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Cell-Cycle Arrest in TrkA-expressing NIH3T3 Cells Involves Nitric Oxide Synthase

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Abstract We have examined nerve growth factor (NGF)-triggered signaling in two NIH3T3 cell lines exogenously expressing the NGF receptor, TrkA. TRK1 cells cease to proliferate and extend long processes in response to NGF, while E25 cells continue to proliferate in the presence of NGF. These two cell lines express similar levels of TrkA and respond to NGF with rapid elevation of mitogen-activated protein kinase (MAPK) activity. MAPK activation is slightly more sustained for E25 cells than for TRK1 cells, although sustained activation of MAPK has been suggested to cause cell-cycle arrest. As judged by NADPH-diaphorase staining, nitric oxide synthase (NOS) activity is increased in TRK1 cells upon exposure to NGF. In contrast, diaphorase staining in E25 cells is unaffected by NGF treatment. Immunocytochemistry shows that levels of the brain NOS (bNOS) isoform are increased in TRK1, but not E25, cells exposed to NGF. Furthermore, Western blots show that NGF elevated cyclin-dependent kinase inhibitor, $p21^{WAF1}$, in TRK1 cells only. NGF-induced $p21^{WAF1}$ expression, cell-cycle arrest and process extension are abolished by *N*-nitro-L-arginine methyl ester (L-NAME), a competitive inhibitor of NOS. The inactive enantiomer, D-NAME, did not inhibit these responses. Furthermore, even though E25 cells do not respond to NGF or nitric oxide donors, they do undergo a morphological change in response to NGF plus a nitric oxide donor. Therefore, NOS and $p21^{WAF1}$ are induced only in the TrkA-expressing NIH3T3 cell line that undergoes cell-cycle arrest and morphological changes in response to NGF. These results demonstrate that sustained activation of MAPK is not the sole determining factor for NGF-induced cell-cycle arrest and implicate NO in the cascade of events leading to NGF-induced morphological changes and cell-cycle arrest. J. Cell. Biochem. 81:193–204, 2001.

Key words: TrkA; nerve growth factor; nitric oxide synthase; signal transduction; proliferation; differentiation; $p21^{WAF1}$

Even though receptor tyrosine kinases (RTK) commonly activate many of the same signal transduction pathways (mitogen-activated protein kinase (MAPK), phospholipase Cγ, and phosphatidylinositol 3-kinase), RTKs can induce either proliferation or differentiation [Marshall, 1995]. Epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) are mitogenic for many cell types, while nerve growth factor (NGF) induces differentiation of neuronal cells, such as PC12 pheochromocytoma cells [Greene and Tischler, 1976]. In addition, the same RTK can induce either proliferation or differentiation depending on the cellular context [Jiang et al., 1997]. Fibroblast growth factor (FGF) stimulates proliferation in fibroblasts but induces differentiation of PC12 cells [Koizumi et al., 1988; Baetge and Hammang, 1991]. NGF stimulates differentiation of PC12 cells but can induce proliferation of glioma cells expressing exogenous TrkA, the NGF-activated RTK [Lachyankar et al., 1997]. NGF is mitogenic for breast cancer cells but not for normal breast epithelial cells [Descamps et al., 1998]. These biological responses are also influenced by levels of RTK expression. Overexpression of TrkA in PC12 cells results in a more rapid differentiation

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in response to NGF [Hempstead et al., 1992]. Overexpression of EGF receptor or insulin receptor in PC12 cells allows EGF or insulin, respectively, induced differentiation [Dikic et al., 1994; Traverse et al., 1994].

The MAPK pathway has been suggested to play a particularly important role in determining whether RTK signaling induces cell proliferation or cell-cycle arrest [Cowley et al., 1994; Marshall, 1995]. In PC12 cells, MAPK activation by NGF is more sustained compared with activation by EGF, and this sustained activation correlates with NGF-induced differentiation [Qiu and Green, 1992; Nguyen et al., 1993; Traverse et al., 1994; Obermeier et al., 1996]. For PC12 cells stimulated with NGF, MAPK activity peaks at about 5 min and is largely undiminished for at least 90 min (example of sustained activation) [Traverse et al., 1992]. With EGF treatment, MAPK activity peaks at 2 min and returns to basal levels by 60–90 min (example of transient activation). Overexpression of a constitutively active form of MAPK kinase (MEK) which is upstream of MAPK, is sufficient to induce PC12 differentiation [Cowley et al., 1994]. However, the sustained activation of MAPK proteins expressed at normal levels appears to be insufficient for neuronal differentiation [Vaillancourt et al., 1995]. These complex results led to the suggestion that both sustained MAPK activation and additional pathway(s) are required for NGF-induced differentiation [Kaplan and Miller, 1997].

Recently, a signal transduction pathway involving nitric oxide synthase (NOS) has been shown to play a role in cell-cycle arrest during development. The brain isoform of NOS (bNOS) is expressed transiently during neurogenesis in both the central and peripheral nervous systems [Bredt and Snyder, 1994; Dawson and Snyder, 1994; Ientile et al., 1996; Goureau et al., 1997]. NO acts as an anti-proliferative agent in *Drosophila* larvae. Inhibition of *Drosophila* NOS results in excessive cell proliferation in imaginal discs, and elevated NOS expression stunts development [Kuzin et al., 1996]. In rats, NGF enhances expression of bNOS by cholinergic neurons of the basal forebrain [Holtzman et al., 1996]. Induction of NOS is associated with differentiation and cell-cycle arrest in NGF-treated PC12 cells [Hirsch et al., 1993; Peunova and Enikolopov, 1995; Sheehy et al., 1997]. Two studies [Hirsch et al., 1993; Sheehy et al., 1997] reported that the brain (bNOS) isoform was induced by NGF, but a third study [Peunova and Enikolopov, 1995] reported that bNOS, endothelial NOS (eNOS) and inducible NOS (iNOS) were induced. Nitric oxide may influence survival of PC12 cells by activating ras [Teng et al., 1999]. This mechanism may also affect ischemic neurons [Gonzalez-Zulueta et al., 2000]. In addition, we [Poluha et al., 1997] have proposed that NO triggers cell-cycle arrest by induction of $p21^{WAF1}$, an inhibitor of cyclin-dependent kinases [Elledge et al., 1996]. Expression of exogenous $p21^{WAF1}$ in PC12 cells blocks cell proliferation [Poluha et al., 1997; Erhardt and Pittman, 1998]. $p21^{WAF1}$ is not expressed in the mitotic germinal layer of the olfactory epithelium but is expressed by olfactory neurons [Parker et al., 1995]. We have demonstrated that $p21^{WAF1}$ is required for differentiation of neuroblastoma cells [Poluha et al., 1996] and PC12 cells [Poluha et al., 1997]. Since continued expression of $p21^{WAF1}$ after differentiation is not required to maintain the differentiated phenotype of PC12 cells [van Grunsven et al., 1996; Erhardt and Pittman, 1998], it may be that $p21^{WAF1}$ plays a particularly important role during differentiation.

The goal of this study is to determine whether sustained MAPK activation, NOS induction, or both are required for NGF-induced cell-cycle arrest. To resolve this question, we have taken advantage of two NIH3T3 fibroblast lines expressing exogenous TrkA. TRK1 cells extend processes, express $p21^{WAF1}$ and cease to proliferate in response to NGF treatment [Decker, 1995]. In contrast, E25 cells continue to proliferate in the presence of NGF and do not display morphological changes or induction of $p21^{WAF1}$ [Cordon-Cardo et al., 1991]. Addition of NGF to quiescent E25 cells is mitogenic. We find that bNOS is induced in TRK1 cells undergoing NGF-induced cell-cycle arrest but not in E25 cells. MAPK activation for TRK1 cells is slightly less sustained than that for E25 cells. Even though E25 cells do not respond to NGF or a nitric oxide donor, they undergo a morphological change in response to NGF plus a nitric oxide donor. These results demonstrate that sustained activation of MAPK is not the sole determining factor for NGF-induced cell-cycle arrest and implicate NO as a second messenger for both NGF-induced morphological changes and cell-cycle arrest.

MATERIALS AND METHODS

Cell Culture

TrkA-expressing NIH3T3 cells were provided by Dr. Stuart Decker (Parke-Davis Pharmaceuticals) [Decker, 1995] and Dr. Mariano Barbacid (National Center for Oncological Investigation, Madrid, Spain) [Cordon-Cardo et al., 1991]. TrkA-expressing NIH3T3 cells from Decker (TRK1) and Barbacid (E25) were grown at 37°C under 5% CO_2 in Dulbecco's modified Eagle's media (DMEM, Gibco-BRL) with 100 μg/ml gentamycin, 2 mM glutamine and supplemented with 5% or 10% heat-inactivated newborn calf serum (Gibco-BRL), respectively. Antibiotic selection with either 50 U/ml hygromycin B (TRK1, Calbiochem) or 0.5 mg/ml G418 (E25, Calbiochem) was used to maintain expression of TrkA. Both TRK1 and E25 cells exhibited contact-inhibition of cell proliferation (data not shown), consistent with normal untransformed cell phenotypes. For experiments, 100 ng/ml of 2.5S NGF (Bioproducts for Science) was added to the cell culture media after allowing cells to attach. All results reported were obtained by treating cells with NGF in cell culture media with 5% (TRK1) or 10% heat-inactivated newborn calf serum (E25). However, control experiments indicated that treatment of E25 cells with NGF in 5% heat-inactivated newborn calf serum yielded similar results.

Diaphorase, Immunostaining, and BrdU Staining

NGF-induced NOS activity was assessed with NADPH-diaphorase staining, which closely correlates with NOS activity [Dawson et al., 1991]. Cells were plated in 35 mm dishes, washed twice with PBS and fixed for 20 minutes at room temperature with 4% paraformaldehyde in PBS. Staining was performed by incubating fixed cells in 1 mM NADPH, 0.5 mM nitro blue tetrazolium, 0.2% Triton X-100, 50 mM Tris–HCl buffer (pH 8.0) at 37°C for 30–60 minutes. Cells were washed with PBS prior to storage and photography.

Immunostaining was used to assess TrkA expression levels as well as to detect NOS isoforms. Cells were fixed in 4% paraformaldehyde in PBS, followed by additional fixation in MeOH at −20°C for 20 min. Samples were dried, then blocked with 1% BSA in PBS overnight at 4°C. TrkA expression was characterized by incubating cells for 60 min in PBS with 0.5% BSA and a monoclonal antibody (TA-1; ascites 1:200) made in our laboratory [Ross et al., 1996], followed by a fluorescein-conjugated secondary antibody. NOS isotype expression was characterized with monoclonal antibodies (1 μg/ml) to iNOS (Transduction Laboratories, Catalog No. N32020) and eNOS (Catalog No. N30020), and rabbit antibody (1 μg/ml) to bNOS (Catalog No. N31030), followed by fluorescein- or rhodamine-conjugated secondary antibodies against mouse or rabbit antibody, respectively. Samples were mounted in Citifluor (Ted Pella, Inc.) and viewed with a Zeiss Axioskop microscope with a 25 × objective using bright field (for diaphorase staining) and fluorescence (for immunostaining) optics. Cell proliferation was assessed by bromodeoxyuridine (BrdU) labeling, as previously described [LoPresti et al., 1992]. The samples were mounted in a DAPI-containing solution to facilitate counting of nuclei.

Flow Cytometry

Flow cytometry was used to quantify TrkA expression levels in TRK1 and E25 NIH3T3 cells. Cells were harvested from 25 cm^2 tissue culture flasks with 1 mM EDTA in Hank's buffered saline lacking divalent cations, fixed with 4% paraformaldehyde (in PBS) in suspension, blocked with 1% BSA in PBS, and then stained with TA-1 monoclonal antibody for 1 h. Cells were washed with PBS, and incubated with fluorescein-conjugated secondary antibodies for 30 min. Cells were washed, resuspended in PBS and filtered through a 35 μm cell strainer cap (Falcon) before flow cytometry was conducted with a MoFlo Flow Cytometer (Cytomation) at the Cancer Center of the University of Massachusetts Medical Center.

Western Blots and MAPK Activation

NGF induction of bNOS and p21^{WAF1} was characterized for TRK1 and E25 cells. Cells were plated on 25 cm^2 tissue culture flasks and treated with 100 ng/ml NGF. Control and treated cells (4×10^5 cells per sample) were harvested, boiled under reducing conditions in SDS-PAGE sample buffer, and subjected to electrophoresis on a 7.5% (bNOS) or 12.5% (p21^{WAF1}) polyacrylamide gel. Western blots were obtained by electrotransfer of separated proteins to an Immobilon-P membrane (Millipore). Even loading was assessed by Coomassie Blue staining of residual proteins in the gel or

by staining membrane-bound proteins with 0.1% India ink in Tris–buffered saline plus 0.1% Tween-20 (TTBS). Membranes were blocked for 1 h in 5% powdered milk in TTBS and then incubated with monoclonal anti-bNOS antibody NOS-B1 (1:3,000, Sigma) or 1 μg/ml rabbit anti-$p21^{WAF1}$ antibody (Santa Cruz Biotechnology) followed by HRP-conjugated secondary antibody (Amersham). Western blots were visualized with a chemiluminescence detection reagent prepared as described [Matthews et al., 1985] or purchased from New England Nuclear.

Activation of MAPK was determined by Western blotting using antibodies (New England Biolabs) specific for phosphorylated, activated MAPK (p42 and p44 forms) as well as by phosphorylation of myelin basic protein (MBP). Protein loads were normalized with Bradford protein assays (Bio-Rad). Activated MAPK was detected with 1:1,000 dilution of primary antibody, followed by HRP-conjugated secondary antibody and chemiluminescence detection.

For MAPK assays, TRK1 and E25 cells were plated on 25 cm^2 tissue culture flasks and treated with NGF for the indicated times. Cells were harvested by extraction at 4°C with 1 ml Lysis Buffer (20 mM Tris (pH 7.4), 137 mM NaCl, 2 mM EDTA, 1 mM Na_3VO_4, 1% Triton X-100, 1 mM PMSF, 5 μg/ml aprotinin, and 0.5 mM DTT) on a rocker table for 10 min. Lysates were transferred to microfuge tubes and centrifuged for 15 min at 16,000*g* in a microcentrifuge. Protein concentrations were adjusted after Bradford protein assays (Bio-Rad) to ensure equal loading of lysates. To immunoprecipitate ERK2, 10 μl Protein-A agarose beads (Sigma) were pre-equilibrated with 2 μl anti-ERK2 antiserum (Santa Cruz Biotechnology) in 500 μl of Lysis Buffer, combined with sample extracts and rotated for 2 h at 4°C. Samples were centrifuged, washed three times with Lysis Buffer, once with Kinase Assay Buffer (25 mM HEPES (pH 7.4), 25 mM $MgCl_2$, 0.1 mM Na_3VO_4, and 0.5 mM DTT), and resuspended in 50 μl Kinase Assay Buffer. Ten microliter of this sample was added to 45 μl Kinase Assay Buffer, 5 μl MBP (10 μg) and reactions initiated by addition of 0.5 μl ^{32}P-ATP (5 μCi). Samples were incubated at room temperature for 15 min, and reactions were stopped by boiling in 120 μl SDS sample buffer for 5 min. Phosphorylated proteins were separated on a 10% SDS polyacrylamide gel, and autoradiograms were obtained after drying the gel and exposing films for 8 to 24 h.

RESULTS

As a first step to comparing the two TrkA-expressing NIH3T3 lines, we treated TRK1 and E25 cells with NGF. Consistent with earlier studies, we found that E25 cells exposed to NGF, continued to proliferate [Cordon-Cardo et al., 1991; Jing et al., 1992]. In contrast, TRK1 cells ceased to proliferate within 36–48 h when exposed to NGF, and cells became flattened and extended long processes [Decker, 1995]. We have used these TrkA-expressing NIH3T3 cell lines to delineate the mechanisms by which TrkA signaling triggers proliferation or cell-cycle arrest.

To determine if TrkA expression levels were similar in TRK1 and E25 cells, we stained cells with TA-1, a monoclonal antibody that recognizes TrkA [Ross et al., 1996]. TRK1 and E25 cells exhibited similar immunofluorescence for TrkA (not shown). Further support of these results was obtained with flow cytometry (Fig. 1), demonstrating that TRK1 and E25 cells express similar levels of TrkA.

NGF Induces MAPK Activity in Both Cell Lines

We next analyzed activation of MAPK in these cell lines. By MBP phosphorylation, MAPK was rapidly activated in both TRK1 and E25 cells (Fig. 2), indicating that both of these cell lines were competent for NGF

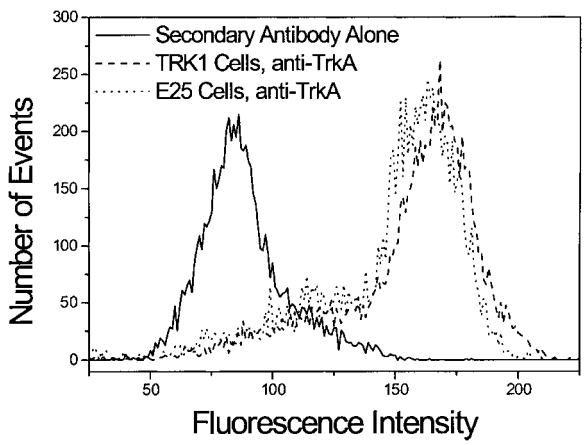

Fig. 1. TrkA receptor levels are similar for E25 and TRK1 lines. TRK1 and E25 cells were analyzed by flow cytometry (n = 2). TRK1 cells show specific immunofluorescence that is similar in magnitude to that observed for E25 cells.

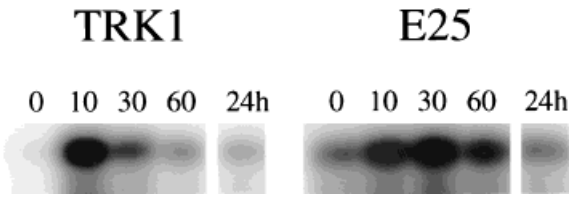

Fig. 2. MAPK in both TRK1 and E25 lines is activated by NGF. MAPK activity was assessed by phosphorylation of MBP after 10, 30, 60 min and 24 h of NGF treatment as described in the Materials and Methods. The autoradiogram shown is from a representative experiment (n = 3).

activation of TrkA and subsequent, downstream activation of MAPK. These findings indicated that activation of MAPK occurred similarly in TRK1 and E25 cells. Similar results (n = 2, not shown) were obtained using antibodies specific for phosphorylated MAPK (see Methods for details). Downregulation of MAPK in E25 cells was slightly slower than that in TRK1 cells.

NOS is Induced in TRK1 but not in E25 Cells

To evaluate the relationship between cell cycle arrest and NOS expression, we utilized NADPH-diaphorase staining, which allows visualization of NOS activity. As judged by diaphorase staining, NGF induced NOS activity in TRK1 cells, but had no effect on NOS activity in E25 cells (Fig. 3). Both parental NIH3T3 cell lines used to derive these TrkA-expressing clones did not exhibit increased NADPH-diaphorase staining after exposure to NGF (data not shown). Greater than 75% of the TRK1 cells showed NADPH-diaphorase activity within 24 h of exposure to NGF (Fig. 3B), while greater than 90% of the cells stained for NOS activity after 72 h (Fig. 3D). Onset of NADPH-diaphorase activity was observed after 6–12 h of NGF treatment (not shown). Hence, in TRK1 cells, induction of NOS activity correlated with cell-cycle arrest.

Identification of NOS Isotypes Induced by NGF

We used immunofluorescence to study the effects of NGF on iNOS, bNOS, and eNOS expression in TRK1 and E25 cells. Untreated TRK1 cells exhibited slight, but specific immunoreactivity for iNOS, bNOS, and eNOS (bNOS in Fig. 4, iNOS and eNOS not shown). TRK1 cells exposed to NGF for 2 days exhibited increased immunofluorescence for bNOS. No clear, consistent changes in the levels of iNOS

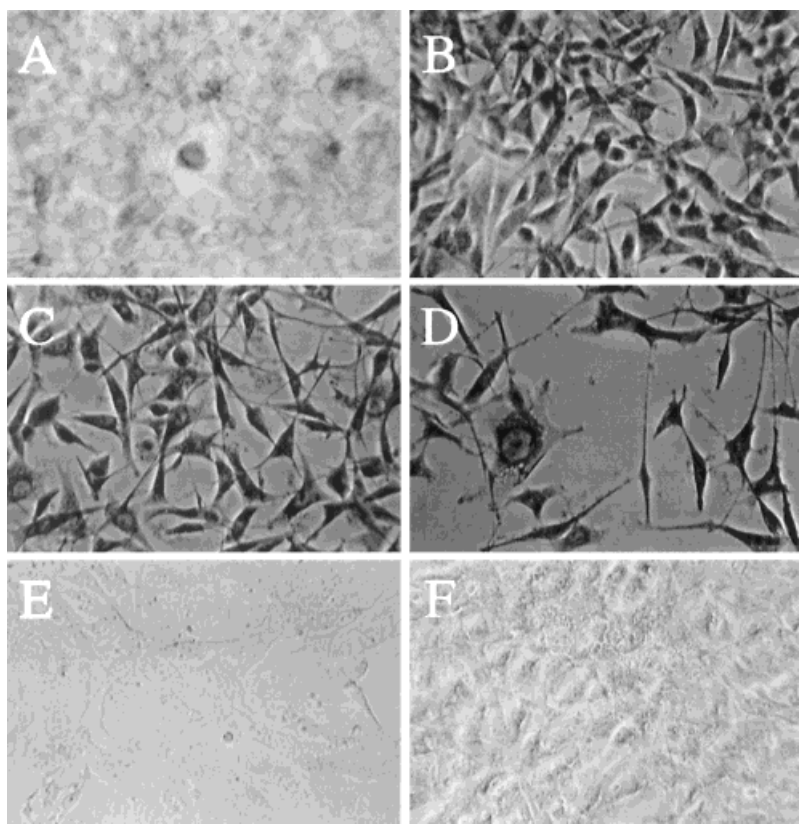

Fig. 3. NOS activity is elevated in NIH3T3 TrkA-expressing cells that undergo cell cycle arrest in response to NGF. TRK1 and E25 cells were stained for NADPH diaphorase as described in Materials and Methods. Staining is representative of the results observed for TRK1 (n = 12) and E25 (n = 6) cells. Parental cell lines used to derive these TrkA expressing NIH3T3 clones did not exhibit any NGF-induced increase in NADPH diaphorase staining (data not shown). TRK1 cells untreated (**A**) or exposed to 100 ng/ml of NGF for 24 (**B**), 48 (**C**), or 72 (**D**) hrs prior to NADPH diaphorase staining. E25 cells untreated (**E**) or treated with 100 ng/ml of NGF for 48 h (**F**).

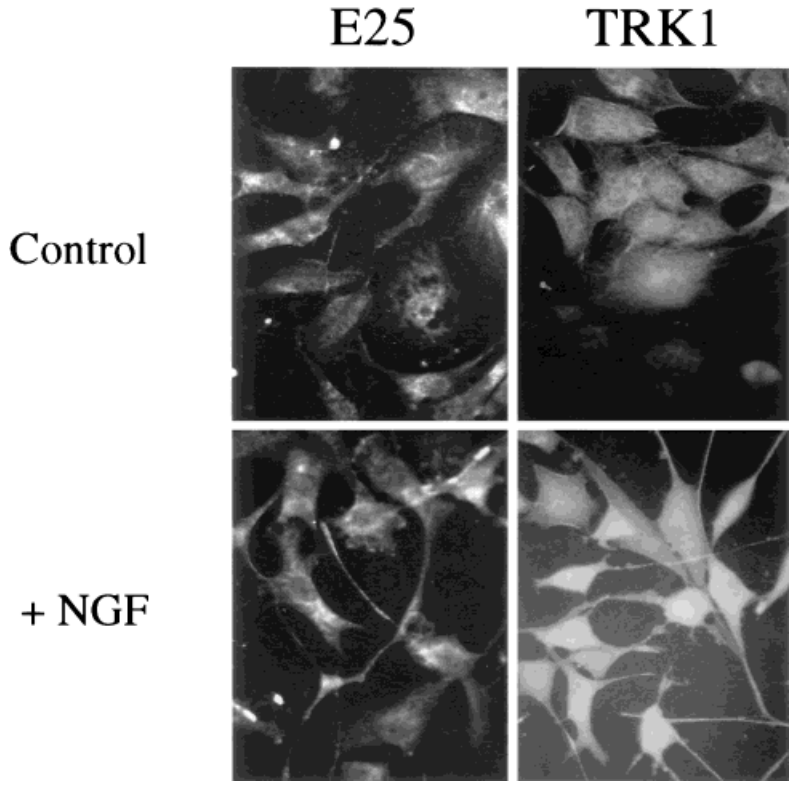

Fig. 4. Identification of NOS isoform induced by treatment with NGF for 48 h. TRK1 cells were immunostained for bNOS as described in Materials and Methods. Untreated cells (top row) show weak, but specific, staining for bNOS and NGF treatment (bottom row) increases staining intensity for TRK1 cells but not for E25 cells. TRK1 and E25 cells exposed to secondary antibody alone showed no appreciable immunostaining (data not shown).

and eNOS were observed (not shown). Both untreated and NGF-treated E25 cells showed barely detectable immunostaining for bNOS, iNOS and eNOS. Cells exposed to secondary antibody alone did not exhibit significant immunofluorescence (data not shown). These results indicate that E25 cells express lower levels of NOS than TRK1 cells. bNOS induction correlates with NGF-induced cell-cycle arrest and may play an important role in signal transduction.

NOS-Dependent Induction of Process Extension, Cell-Cycle Arrest and $p21^{WAF1}$ in TRK1 but not in E25 Cells

We next tested whether NOS activity is required for the effects of NGF on these lines. TRK1, but not E25, cells extended long processes ($\geq$3 cell diameters) in response to NGF (Fig. 5A,B). Extension of processes was evident after about 24 h of NGF treatment and became more pronounced in the subsequent 48 h. L-NAME, a specific NOS inhibitor, inhibited this response ($P \leq 0.01$, Bonferroni Multiple Comparisons Test). There was no significant effect of D-NAME, an inactive enantiomer. In addition, NGF blocked TRK1 cell proliferation as judged by BrdU labeling (Figs. 5C,E and 6). L-NAME, but not D-NAME, blocked NGF-induced cell-cycle arrest. NGF, L-NAME, and D-NAME had little or no effect on BrdU labeling of E25 cells. About one-half of TRK1 cells were cell-cycle arrested after 30–35 h of NGF treatment, and arrest was essentially complete after 40–50 h (Fig. 5E).

Since $p21^{WAF1}$ expression is induced in cells that cell-cycle arrest in response to NGF [Decker, 1995; Yan and Ziff, 1995, 1997; Billon et al., 1996; van Grunsven et al., 1996; Gollapudi and Neet, 1997; Poluha et al., 1997], we studied the effects of NGF on the expression of $p21^{WAF1}$ in TRK1 and E25 cells. Figure 7 shows that treatment of E25 cells with 100 ng/ml NGF for three days did not induce expression of $p21^{WAF1}$, as characterized by Western blots. Furthermore, addition of 20 mM L-NAME or D-NAME in the presence of NGF had no affect on $p21^{WAF1}$ expression in E25 cells. In contrast, 100 ng/ml of NGF induced $p21^{WAF1}$ levels in TRK1 cells by 3–5 fold; the induction of $p21^{WAF1}$ was blocked by L-NAME but not by D-NAME. The $p21^{WAF1}$ appeared on the blot as a doublet, probably due to partial C-terminal proteolysis [Poon and Hunter, 1998].

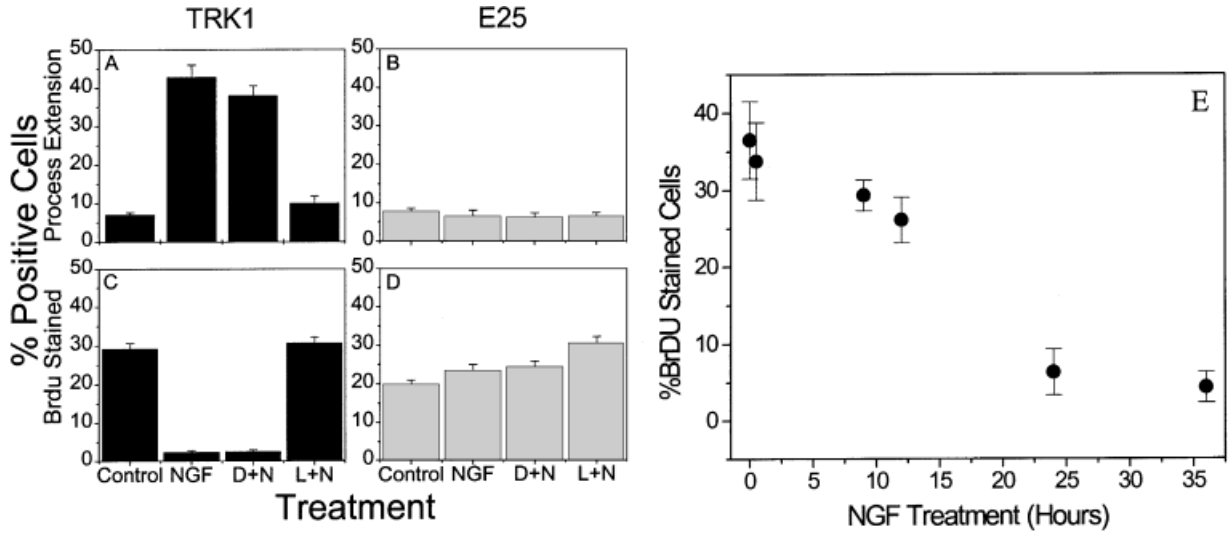

Fig. 5. L-NAME-sensitive process extension and cell-cycle arrest for TRK1 cells but not E25 cells. For 2 days, cells were left untreated (control) or treated with 100 ng/ml of NGF, NGF+10 mM D-NAME (D+N), or NGF+10 mM L-NAME (L+N). Process extension (>3 cell diameters) for TRK1 cells (panel **A**, n = 15) and E25 cells (**B**, n = 9) was measured. Proliferation of TRK1 cells (**C**, n = 18) and E25 cells (**D**, n = 18) was measured by BrdU incorporation. (**E**) The time course of NGF-stimulated cell-cycle arrest in TRK1 cells was determined by BrdU incorporation. TRK1 cells were treated with NGF, followed by incubation with BrdU for 18 h. Except for the 0 h point, NGF was included during BrdU labeling. The total time in the presence of NGF is reported. The average % of BrdU stained cells ± S.D. (4 experiments) is plotted.

These findings indicate that each of the effects of NGF on TRK1 cells—process elongation, cell-cycle arrest and elevation of p21^{WAF1} levels—are dependent on NOS activity.

The time course of bNOS and p21^{WAF1} induction was assessed by Western blotting (Fig. 8). Induction of both bNOS and p21^{WAF1} clearly preceded cell-cycle arrest (Fig. 5E). bNOS was induced in 6–12 h and then partially decreased. p21^{WAF1} was induced in 12–24 h. These data support the proposed sequence of events in the NGF-NOS-p21^{WAF1} pathway [Poluha et al., 1997].

Effects of NO Donor on TRK1 and E25 Cells

To determine if the NO donor NOC-18 (DETA NONOate, 2,2′-(hydroxynitrosohydrazino)*bis*-ethanamine) can reverse L-NAME inhibition of NGF-induced process formation, PC12, TRK1, and E25 cells were treated with 100 μM NOC-18. NOC-18 alone had no apparent effect on any of the cell lines tested (Fig. 9). For PC12 and TRK1 cells, addition of NOC-18 resulted in reversal of L-NAME inhibition of NGF-induced process formation. About 4% of the PC12 cells treated with NGF+L-NAME extended processes, while the addition of NOC-18 increased this to about 55% (Fig. 9A). In TRK1 cells, addition of NOC-18 increased the fraction of cells extending processes from about 8% (in NGF+L-NAME treated cells) to about 50% (Fig. 9B). Furthermore, we observed that addition of NOC-18 to E25 cells treated with NGF+L-NAME or NGF+D-NAME resulted in about 31% and 63% of cells extending processes, respectively. Note that for E25 cells exposed only to NGF, we observed around 4% of cells extending processes, which did not differ significantly from untreated cells (3%). These results demonstrate that inhibition of process extension by L-NAME is due to its inhibition of NOS and that the lack of responsiveness of E25 cells is at least partly due to the lack of NO production.

To further characterize the effects of NOC-18 on E25 cells, we determined dose-response curves in the presence and absence of NGF. In the absence of NGF, NOC-18 did not induce process formation at any dose tested (data not shown). When NOC-18 was added with 4 nM NGF, we observed increased process formation for E25 cells (Fig. 10). The EC50 for this response was 51 ± 16 μM NOC-18, with a maximum of about 67 ± 7% of cells forming processes. The NO donor NOC-18 is commonly used in this concentration range [Clementi et al., 1998]. We also determined NGF dose-response curves for TRK1 process extension in

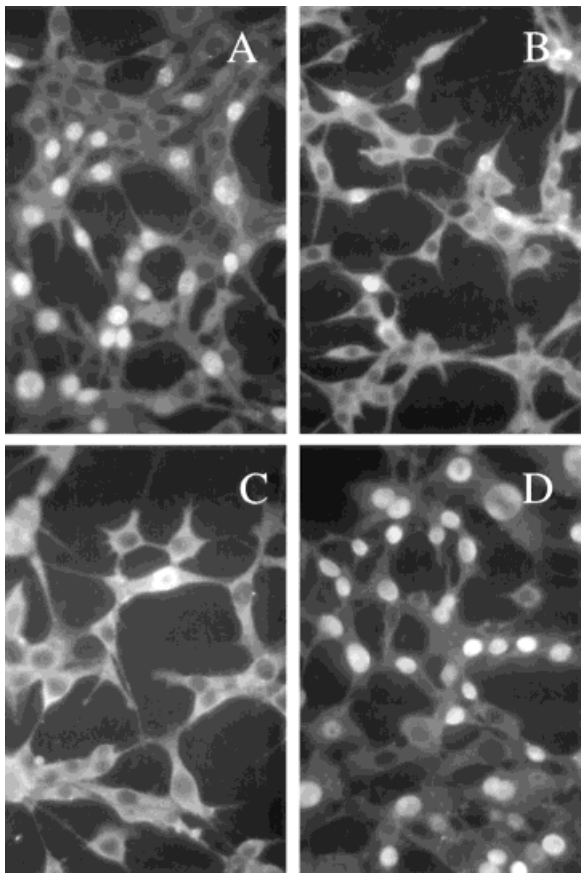

Fig. 6. Cell-cycle arrest of TRK1 cells by NGF is inhibited by the NOS inhibitor, L-NAME. Proliferation of TRK1 cells was measured by BrdU labeling after treatment for 2 days. (**A**) No treatment. (**B**) 100 ng/ml NGF. (**C**) 100 ng/ml NGF and 10 mM D-NAME. (**D**) 100 ng/ml NGF and 10 mM L-NAME.

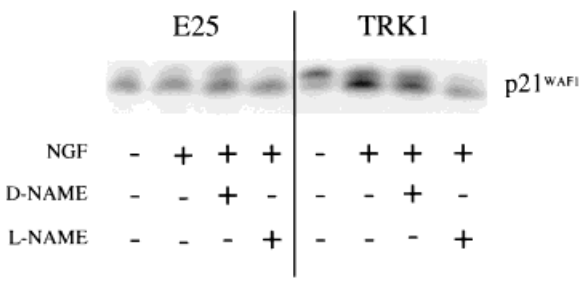

Fig. 7. Expression of $p21^{WAF1}$ is increased in TrkA-expressing NIH3T3 cells that cell-cycle arrest in response to NGF treatment. TRK1 and E25 cells were untreated, treated with 100 ng/ml NGF alone, or exposed to 20 mM D-NAME or 20 mM L-NAME for 3 days in the presence of NGF. Levels of $p21^{WAF1}$ expression were assessed by Western blotting as described in the Materials and Methods. TRK1 cells exhibited an increase in $p21^{WAF1}$ expression that is inhibited by L-NAME but is not affected by D-NAME. $p21^{WAF1}$ expression was not affected by experimental treatments of E25 cells.

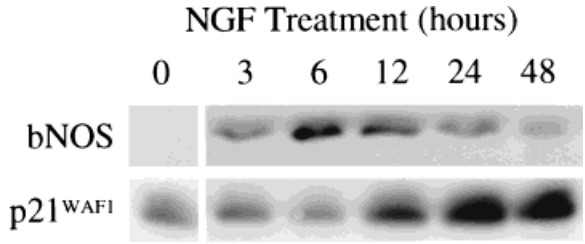

Fig. 8. The time course of NGF-induced bNOS and $p21^{WAF1}$ expression was measured by Western blotting. TRK1 cells were untreated, or treated with NGF for 3, 6, 12, 24, or 48 h. Samples were extracted in SDS sample buffer, normalized to ensure equal loading, and analyzed on 7.5% (bNOS) or 12.5% ($p21^{WAF1}$) polyacrylamide gels. The data are from the same experiment and autoradiogram film.

the presence and absence of 100 μM NOC-18 (data not shown). NOC-18 did not affect these dose-response curves ($n = 2$) and, therefore, does not increase the potency of NGF on TRK1 cells, perhaps, because the nNOS induced by NGF is already sufficient for process extension.

DISCUSSION

In this study, we examine NGF-triggered signaling in two TrkA-expressing NIH3T3 cell lines. These independently derived cell lines differ in their responses to NGF. Since the lines do not express the p75 NGF receptor, these responses are initiated by the TrkA receptor. NGF treatment of TRK1 cells induces NOS, $p21^{WAF1}$, process extension and cell-cycle arrest. Cell-cycle arrest, $p21^{WAF1}$ induction and process extension are sensitive to L-NAME and, therefore, are dependent on NOS activity. In contrast, NGF treatment of E25 cells does not cause cell-cycle arrest and induces neither NOS nor $p21^{WAF1}$. E25 cells treated with NGF and the NO donor NOC-18 undergo a morphological change. These lines offer a unique system for examination of TrkA signaling and provide further evidence for the role of a TrkA-NOS-$p21^{WAF1}$ signal transduction pathway in NGF-induced cell-cycle arrest. Induction of bNOS appears to be the critical signaling event that distinguishes NGF responses in these two lines.

There are a number of possible explanations why bNOS induction varies between these two NIH3T3 lines. NIH3T3 clones are known to be heterogenous [Jainchill et al., 1969], although we have not noted any obvious differences in morphology or growth rate between the two

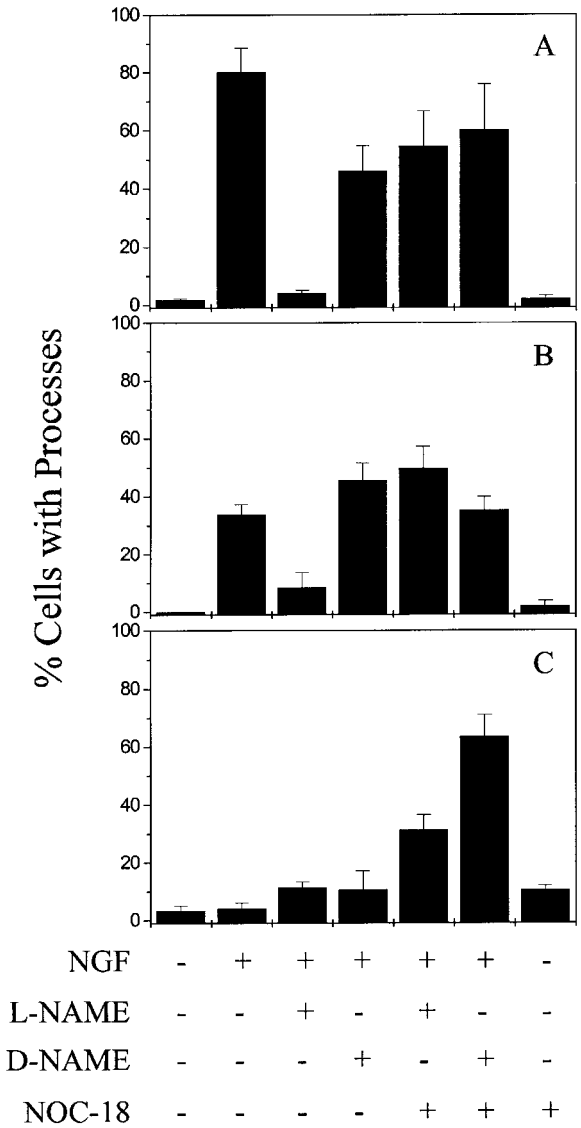

Fig. 9. Reversal of L-NAME inhibition. PC12 (**A**), TRK1 (**B**), and E25 (**C**) cells were treated with 100 μM NOC-18, 100 ng/ml of NGF, and 10 mM L-NAME or D-NAME. After 3 days, process formation was scored as described in Materials and Methods. The data shown are from representative experiments for PC12 (n = 3), TRK1 (n = 5) and E25 (n = 4) cells.

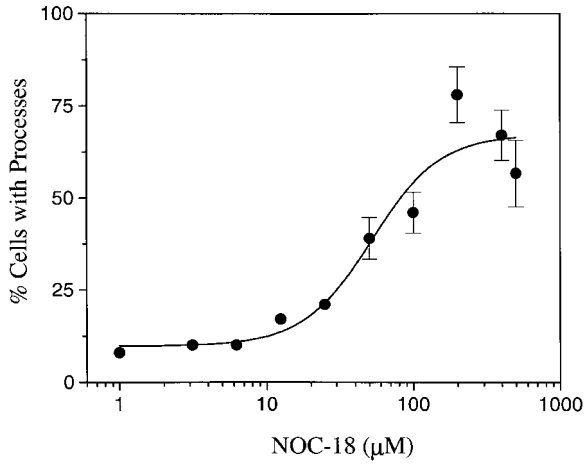

Fig. 10. NOC-18 dose-response curve for E25 cells. E25 cells were exposed to increasing concentrations of NOC-18 in the presence of 4 nM NGF for 3 days, and process formation was scored as described in Materials and Methods. The data shown are from a representative experiment (n = 4). Results of non-linear curve fitting are presented in the text.

parental lines. Alternatively, this difference in NGF responsiveness may relate to selection and passaging of these lines. NIH3T3 lines are "plastic" and adapt to different culture conditions [Rubin and Xu, 1989]. Three other possible explanations can be ruled out. First, the differences between these two lines is not due to a difference in expression levels of TrkA. Flow cytometry measurements showed very similar levels. Second, the difference is not due to expression of the other NGF receptor, p75, since p75 is not expressed by either of these lines. Third, the difference is not due to insertion of the TrkA expression vector into a gene critical for this response, e.g., bNOS gene. Both E25 and TRK1 are representative of multiple independent clones and, hence, are not dependent on any single insertion site. In addition, the TRK1 line was derived with an episomal vector. Although we do not know the primary reason for this difference in bNOS induction, these lines, nonetheless, provide a system for analyzing how TrkA produces radically different responses in similar cellular environments.

NGF activates MAPK for both E25 and TRK1. Furthermore, a MEK inhibitor blocks NGF-induced cell-cycle arrest of TRK1 cells [Pumiglia and Decker, 1997], demonstrating a requirement for MAPK activation. However, activation of MAPK for E25 cells was slightly more sustained than that for TRK1 cells, even though sustained MAPK activation has been associated with cell cycle arrest [Traverse et al., 1992, 1994; Qiu and Green, 1992; Nguyen et al., 1993; Obermeier et al., 1996]. Hence, sustained activation of MAPK is not the sole determining factor for cell-cycle arrest. One possible target of the MAPK pathway might be induction of NOS. It has previously been reported that MAPK is a critical pathway for induction of iNOS [Singh et al., 1996], but its effect on bNOS expression has not been reported.

NO may act through a number of mechanisms to mediate the effects of NGF on TRK1 cells. We have proposed an NGF-triggered signal transduction pathway in which NO activates the $p21^{WAF1}$ promoter, increases $p21^{WAF1}$ protein levels and, thereby, induces cell-cycle arrest [Poluha et al., 1997]. It is likely that this mechanism occurs in TRK1 cells since the NOS inhibitor, L-NAME, blocks $p21^{WAF1}$ induction. The cytoskeleton is another possible target for NO, consistent with NGF-induced morphological changes in TRK1 cells. NGF treatment of TRK1 cells, but not E25 cells, induced process elongation, and this response was blocked by L-NAME. NO has been suggested to influence the cytoskeleton by several mechanisms. Even very low doses of NO lead to ADP-ribosylation of actin [Clancy et al., 1993], which can affect actin polymerization [Clancy et al., 1995]. Some integrin-induced rearrangments of the cytoskeleton are blocked by NO-induced increases in cGMP [Clancy et al., 1997]. Further studies are required to distinguish among these possibilities.

In this study, we have analyzed the sequence of events leading to cell-cycle arrest of TRK1 cells. The most rapid events measured in this study are TrkA autophosphorylation and MAPK activation (15 min of NGF treatment). Induction of bNOS (6–12 h) and $p21^{WAF1}$ (12–24 h) proteins is slower with bNOS induction slightly preceding that of $p21^{WAF1}$. Diaphorase staining, indicating NOS activity, is also evident after 6–12 h of NGF treatment. Process extension is extensive by 24 h and complete by 72 h. Cell-cycle arrest is one-half maximal after 36 h and complete by 72 h. This sequence of events, combined with the sensitivity of $p21^{WAF1}$ induction, process extension, and cell-cycle arrest to L-NAME, strongly supports our proposed NGF-NOS-$p21^{WAF1}$ pathway.

This study adds to the increasingly strong evidence for a role of NOS in cell-cycle arrest during development. bNOS is expressed in regions of the PNS and CNS undergoing neurogenesis [Bredt and Snyder, 1994]. There also is a requirement for NO in NGF-induced cell-cycle arrest of PC12 cells and *Drosophila* cells in imaginal discs [Peunova and Enikolopov, 1995; Kuzin et al., 1996]. For PC12 cells, we proposed an NGF-activated pathway involving NOS, p53, and $p21^{WAF1}$ [Poluha et al., 1997]. For both TRK1 and a neuroblastoma cell line [LoPresti et al., 1992] [Poluha et al., unpublished], similar pathways are required for cell-cycle arrest. Although many details of this pathway remain to be elucidated, these data demonstrate that this pathway is not peculiar to any single cell culture model, rather it is a general mechanism for initiating cell-cycle arrest.

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