further structural studies would be required to resolve this question.

Is Hdm2 nitrosylation a physiologically relevant form of p53 regulation?

Previously, we have shown that NO induces p53 accumulation during neuronal differentiation (84). We also know that NO acts in a cGMP independent manner during PC12 differentiation (184) and (Chapter IV). NO is also known to induce p53 during apoptosis. Activation of iNOS in RAW 246.7 macrophages results in p53 accumulation.

A

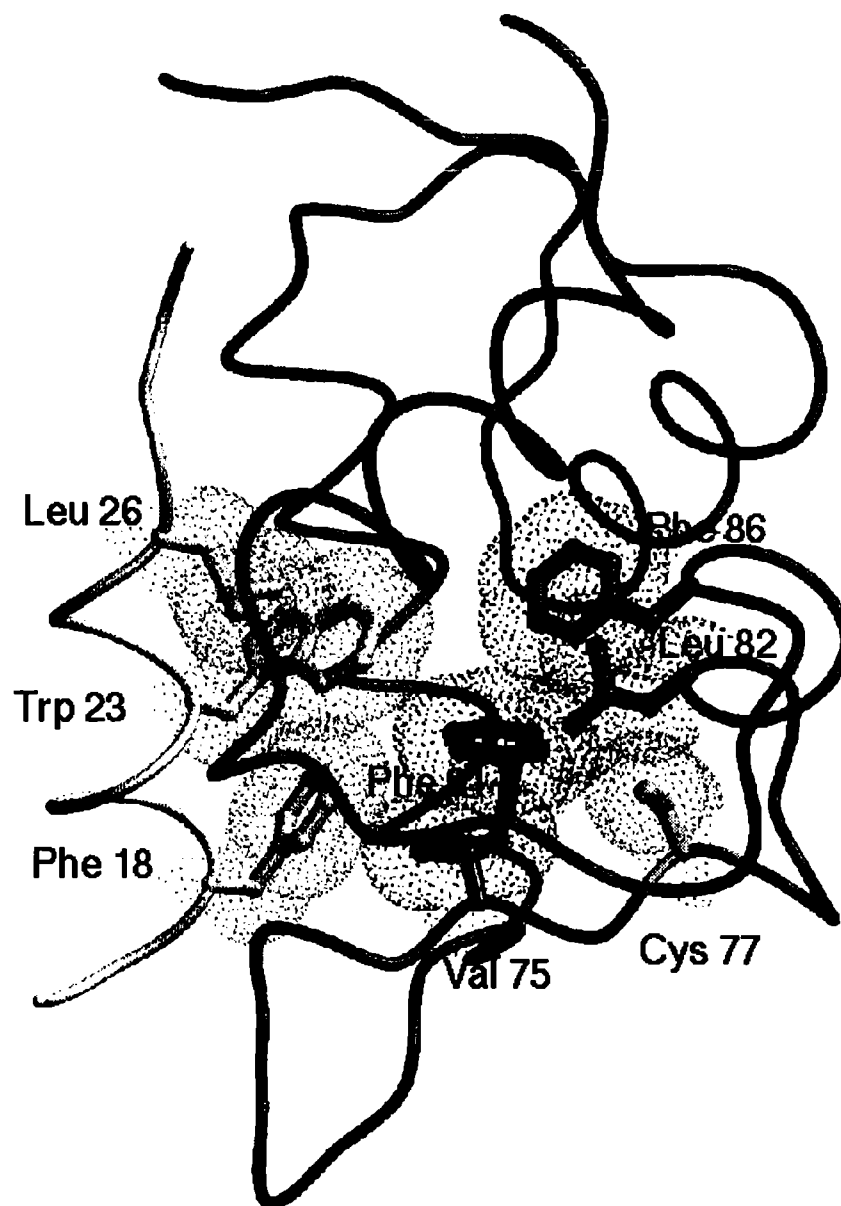


Figure 5-8 A. Cys77 (yellow) is in close proximity to other Hdm2 residues (gray) that bind p53 (green). The crystal structure (194) was used with the MIDAS display system (195) to create this model.

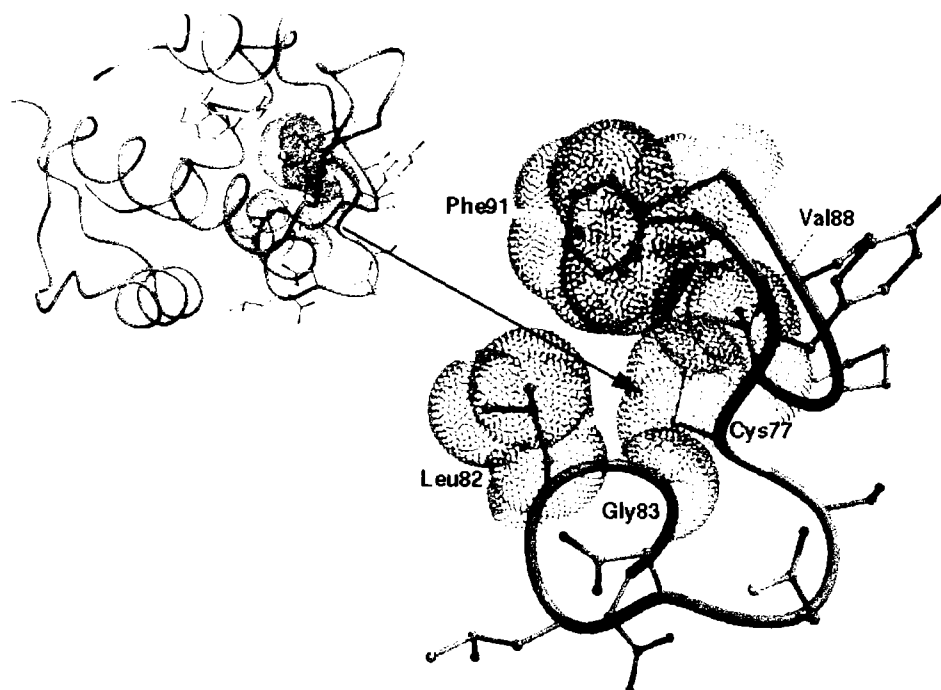
B

Figure 5-8 B. Cys77 is located in a hydrophobic pocket.

Moreover, work with NO donors showed a clear increase in p53 levels while NO removal blocked p53 induction (76,111,112,197). Despite the clear link between NO and p53 induction during apoptosis the molecular mechanism is not understood. In both cases, neuronal differentiation and apoptosis, NO might increase p53 levels by inactivating Hdm2.

CHAPTER VI

ADDITIONAL DISCUSSION

Taken in their entirety, the results from this thesis describe the importance of a signal transduction pathway activated by NGF that involves nitric oxide, p53 and WAF1 which leads to cell cycle arrest and ultimately neuronal differentiation in PC12 cells (Chapter II). In addition, it is now known that NGF induces NOS through activation of the Ras-ERK MAP kinase pathway (Chapter III). Moreover, nitric oxide exerts its actions in a cyclic GMP-independent manner in this system (Chapter IV). Finally, we present *in vitro* evidence that nitric oxide could regulate p53 levels by blocking the p53 regulator, Hdm2, from binding to p53 (Chapter V).

When presented in total these data suggest an appealing pathway important for the control of neuronal differentiation (Figure 6-1). However, several caveats must be considered. One key aspect to consider was the culture conditions used to differentiate PC12 cells. In Chapters II and IV we used defined media to quicken the speed of differentiation. In Chapter III, however, serum-containing media was used to optimize the assays used for NOS detection. It is important to note that the timing of NOS, p53 and p21 WAF1 induction is different between the two culture conditions. Another group has looked carefully at the NGF-induction of p53 and p21 WAF1 in serum and serum-free media (129). The induction of NOS as viewed by diaphorase staining in serum-free conditions (three days) or by RT-PCR, western blotting and citrulline assay (from two to

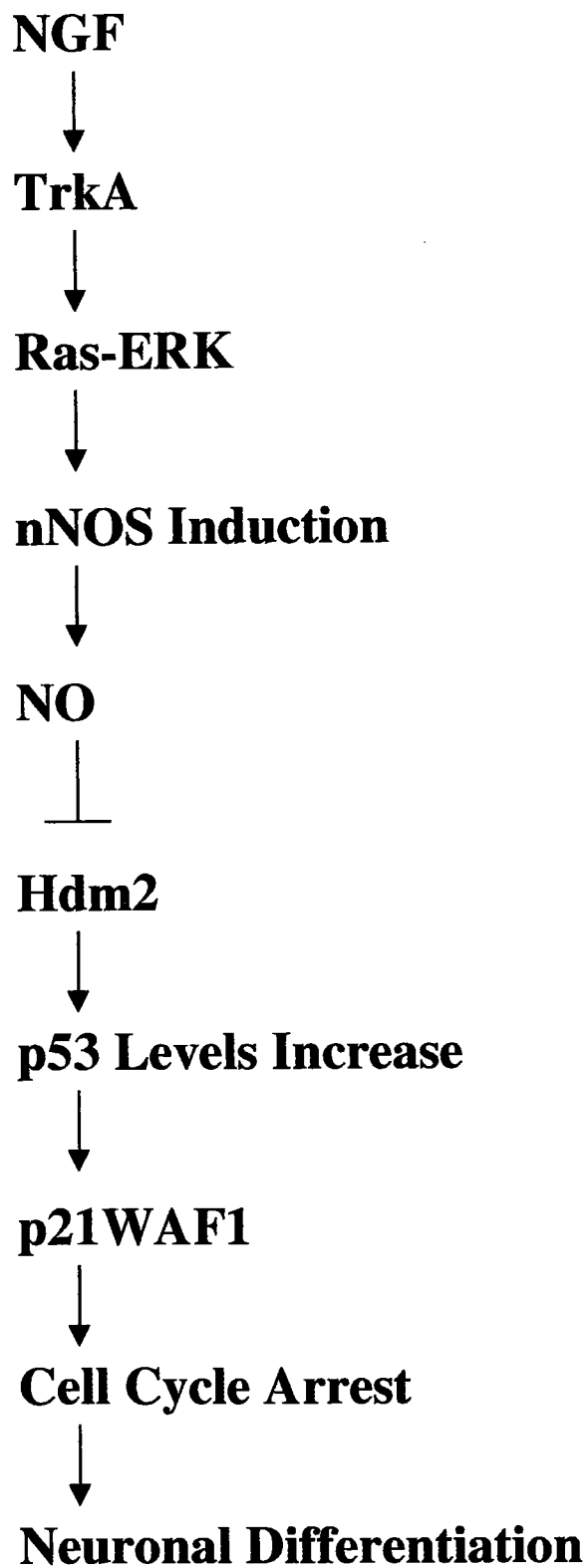


Figure 6-1. A signal transduction pathway important for the control of neuronal differentiation.

six days) in serum-containing conditions are consistent with the timing of the induction of total p53 levels seen by us (Chapter II) and others (129).

A significant amount of the data in this thesis relies on the specificity of various chemical inhibitors. Recently, a study has been published investigating the specificity and limitation of some of these drugs (198). The three kinase inhibitors used in this thesis, PD98059, U0126 and SB203580, are all extremely specific for their substrates. Nonetheless, in the case of PD98059 and U0126 we found varying results due to their different potencies (Chapter III). An effect on neurite extension but not NOS induction was seen by SB203580 (Chapter III). While the guanylate cyclase inhibitor LY83583 did not have an effect on the induction of a differentiation marker (Chapter IV), it was used at a concentration that has been used by others to block guanylate cyclase activity in PC12 cells (184). The use of chemical inhibitors has been a very important tool in biochemical research. However, an over-reliance on these drugs can lead to faulty conclusions. When available, other means should be used to bolster results obtained with chemical inhibitors. The use of a dominant negative expressing protein, as we did in Chapter III, is one convenient alternative to a chemical inhibitor. Finally, if no effect is seen with a chemical inhibitor, the ideal control is to assay the activity of the proposed substrate of the drug.

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