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# Antiproliferative effect of nitric oxide on epidermal growth factor-responsive human neuroblastoma cells

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**Abstract**

Addition of nitric oxide (NO) donors to NB69 neuroblastoma cells produced a cGMP-independent decrease in cell proliferation, without affecting cell viability or apoptosis. The potency of short half-life NO donors was higher when cell proliferation was stimulated by epidermal growth factor (EGF), as compared with cultures exposed to fetal calf serum (FCS). Immunoprecipitation and western blot analysis of the EGF receptor (EGFR) revealed a significant reduction of its EGF-induced tyrosine phosphorylation in cells treated with the NO donor 2-(*N*,*N*-diethylamino)-diazenolate-2-oxide (DEA-NO). When total cell lysates were subjected to western blotting, we observed that DEA-NO also reduced tyrosine phosphorylation in EGF-activated phosphoproteins, but not in those proteins whose tyrosine phosphorylation was evident in the absence of EGF. The effect of NO on EGFR transphosphorylation was concentration-dependent and transient, with a total recovery observed between 1.5 and 3 h after addition of DEA-NO to the cells. When cells were incubated for 15 min with DEA-NO and then washed, the EGFR transphosphorylation returned to control levels immediately, indicating that the interaction of NO with the receptor molecule was fully reversible. NB69 cells expressed both the neuronal and the inducible isoforms of NO synthase (NOS) when cultured in the presence of FCS; under this condition, the NOS inhibitor, $N^{\omega}$-nitro-L-arginine methyl ester, produced a small but significant increase in cell proliferation. The results suggest that NO is an endogenous antimitotic agent and that its interaction with EGFR contributes to cytostasis in NB69 cells.

**Keywords:** cell proliferation, DNA synthesis, NB69 cell line, neuroblasts, nitric oxide donors, nitric oxide synthase.

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Nitric oxide (NO) is a strong inhibitor of cell proliferation in many cell types. Both cGMP-dependent and cGMP-independent mechanisms seem to be involved in NO-mediated cytostasis (Garg and Hassid 1989, 1990). Several proteins and molecular pathways have been proposed to directly or indirectly account for the NO antiproliferative effect. Among these, ribonucleotide reductase (Lepoivre *et al.* 1994), ornithine decarboxylase (Buga *et al*. 1998; Bauer *et al*. 2001) and the epidermal growth factor (EGF) receptor (EGFR; Estrada *et al.* 1997) are inhibited by NO, whereas p38 mitogen-activated protein kinase (Browning *et al*. 1999), and the p53 (Ho *et al*. 1996; Gu *et al*. 2000; Nakaya *et al*. 2000) and retinoblastoma pathways (Kuzin *et al*. 2000) are activated.

A role for NO in neural cell differentiation has also been proposed. Several studies using murine and human neuroblastoma, as well as PC12 cell lines, have demonstrated that these cells express different isoforms of NO synthase (NOS) when their differentiation to a neuronal phenotype is induced by certain cytokines or neurotrophic factors (Peunova and

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*Abbreviations used*: BSA, bovine serum albumin; DETA-NO, (*Z*)-1-[2-(2-aminoethyl)-*N*-(2-ammonioethyl)amino]diazen-1-ium-1,2-diolate]; DEA-NO, 2-(*N*,*N*-diethylamino)-diazenolate-2-oxide; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethylsulphoxide; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; FCS, fetal calf serum; L-NAME, $N^{\omega}$-nitro-L-arginine methyl ester; NO, nitric oxide; NOS, nitric oxide synthase; ODQ, 1-*H*-oxodiazol-[1,2,4]-[4,3] quinoxaline-1-one; PBS, phosphate-buffered saline; PI, propidium iodide; SDS–PAGE, sodium dodecyl sulphate–polyacrylamide gel electrophoresis; SNAP, *S*-nitroso-*N*-acetylpenicillamine; TBS, tris-buffered saline; TCA, trichloroacetic acid; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end DNA labelling.

 

Enikolopov 1995; Ogura and Esumi 1996; Obregon *et al*. 1997; Phung *et al*. 1999); the NOS-derived NO triggers a growth arrest that allows the subsequent action of the differentiating agents. These findings correlate well with recent studies in *Drosophila* and *Xenopus* embryos, showing a role for NO in development of the nervous system (Kuzin *et al*. 1996; Peunova *et al*. 2001). A similar mechanism probably operates during neurogenesis in the mammalian brain, either during development, when transient expression of NOS occurs in different brain areas (Bredt and Snyder 1994; Giuili *et al*. 1994; Keilhoff *et al*. 1996), or in the remaining neurogenic regions in the adult, where nitrergic neurones provide a NO source in the proximity of neural precursors (Moreno-López *et al*. 2000).

The control exerted by NO on the balance between proliferation and differentiation in normal and tumoral cells of neural origin is probably mediated through its concomitant interaction with different molecules, among which the inhibition of growth factor receptors could be one of the earliest events, as it would directly stop the mitogenic signals coming from the extracellular matrix. In this context, the EGFR is of special interest because neural precursors in the developing and adult brain are highly responsive to the proliferative action of EGF, both *in vivo* (Craig *et al*. 1996; Khun *et al*. 1997) and *in vitro* (Reynolds and Weiss 1992; Gritti *et al*. 1999).

The EGFR is a plasma membrane tyrosine kinase that belongs to the ErbB family of receptors. These molecules remain as monomers until they are activated by a specific ligand that induces homodimerization or heterodimerization among themselves, and this triggers the activation of their intrinsic tyrosine kinases and their cross-phosphorylation of tyrosine residues (Weiss and Schlessinger 1998). In a previous work using permeabilized EGFR-T17 fibroblasts, a transfected murine cell line which overexpresses the human EGFR, we showed that NO donors inhibited EGF-induced EGFR transphosphorylation without affecting the binding of the ligand to the receptor (Estrada *et al*. 1997).

We show now that exogenous and endogenously produced NO can decrease cell proliferation in a neuronal tumoral cell line (NB69) that naturally expresses the EGFR, and that NO inhibits the mitogenic signal initiated by EGF.

## Materials and methods

### Materials

The following products were used: [*methyl*-$^3$H]thymidine and EcoLite(+)™ liquid scintillation cocktail from ICN Pharmaceuticals (Costa Mesa, CA); *S*-nitroso-*N*-acetylpenicillamine (SNAP) (*Z*)-1-[2-(2-aminoethyl)-*N*-(2-ammonioethyl)amino]diazen-1-ium-1,2-diolate] (DETA-NO) and 2-(*N*,*N*-diethylamino)-diazenolate-2-oxide (DEA-NO) from Sigma or RBI (Natick, MA, USA) (Sigma-Aldrich, St. Louis, MO, USA); 1-H-oxodiazol-[1,2,4]-[4,3] quinoxaline-1-one (ODQ) from Alexis Corporation (San Diego, CA, USA); tyrphostin AG 1478 (EGFR inhibitor) from Calbiochem (Darmstadt, Germany); EGF (human recombinant) from Upstate Biotechnology (Lake Placid, NY, USA); apoptosis detection kits from Promega (Madison, WI, USA); polyvinylidene difluoride (PVDF) membranes (Immobilon™-P) from Millipore Corporation (Bedford, MA, USA); culture media, saline solutions, fetal calf serum (FCS), trypsin/EDTA, gentamicin and bovine serum albumin (BSA) from Life Technologies (Gaithersburg, MD, USA); trichloroacetic acid (TCA), glycerol, inorganic salts, and concentrated acids, bases and alcohols from Merck (Darmstadt, Germany); sodium dodecyl sulphate (SDS), acrylamide:bis-acrylamide solutions and Bio-Rad protein assay from Bio-Rad Laboratories (Hercules, CA, USA); Supersignal® chemiluminiscent-substrate (ECL) kit and ABC staining kit from Pierce (Rockford, IL, USA); and Vectashield® mounting medium from Vector Laboratories (Burlingame, CA, USA). All the other chemicals used were obtained from Sigma-Aldrich).

### Cell culture and drug treatments

Human neuroblastoma NB69 cells were obtained from Dr Mena (Hospital Ramón y Cajal, Madrid). Cells were grown in 75-cm$^2$ culture flasks with 10 mL of Dulbecco's modified Eagle's medium supplemented with 15% (vol/vol) FCS, 2 mM L-glutamine and 40 μg/mL gentamicin (DMEM-FCS), in a humidified atmosphere of 5% $CO_2$ in air at 37°C. Once a week, cells were detached with trypsin/EDTA, counted using a Neubauer chamber and seeded at a density of 9000 cells/cm$^2$, and the medium was changed 3 days afterwards. Experiments were always performed with cells subcultured two to seven times after thawing.

Drug treatments were always applied to cells that had been maintained during the preceding 24 h in a serum-free medium containing 1% (wt/vol) BSA (DMEM-BSA). Three different NO donors, with distinct mechanisms and half-lives for the release of NO, were used. DETA-NO and DEA-NO belong to the NONOate family of NO adducts, which spontaneously decompose to produce NO when dissolved in aqueous solution, and their half-lives are 20 h and 2.1 min, respectively (Feelisch and Stamler 1996). The other NO donor used was SNAP, which is a tertiary *S*-nitrosothiol; its decomposition to produce NO is both spontaneous and enhanced by biological thiol groups and metals, and it has a half-life which is known to be 4–5 h in solution but is unlikely to exceed 30 min when added to the cell culture (Janssens *et al*. 1999). All three drugs were dissolved immediately before addition to the cultures.

All the substances used were dissolved in culture medium, except for SNAP and ODQ, which were dissolved in dimethylsulphoxide (DMSO). Control cultures, in the presence of vehicle alone, were performed.

### Immunocytochemical studies

Cells were grown onto 12 mm-diameter round coverslips for immunocytochemical detections. After fixation with 4% (wt/vol) paraformaldehyde in 0.1 M phosphate buffer, cells were incubated for 30 min in a solution containing 2.5% (wt/vol) BSA in phosphate-buffered saline (PBS) to prevent non-specific antibody binding. The same solution was used to dilute the different antibodies. For EGFR immunocytochemical staining, cells were successively incubated with a mouse monoclonal antibody raised against the EGFR (1 : 200; Upstate Biotechnology) for 16 h at 4°C,

and an anti-mouse IgG labelled with Cy3 (1 : 1000; Amersham Life Science, Little Chalfont, Buckinghamshire, UK) for 1.5 h at room temperature. After washing with PBS, coverslips were mounted on slides with Vectashield and fluorescent signals were detected using a BX60 Olympus microscope (Olympus, Hamburg, Germany) fitted with appropriate filters. A similar protocol was used for detection of the neurone-specific intermediate filament βIII-tubulin, and for the astrocyte-specific glial fibrillary acidic protein (GFAP), using mouse monoclonal antibodies (1 : 1000, Promega; and 1 : 300, Sigma, respectively). Nestin immunostaining was carried out by means of a 1 : 5000 dilution of a rabbit antiserum kindly provided by Dr M. Vallejo (Madrid, Spain; McManus *et al.* 1999), followed by an anti-rabbit IgG labelled with FITC (1 : 200; Jackson ImmunoResearch, West Grove, PA, USA).

### [*methyl*-$^3$H]Thymidine incorporation

Cell proliferation was analysed by quantifying the incorporation of [*methyl*-$^3$H]thymidine into the DNA of dividing cells, as previously described (Estrada *et al*. 1997). Essentially, cells were seeded in 24-well culture plates at a density of 40 000 cells/well in DMEM-FCS. Three days later, when cells had reached 40–50% confluence, the cultures were maintained in serum-free medium for 24 h. Cells were then exposed to DMEM-FCS, DMEM-BSA, or DMEM-BSA plus 10 ng/mL EGF; any test drug and [*methyl*-$^3$H]thymidine (1.51 μM, specific activity 0.33 Ci/mmol) were added immediately. Preliminary experiments showed that the effect of EGF was evident 24 h later, reaching a maximum at 48 h. As our goal was to analyse a possible interference of NO with EGFR activation, [*methyl*-$^3$H]thymidine incorporation into DNA was measured after 48 h of treatment. Cells were then washed twice with Dulbecco's phosphate-buffered saline (DPBS), treated with ice-cold 10% (wt/vol) TCA for 10 min, solubilized overnight with 0.2 M NaOH, and neutralized with 0.2 M HCl. Finally, aliquots of the samples were diluted in scintillation fluid and counted in a scintillation counter.

### Cell growth curves

Immediately after initiation of the cell treatments, phase-contrast microscopy images of randomly selected fields (five to eight fields per culture) were captured with a video camera coupled to an inverted microscope (IX-70; Olympus). Images of these same fields were also captured at different time-points up to 48 h. The number of cells was counted with the aid of Microimage software (Olympus) and expressed as cells × cm$^{-2}$, or as cell increment × cm$^{-2}$.

### Determination of the cell cycle phases by flow cytometry

To analyse cell DNA content, cultures were maintained in serum-free medium for 24 h and exposed to the different experimental conditions for an additional 24-h time-period. Cells were then collected in PBS (0.5–1 × 10$^7$ cells/mL), fixed with 70% ethanol at 4°C for 1 h, washed twice with cold PBS, and incubated with 0.1 mg/mL propidium iodide and 40 U/mL RNase in PBS, for 15 min at 37°C, to label DNA. Fluorescence-activated cell sorting analysis was performed on a FACScalibur instrument (Becton-Dickinson, San Jose, CA, USA), as previously described (Telford *et al*. 1994). Cell samples were analysed for propidium iodide width versus area fluorescence (which allowed the exclusion of weakly fluorescent debris and doublet cells), followed by gating into a red fluorescence area histogram. Two peaks were observed, the larger one corresponding to cells in the $G_0/G_1$ cell-cycle phases and the other one, with double-fluorescence intensity, formed by cells in $G_2$/M phases. The ratio between the number of cells in the $G_2$/M region and those in the $G_0/G_1$ region was calculated for each experimental condition.

### Cell viability

Cell viability was analysed using the Trypan Blue exclusion test. After 1, 8 and 48 h of treatment, with or without different NO donors, the culture medium was removed and the cells were scraped and resuspended in PBS. Aliquots of the cell suspensions were mixed with an equal volume of 0.4% (wt/vol) Trypan Blue, and the percentage of viable cells was calculated using a Neubauer chamber.

### Apoptosis detection

The detection and quantification of apoptotic cells was performed using the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling (TUNEL) staining method, which takes advantage of the DNA fragmentation that occurs in cells undergoing programmed cell death. A commercial kit from Promega was used and the standard protocol provided by the supplier was followed. Apoptotic cells (fluorescein-12-dUTP DNA-labelled cells) were counted directly under a fluorescence microscope (BX-60; Olympus) and were expressed as percentage of the total number of cells, counterstained with propidium iodide. Along with the samples, negative and positive controls were simultaneously processed, which consisted, respectively, of: (i) NB69 cells in the absence of terminal deoxynucleotidyl transferase, to detect non-specific staining (which was never found); and (ii) cells treated with DNase I to generate DNA fragments that are targets for positive labelling.

### Inmunoprecipitation of the epidermal growth factor receptor

NB69 cells were seeded in 100 mm-diameter culture dishes at a density of 1–1.5 × 10$^6$ cells/dish, and were cultured for 3 days to reach cellular confluence. After a 24-h period in serum-free medium, cells were incubated with or without the NO donor DEA-NO for 15–120 min, followed by a 2-min pulse addition of 10 ng/mL EGF. Controls in the absence of EGF were also included. Cells were immediately lysed with ice-cold lysis buffer [50 mM Tris–HCl, pH 7.4, 1% (vol/vol) Triton®-X-100, 0.5% (wt/vol) sodium deoxycholate, 1 mM phenylmethylsulphonyl fluoride (PMSF), 10 μg/mL leupeptin, 10 μg/mL pepstatin A, 10 μg/mL aprotinin and 2 mM sodium orthovanadate], detached from the dishes using a cell scraper and left for 30 min on ice. Afterwards, samples were centrifuged at 7500 *g* for 30 min; supernatants were collected and their protein content was measured using the Bradford-based Bio-Rad microassay method. Equal amounts of protein from each cellular extract were mixed with 1 mL of protein A–Sepharose, which had been incubated overnight at 4°C with the anti-EGFR antibody (4 μg/mL; Upstate Biotechnology), and the precipitation reaction was allowed to occur overnight at 4°C. Pellets were collected by centrifugation at 7000 *g* and the immunoprecipitation complexes were then denatured by boiling for 5 min in loading buffer, which contained 0.02% (wt/vol) Bromophenol Blue, 5% (vol/vol) 2-mercaptoethanol, 20% (wt/vol) glycerol, 60 mM Tris–HCl, pH 6.8, and 10% (wt/vol) SDS. The samples were loaded and electrophoresed (Laemmli 1970) in SDS–polyacrylamide minigels at a 23-mA constant

current. Proteins were transferred onto a PVDF membrane and processed for anti-phosphotyrosine immunoblotting.

### Western blot analysis

*Tyrosine phosphorylation of the epidermal growth factor receptor*

For the detection of tyrosine phosphorylation (Basu *et al*. 1984), NB69 cells were seeded in six-well culture clusters at a density of 400 000 cells/well, and grown to confluence for 3 days. Then, after a 24-h deprivation of serum, cells were treated with DEA-NO and EGF as described for immunoprecipitation; the incubations were stopped with ice-cold 10% (wt/vol) TCA. Afterwards, cellular precipitates were scraped, collected by centrifugation (7500 *g*) and each pellet was dissolved in 50 μL of loading buffer. After boiling for 5 min, samples were loaded into 5–20% SDS–polyacrylamide linear-gradient gels and electrophoresed at 30 mA. The proteins were electrotransferred to a PVDF membrane, fixed with 0.2% glutaraldehyde and temporarily stained with the dye Fast Green FCF (Kurien *et al*. 1998). The stained membrane was scanned for later measurements of the protein content in each track. Thereafter, the membrane was rinsed, treated for 2 h with Tris-buffered saline (TBS) containing 5% (wt/vol) BSA (TBS–BSA) to block non-specific binding sites, and incubated overnight at room temperature with a peroxidase-conjugated anti-phosphotyrosine monoclonal antibody (1 : 2000; Transduction Laboratories, Lexington, KY, USA) also diluted in TBS–BSA. The membrane was washed three times for a total of 30 min with 0.1% (vol/vol) Tween-20™ in TBS (T–TBS). It was then immersed in an enhanced chemiluminescent-substrate solution (Supersignal; Pierce), following the manufacturer's guidelines, and the phosphotyrosine-containing proteins were made visible by exposing the membrane to a light-sensitive film. Quantification of the phosphorylated EGFR band was carried out by photodensitometry, using a computer-assisted scanner and the Microimage software. The optical densities measured for the EGFR bands were normalized according to the total protein loaded, as assessed using Fast Green staining, although only minor differences in protein content were occasionally detected. In some cases, after stripping the membrane, a second-round of immunoblotting was carried out using a rabbit polyclonal anti-EGFR antibody (1 : 1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA), following the same protocol described below for NOS isoforms.

*Nitric oxide synthase isoforms*

Cultures of NB69 cells were exposed to three different media, as described for [*methyl*-$^3$H]thymidine incorporation. After 48 h, cells were washed with DPBS and lysed with ice-cold lysis buffer. Supernatants were collected by centrifugation (7500 *g*) and their protein concentration was measured as described above. An aliquot (50 μg of total protein) from each sample was diluted in loading buffer, boiled and loaded onto 9% SDS-polyacrylamide minigels. After transfer, the membrane was blocked with 3% (wt/vol) fat-free dry milk in T–TBS for 30 min, incubated with rabbit polyclonal antibodies against either neuronal NOS (nNOS) (1 : 5000; two antibodies were used, one from Santa Cruz Biotechnology and the other kindly provided by Dr J. Rodrigo, Uttenthal *et al*. 1998) or inducible NOS (iNOS) (1 : 5000; Santa Cruz Biotechnology) for 2 h at room temperature, and with a biotin-conjugated anti-rabbit antibody (1 : 10 000; Sigma) for 1 h. Finally, the bands were made visible using the ABC and ECL kits (Pierce), following the manufacturer's instructions. The specificity of the antibodies was previously tested using lysates of rat pituitary gland and activated macrophages as positive controls for nNOS and iNOS, respectively.

### Data analysis and statistics

Data are presented as the mean ± SEM of values obtained from three or more experiments, each of them performed in quadruplicate cultures. Comparisons between two values were analysed using the Student's *t*-test for paired or unpaired samples according to the type of experiment. When more than one variable was analysed, the one-way analysis of variance was used. Differences were considered significant when $p < 0.05$.

## Results

### Immunocytochemical characterization of the human neuroblastoma cell line NB69

Immunocytochemical staining using antibodies against phenotype-specific antigens revealed that NB69 cells (Fig. 1a) contain the neuroblast-specific protein, βIII-tubulin (Fig. 1c), but not the neuroepithelial marker, nestin, which is present in immature progenitors and in some neuroblastoma cells (Kashima *et al*. 1995), or the astrocyte-specific antigen, GFAP. In addition, these cells express the EGFR protein in their plasma membrane, as assessed by immunocytochemistry using a specific anti-human EGFR antibody (Fig. 1d). Throughout the study, the cells remained in an undifferentiated state; none of the treatments induced neurite extension or other morphological changes, as shown by SNAP treatment in Fig. 1(b).

### NB69 cells proliferate in response to epidermal growth factor

The effect of EGF on NB69 human neuroblastoma cell proliferation was estimated by measuring the incorporation of [*methyl*-$^3$H]thymidine into DNA. After 48 h of culture, EGF (10 ng/mL) produced a 2.14-fold increase in the proliferation of NB69 cells, when compared with control cultures in the absence of exogenous growth factors (Fig. 2a). The maximum proliferation rate (3.3-fold increase when compared with controls) was obtained when cells were grown in the presence of 15% FCS, the medium used for standard expansion of the cell line (Fig. 2a). Similar results were obtained when the relative number of cells in the $G_2$/M cell-cycle phases was estimated by flow cytometry (Fig. 2b). The proliferative action of EGF and FCS on NB69 cells was also confirmed by analysis of the cell number over time (Fig. 2c).

In the presence of FCS, addition of EGF did not significantly increase [*methyl*-$^3$H]thymidine incorporation into DNA (110.8 ± 20% of the value with FCS alone, $n = 5$). The use of an anti-EGFR neutralizing antibody

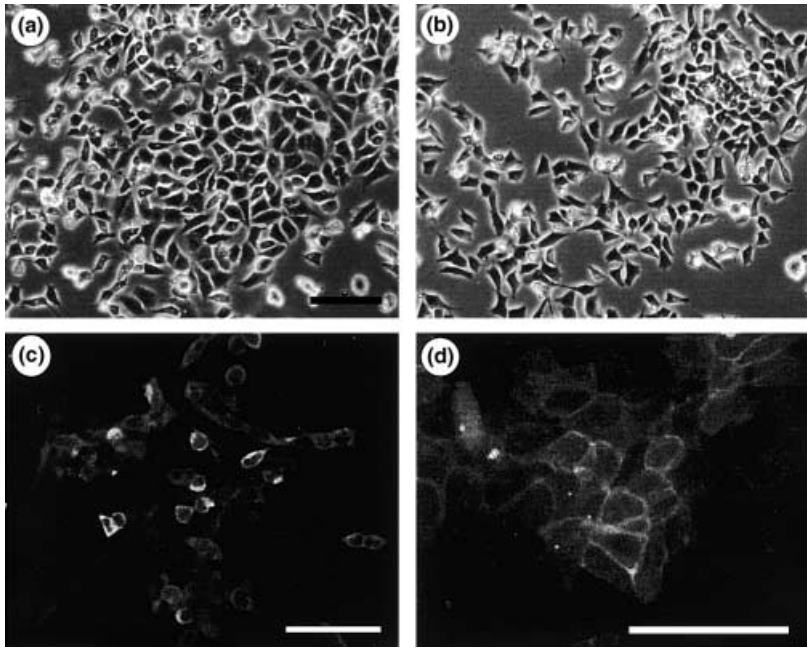


**Fig. 1** (a) and (b). Phase-contrast photomicrographs of NB69 human neuroblastoma live cells grown for 48 h in a medium containing 10 ng/mL epidermal growth factor (EGF), in the absence (a) or presence (b) of 0.1 mM *S*-nitroso-*N*-acetylpenicillamine (SNAP). (c) Immunostaining of βIII-tubulin in NB69 cells. Immunofluorescence is observed in the cell cytoplam. (d) Immunostaining of epidermal growth factor receptor in NB69 cells. Immunofluorescence is observed in the cell membrane. Calibration bars: 100 μm in (a) and (b); 50 μm in (c) and (d).

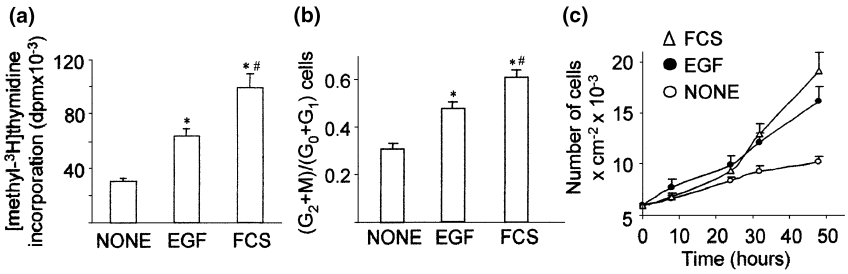


**Fig. 2** Effect of epidermal growth factor (EGF) and fetal calf serum (FCS) on NB69 cell proliferation. NB69 cells were grown for 48 h (a and c) or 24 h (b) in a medium devoid of growth factors, or containing either 10 ng/mL EGF or 15% FCS. (a) [*methyl*-$^3$H]Thymidine incorporation into DNA. (b) The ratio between the number of cells containing double and single fluorescence intensity (propidium iodide (PI)-staining), as detected by fluorescence-activated cell sorter (FACS) analysis, which represents the number of cells in $G_2$/M and $G_0/G_1$ cell-cycle phases, respectively. (c) Growth curves showing the number of cells $\times$ cm$^{-2}$ at different time-points, under the three culture conditions tested. Data are presented as mean ± SEM; $n = 20$–25 in (a), $n = 3$ in (b) and $n = 4$ in (c). *$p < 0.05$ when compared with the control condition; #$p < 0.05$ when compared with EGF-treated cultures.

(5 μg/mL), or the EGFR inhibitor AG 1478 (10 μM), which completely prevented the proliferative action of EGF in NB69 cells (data not shown), modified only slightly the proliferation induced by FCS (90.4 ± 3.0% and 94.6 ± 1.3% of FCS values, respectively, $n = 3$), indicating that growth factors other than EGF were responsible for the serum-induced DNA synthesis.

### Different nitric oxide donors inhibit NB69 cell proliferation by a cGMP-independent pathway

Growth curves were analysed in EGF-treated cultures exposed to increasing concentrations of SNAP. As shown in Fig. 3(a), the NO donor prevented dose-dependently the mitogenic action of EGF. Accordingly, in the presence of SNAP, the relative number of cells in the $G_2$/M phase of the cell cycle decreased significantly in a concentration-dependent manner (Fig. 3b).

To determine the influence of the culture conditions on the antimitotic effect of NO, [*methyl*-$^3$H]thymidine incorporation into DNA and total cell number were measured in control and NO-donor treated cells grown for 48 h in the three different media described above (i.e. in the absence of exogenous growth factors, in the presence of EGF, and in the presence of FCS). Figure 4(a, b and c) show that increasing concentrations of three different NO donors progressively inhibited [*methyl*-$^3$H]thymidine incorporation into the DNA of NB69 cells, in all culture conditions tested. We observed that the longer the NO donor half-life for the release of NO, the higher potency of its antiproliferative effect. One interesting finding was that, for short half-life donors,

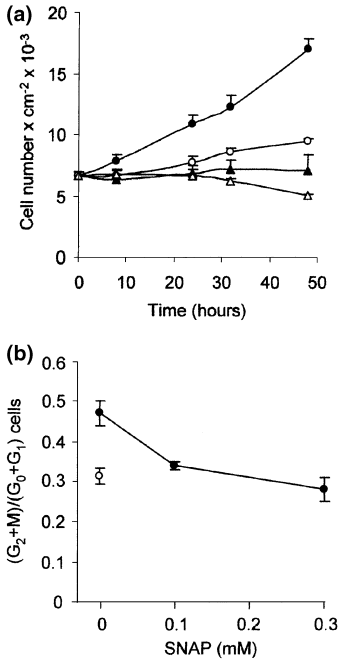


**Fig. 3** Effect of *S*-nitroso-*N*-acetylpenicillamine (SNAP) on cell growth and cell cycle in epidermal growth factor (EGF)-treated NB69 cells. Cells were cultured for 24 h in serum-free medium and then supplemented with 10 ng/mL EGF, with or without the nitric oxide (NO) donor, SNAP, at different concentrations. (a) Growth curves in the absence of NO donor (●), and in the presence of 0.03 mM (○), 0.1 mM (▲) and 0.3 mM (△) SNAP. (b) Fluorescence-activated cell sorter (FACS) analysis of propidium iodide (PI)-stained cells, after 24 h of treatment, revealed that SNAP prevents the cell cycle progression promoted by EGF. The mean value corresponding to cultures in the absence of added growth factors is represented by the open circle (○). Data are presented as mean values ± SE; $n = 3$.

EGF-treated cultures were much more sensitive to the antimitotic effect of NO than cultures grown either with FCS or in the absence of any added growth factor. This is clearly shown in Fig. 4(d), where the $IC_{50}$ for the different NO donors in the three culture media tested are represented. The higher sensitivity to the NO action in cells growing in the presence of EGF was also evident when proliferation was estimated by culture cell density analysis (Fig. 4e).

To study the involvement of cGMP on the antimitotic action of NO, we tested the effects of SNAP in the presence of the guanylyl cyclase inhibitor, ODQ, at a concentration (10 μM) that, in our hands, was able to inhibit NO-mediated neurogenic relaxation of cerebral blood vessels (Gonzalez *et al*. 1997). Figure 5 shows that ODQ did not modify the effect on proliferation exerted by SNAP. This means that, in NB69 cells, the NO mechanism of action leading to growth arrest was cGMP-independent. However, in the absence of SNAP, ODQ had a small antiproliferative effect by itself in EGF-treated cells.

### Nitric oxide donors did not reduce the viability of NB69 cells

To assess whether the decrease in cell number and [*methyl*-$^3$H]thymidine incorporation, produced by NO donors, was indeed an antiproliferative effect and not a consequence of cell death owing to NO cytotoxicity, cell viability and/or apoptosis were analysed at different time-points after addition of SNAP or DETA-NO to NB69 cells. Table 1 shows that neither cell viability (as measured using the Trypan Blue exclusion test) nor the percentage of apoptotic cells (detected using the TUNEL technique) changed significantly in any of the conditions tested, when cells were incubated with concentrations of the NO donors able to produce a dramatic reduction in [*methyl*-$^3$H]thymidine incorporation (see Figs 4a and b). In addition, flow cytometry analysis showed that hypodiploid cells did not accumulate after 24 h of incubation with 0.1–0.3 mM SNAP, indicating that apoptosis did not occur under these conditions (data not shown).

### Nitric oxide inhibits epidermal growth factor-induced epidermal growth factor receptor tyrosine kinase activation in NB69 cells

The finding that NB69 cells whose growth was stimulated by EGF were more sensitive to the NO antiproliferative action raised the possibility that the mechanism of action of NO in this system might include inhibition of the EGFR tyrosine kinase activity. To explore this possibility, we examined whether NO modified the tyrosine transphosphorylation of the EGF receptor. To achieve this, NB69 cells were incubated with or without the NO donor DEA-NO (0.1–1 mM) for increasing periods of time (from 15 to 120 min) and then stimulated for 2 min with EGF. After immunoprecipitation using an anti-EGFR antibody, the EGFR was electrophoresed and immunoblotted using an antiphosphotyrosine antibody to detect the active protein fraction. Figure 6 shows that DEA-NO produced significant decreases in the tyrosine phosphorylation of the EGFR and that this inhibition was concentration- and time-dependent.

To exclude the possibility that NO may modify the EGFR antigenic properties and therefore decrease the affinity of the anti-EGFR antibody used in the immunoprecipitation step, we also performed denaturing SDS–polyacylamide gel electrophoresis (PAGE) and western blot using total cell lysates from NB69 cells treated in the same way as for immunoprecipitation. When cells had received a 2-min pulse of EGF, immunoblotting with the anti-phosphotyrosine antibody revealed a band of 170 kDa molecular weight, which corresponded to the EGF receptor (as assessed by a second round of immunoblotting utilizing an EGFR antibody) (Fig. 7a). As described for immunoprecipitation experiments, phosphorylation of the EGFR band was decreased in cultures treated with NO donors. In addition, Fig. 7(b) shows how the anti-phosphotyrosine antibody also

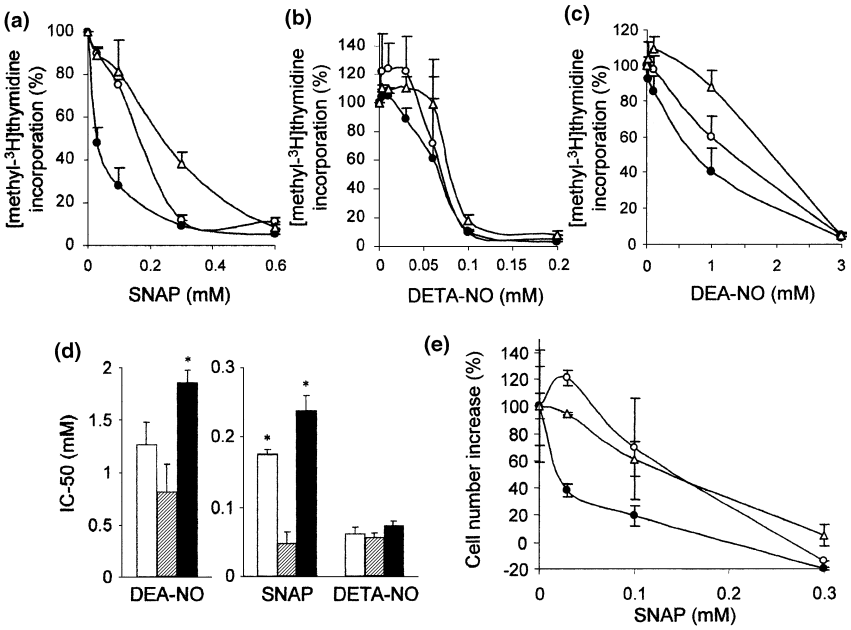


**Fig. 4** Effect of nitric oxide (NO) donors on NB69 cell proliferation under different culture conditions. Incorporation of [*methyl*-$^3$H]thymidine into DNA by NB69 cells grown for 48 h in the absence of added growth factors (○), or in a medium containing either 10 ng/mL epidermal growth factor (EGF) (●) or 15% fetal calf serum (FCS) (△), with increasing concentrations of the NO donors: *S*-nitroso-*N*-acetylpenicillamine (SNAP) (a); (*Z*)-1-[2-(2-aminoethyl)-N-(2-ammonioethyl)amino]diazen-1-ium-1,2-diolate] (DETA-NO) (b); and 2-(*N*,*N*-diethylamino)-diazenolate-2-oxide (DEA-NO) (c). Data are expressed as percentage of the values measured in the absence of NO donors in each experimental condition. (d) Calculated concentrations of NO donors required to inhibit by 50% [*methyl*-$^3$H] thymidine incorporation ($IC_{50}$) in NB69 cells cultured in the absence of added growth factors (open bars), or in a medium containing either 10 ng/mL EGF (striped bars) or 15% FCS (filled bars). *$p < 0.05$ as compared with cells grown with EGF. (e) Effect of SNAP on cell density increase, produced in cultures grown for 48 h in the three conditions described above. Symbols and result expression are as described in (a), (b) and (c). Data are presented as mean values ± SEM from three experiments.

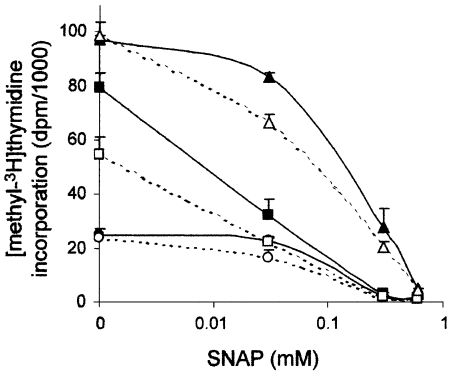


**Fig. 5** Effect of guanylyl cyclase inhibition on the cytostatic action of *S*-nitroso-*N*-acetylpenicillamine (SNAP). Incorporation of [*methyl*-$^3$H]thymidine was studied in cells grown for 48 h in the absence of added growth factors (○, ●), or in a medium supplemented with either 10 ng/mL epidermal growth factor (EGF) (□, ■) or 15% fetal calf serum (FCS) (△, ▲) and exposed to increasing concentrations of SNAP, both in the absence (solid line) or presence (dashed line) of the guanylyl cyclase inhibitor, ODQ (10 μM). Data are given in dpm and are presented as the mean ± SEM from two experiments performed in quadruplicate samples.

labelled a series of protein bands, most of which were phosphorylated in tyrosine as a consequence of the EGF-started cascade of events, as they were silent in the absence of EGF. When cells where pre-incubated with DEA-NO, a time-dependent inhibition of the phosphorylation of the EGFR and of all the EGF-activated phosphoproteins was observed. However, the bands whose tyrosine phosphorylation was evident in the absence of EGF were not inhibited at all by NO (see Fig. 7b).

Although the maximal inhibition produced by 1 mM DEA-NO on the EGFR transphosphorylation was quite reproducible (70.9 ± 12.6%, $n = 8$), the time at which it was reached was variable. We observed two different patterns, as follows: in five out of eight experiments, the maximum inhibition was reached after 15–30 min and then a progressive recovery occurred within the next 2 h (as in the immunoblot shown in Fig. 8); in the three remaining experiments, the inhibitory effect appeared progressively during the first 2 h after addition of the NO donor, reaching a maximum at 120 min (as in the immunoblot shown in Fig. 7b), and finally reverted if longer incubations were allowed. The average kinetics of all the experiments performed is shown in Fig. 7(c). In all

**Table 1** Effects of nitric oxide (NO) donors on NB69 cell viability and apoptosis

| Treatment | Concentration (mM) | Cell viability[a] (%) 1 h | 8 h | 48 h | Apoptotic nuclei[b] (%) 8 h |
|---|---|---|---|---|---|
| None | | 94.6 ± 0.6 | 95.8 ± 0.8 | 90.7 ± 2.6 | 1.04 ± 0.03 |
| SNAP | 0.1 | 96.4 ± 0.3 | 93.6 ± 1.1 | 93.4 ± 0.5 | 1.9 ± 1.0 |
| | 0.3 | – | – | – | 1.4 ± 0.3 |
| | 1.0 | – | – | – | 2.0 ± 0.9 |
| DETA-NO | 0.1 | 93.2 ± 0.8 | 92.7 ± 1.3 | 90.5 ± 0.8 | – |

NB69 cells were deprived of serum for 24 h and then exposed to epidermal growth factor (EGF) in the presence or absence of NO donors for the indicated periods of time. [a]Cell viability was measured using the Trypan Blue exclusion test. The number of cells excluding the dye is expressed as a percentage of the total number of cells. A total of 1600 cells was counted for each condition. The data are presented as the mean ± SEM of two experiments with quadruplicate samples. [b]Apoptosis was evaluated using the terminal deoxynucleotidyl transferase-mediated dUTP nick-end DNA labelling (TUNEL) technique. The number of nuclei containing fragmented DNA is expressed as percentage of the total number of cells. At least 800 cells were counted for each condition. The data are presented as the mean ± SEM of three independent experiments. DETA-NO, (*Z*)-1-[2-(2-aminoethyl)-*N*-(2-ammonioethyl)amino]diazen-1-ium-1,2-diolate]; SNAP, *S*-nitroso-*N*-acetylpenicillamine.

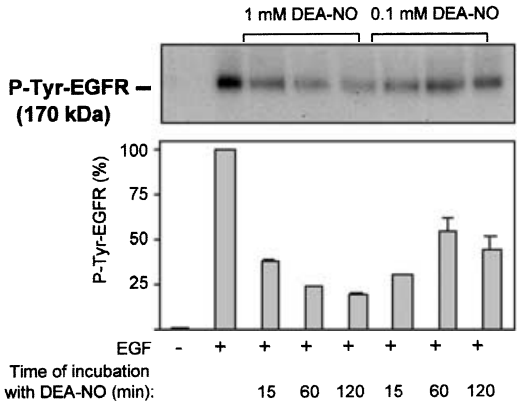


**Fig. 6** Effect of 2-(*N*,*N*-diethylamino)-diazenolate-2-oxide (DEA-NO) on the transphosphorylation of the epidermal growth factor (EGF) receptor (EGFR) isolated by immunoprecipitation from NB69 cells. NB69 cells were incubated with or without DEA-NO (0.1 or 1 mM) for 15–120 min (as indicated). Thereafter, cells received a 2-min pulse addition of 10 ng/mL EGF and were immediately lysed. Protein concentration was determined for each sample and the EGFR was immunoprecipitated and electrophoresed. The level of activation of the EGFR was analysed by western blot, using an anti-phosphotyrosine antibody. Controls in the absence of EGF were also included. The experiment shown is a representative one. In the lower panel, optical densities are presented as a percentage of that measured in the absence of DEA-NO, and are expressed as a percentage of EGFR-transphorylation (P-Tyr-EGFR).

cases, a parallel recovery of all the EGF-activated phosphoproteins was observed (data not shown).

### The effect of nitric oxide on the epidermal growth factor receptor tyrosine phosphorylation is fully reversible

In order to test the reversibility of the NO action on the EGFR, experiments were performed in which cells were incubated with 1 mM DEA-NO for 15 min, then in some cultures the medium was changed to remove the NO donor and, either immediately or 15–75 min later, the cells were stimulated with EGF for 2 min, lysed and processed for immunoblotting as described above. Figure 8 shows that a complete and rapid recovery of the EGF-induced transphosphorylation of the EGFR was observed immediately after NO donor withdrawal. A similar reversion occurred in all EGF-activated phosphoproteins (data not shown).

### Inhibition of nitric oxide synthase activity increased NB69 cell proliferation

To analyse whether endogenous NO production was a physiological mechanism (*in vitro*, at least) participating in the growth control of NB69 cells, we evaluated the effect of the NOS inhibitor $N^{\omega}$-nitro-L-arginine methyl ester (L-NAME) on [*methyl*-$^3$H]thymidine incorporation into DNA and on cell number, in the three different culture media used throughout the present study. A small, but significant, increase on cell proliferation was observed in the presence of L-NAME when cultures had been supplemented with 15% FCS. Surprisingly, this increment was not detected in cells grown in the absence of growth factors or stimulated by EGF (Figs 9a and b).

### NB69 cells show differential expression of nitric oxide synthase isoforms depending on the culture medium composition

To investigate why L-NAME produced a stimulatory effect on NB69 cell proliferation only on cells cultured in the presence of FCS, we analysed the expression of nNOS and iNOS proteins in cells cultured in three different media, as induction of both isoenzymes might vary depending on the culture conditions. Western blots were performed in cells that had been cultured in a medium lacking growth factors or containing either EGF or FCS. As shown in Fig. 10,

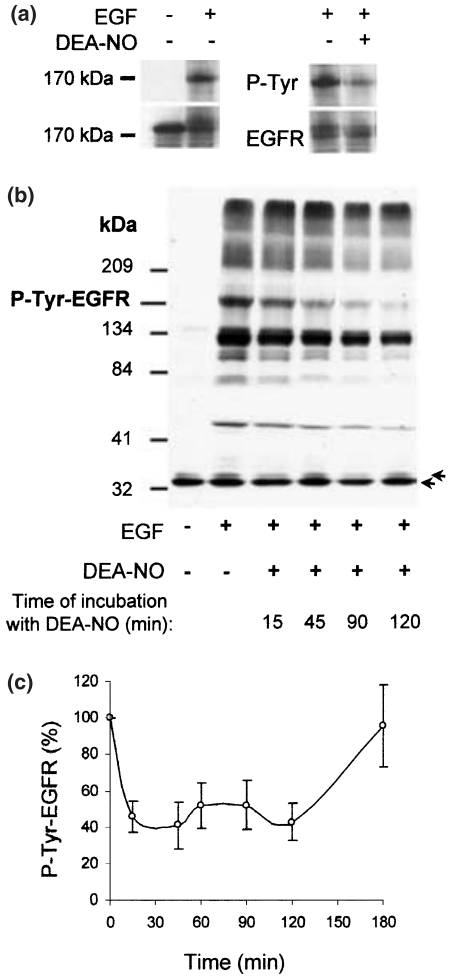


**Fig. 7** Effect of 2-(*N,N*-diethylamino)-diazenolate-2-oxide (DEA-NO) on the epidermal growth factor (EGF)-induced protein phosphorylation cascade. NB69 cells were incubated with or without 1 mM DEA-NO for 15–120 min (as indicated), and then with 10 ng/mL EGF for 2 min. Cells were immediately exposed to ice-cold 10% trichloroacetic acid (TCA) and the precipitates processed for sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and western blot using an anti-phosphotyrosine antibody. Control cells not stimulated by EGF were also included. (a) DEA-NO decreased the tyrosine phosphorylation of the EGF receptor (EGFR), but not the amount of receptor protein, as detected by a second round of immunoblotting using an anti-EGFR antibody. (b) The progressive inhibition of EGFR transphosphorylation observed after longer incubation times with DEA-NO was also extended to all the proteins whose phosphorylation in tyrosine was EGF dependent; however, for those phosphoproteins that were visible in the absence of EGF (pointed with arrowheads), no nitric oxide (NO)-mediated inhibition could be detected. (c) Time course of the EGFR-transphorylation inhibition produced by 1 mM DEA-NO in NB69 cells. EGFR transphorylation is presented as percentage of the optical density measured in the EGFR band in the absence of DEA-NO. Data represent the mean values ± SEM of between three and eight independent experiments.

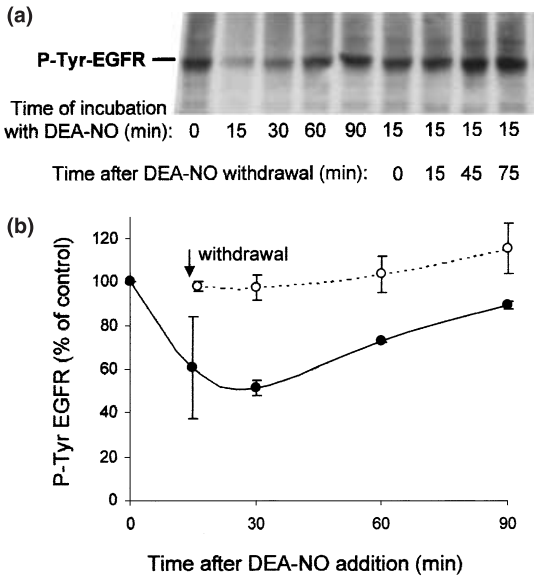


**Fig. 8** Reversibility of the 2-(*N,N*-diethylamino)-diazenolate-2-oxide (DEA-NO) effect on epidermal growth factor (EGF) receptor (EGFR) transphosphorylation. NB69 cells were incubated with 1 mM DEA-NO for 15–90 min (as indicated), or incubated with DEA-NO for 15 min, then washed and further incubated in the absence of nitric oxide (NO) donor for the next 0–75 min. A 2-min pulse addition of 10 ng/mL EGF was performed and the cells were processed for sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and western blot, as described in the legend to Fig. 6. A rapid recovery of the EGFR transphosphorylation was observed immediately after DEA-NO withdrawal. (a) Western blot from one representative experiment. (b) Time course of the reversibility of the DEA-NO effect. Filled circles, data obtained in the presence of DEA-NO; open circles, data obtained after DEA-NO withdrawal. Data are presented as in Fig. 6, and represent the mean values ± SD of two experiments.

expression of both nNOS and iNOS was detected in cells grown in the presence of 15% FCS, but not in those maintained for 48 h either with EGF or in the absence of growth factors. Nevertheless, in serum-treated cultures, the expression of both nNOS and iNOS was probably low because long exposure times were needed in order to detect the protein bands.

## Discussion

Our results show that exposure of NB69 cells to NO resulted in a reduction of cell proliferation, which was independent of cGMP production and more evident when the cell growth was stimulated by EGF. Addition of NO donors to cultured cells produced a rapid and reversible inhibition of the EGFR tyrosine kinase activity, as manifested by the reduced tyrosine phosphorylation of the receptor molecule and of all the proteins whose tyrosine phosphorylation was EGF dependent. In addition, expression of nNOS and iNOS by

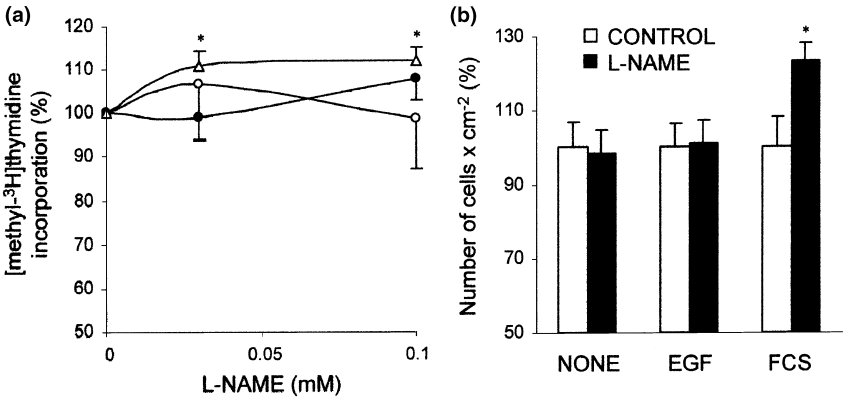


**Fig. 9** Effect of nitric oxide synthase (NOS) inhibition on NB69 cell proliferation. (a) Incorporation of [*methyl*-$^3$H]thymidine in NB69 cells cultured for 48 h in the absence of added growth factors (○), or in a medium supplemented with either 10 ng/mL epidermal growth factor (EGF) (●) or 15% fetal calf serum (FCS) (△), in the presence or absence of the NOS inhibitor, $N^{\omega}$-nitro-L-arginine methyl ester (L-NAME). (b) Effect of 0.1 mM L-NAME on cell density, in cultures grown in the three conditions described above. Data are expressed as percentage of the values measured in the absence of L-NAME. $^*p < 0.05$; $n = 5$–6 in (a) and $n = 3$ in (b).

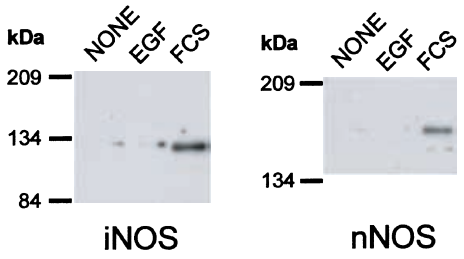


**Fig. 10** Western blot analysis of neuronal nitric oxide synthase (nNOS) and inducible nitric oxide synthase (iNOS). Soluble extracts were obtained from NB69 cells cultured for 48 h in the absence of added growth factors, or in a medium supplemented with either 10 ng/mL epidermal growth factor (EGF) or 15% fetal calf serum (FCS) (as indicated in the panel). Protein concentration was determined in each sample and 50 μg of total protein was loaded per track and electrophoresed. The nNOS and iNOS isoforms were detected using two specific antibodies as described in the Materials and methods. The result of a typical experiment is shown ($n = 3$).

NB69 cells suggests that NO is an endogenous regulator of cell growth in this cell line.

The cytostatic action of NO donors in NB69 cells is in agreement with previously reported results obtained in different cell types of neural origin, including other neuroblastoma cell lines (Peunova and Enikolopov 1995; Obregon *et al*. 1997). The decreased DNA synthesis observed in cells exposed to NO donors was caused by an antimitotic effect and not by a cytotoxic action of NO. In fact, no alteration in cell viability or in the number of apoptotic nuclei was detected in cultures treated, for different periods of time, with NO donors at concentrations capable of producing large decreases in [*methyl*-$^3$H]thymidine incorporation. This is in accordance with a recent report showing that SNAP increases the expression of the antiapoptotic proteins Bcl-2 and Bcl-xL and decreases the expression of the pro-apoptotic protein, Bax, in the NB69 cell line, suggesting that, on the contrary, NO has an antiapoptotic action in these cells (Rodriguez-Martin *et al*. 2000). However, we consider that, at the highest concentrations, cell detachment may contribute to the reduction in cell number or of DNA synthesis produced by NO donors in NB69 cells, as, after 48 h of treatment with 0.3 mM SNAP, cell density was slightly below the starting density.

The antiproliferative effect of NO donors was assayed in three different growth conditions, as a first approximation to elucidate the mechanism of action of NO. Cells whose growth was stimulated by EGF were more sensitive to the cytostatic action of DEA-NO and SNAP than cells growing spontaneously or stimulated by FCS. This finding suggests that NO interacts at a certain point with the mitogenic cascade initiated by activation of the EGFR. However, cell sensitivity to DETA-NO was the same in the three culture media tested. This apparent contradiction can be explained by the different half-lives for NO release of the three drugs used. Whereas DETA-NO, with a half-life of 20 h, produces a long-lasting NO release, the short half-life NO donors DEA-NO and SNAP liberate most NO during the first few minutes after their addition to the cell cultures. As EGF and the NO donors were added at the same time, DEA-NO and SNAP could interact more effectively than DETA-NO with the initial steps of the EGF-induced signalling process. On the contrary, cytostasis produced by DETA-NO may result from the concomitant action of the continuously released NO on a variety of proteins controlling proliferation in different phases of the cell cycle, and this could explain why its effect was not dependent on EGF stimulation.

Previous work from our laboratory revealed that, in permeabilized transfected fibroblasts overexpressing the

EGFR, NO donors inhibited the transphosphorylation of this receptor. Now we show that the naturally expressed EGFR from NB69 cells was largely phosphorylated in tyrosine residues when immunoprecipitated from intact cells exposed to EGF, and that this tyrosine phosphorylation clearly decreased when the EGFR was isolated from cells exposed to both EGF and DEA-NO. Similar results were obtained when tyrosine phosphorylation was analysed in whole cell lysates, which also showed that the inhibition of phosphorylation produced by NO extended to other EGF-dependent proteins but not to those that were already phosphorylated in tyrosine in the absence of EGF. These results strongly support the hypothesis that, in NB69 cells, EGFR is one of the protein targets of the cGMP-independent antiproliferative action of NO.

Although the mechanism by which NO inhibits EGFR transphosphorylation in NB69 cells has not been fully determined, it is unlikely that such a rapid and transient effect was caused by a decrease in the levels of receptor protein. This was confirmed by western blot analysis using an anti-EGFR antibody, which indicated that the total amount of EGFR was not altered by treatment with DEA-NO. Furthermore, the immediate reversibility upon drug removal makes it unlikely that NO could produce internalization of the EGFR and rather suggests a reversible chemical modification of the protein *in situ*, causing a decrease in its transphosphorylation.

A large number of proteins become *S*-nitrosylated when exposed to NO. In many cases, *S*-nitrosylation occurs in cysteine residues that are critical for the protein function, resulting in either increases or decreases of protein activity (Stamler 1995). Recent data have demonstrated that *S*-nitrosylation is a physiological mechanism of action of nNOS-derived NO (Jaffrey *et al*. 2001). *S*-nitrosoproteins are highly labile, with tissue half-lives ranging from seconds to minutes, as a result of their reactivity with other biological thiols (Kashiba-Iwatsuki *et al*. 1997). The reversibility of the DEA-NO effect on the NB69-cell EGFR transphosphorylation is in agreement with a thionitrosylation mechanism. If this were the case, the variable duration of the NO effect could be a consequence of different availability of endogenous nitrosylation substrates (such as glutathione), which may influence the half-life of nitrosothiol groups in the EGFR. Previous data from our laboratory have shown that, indeed, in permeabilized fibroblasts, the NO effect on the EGFR was partially reversed by dithiothreitol, strongly suggesting *S*-nitrosylation of the receptor (Estrada *et al*. 1997).

To be considered as physiological, the inhibition produced by NO donors on NB69 cell proliferation should be provoked also when NO is endogenously produced by the same cells or by their neighbours. We show here that when NB69 cells were cultured in standard conditions, with 15% FCS, both nNOS and iNOS were expressed. Cell-synthetized NO had indeed an autocrine action, as revealed by the small, but significant, increase in DNA synthesis and cell number observed when NOS activity was blocked. In the absence of serum, however, NOS expression was not detected and, as expected, addition of a NOS inhibitor had no effect on cell proliferation. The small response to L-NAME, observed in serum-treated cells, correlates well with the low enzyme expression detected and also with the fact that, although both isoforms were present, only iNOS was probably active, as nNOS needs specific signals leading to intracytoplasmic increases in calcium concentration to synthesize NO from L-arginine (Moncada *et al*. 1991). Previous work with other neuroblastoma cell lines have shown that some of these cells express nNOS (Fujisawa *et al*. 1994; Dotsch *et al*. 2000) and that, under treatment with differentiation inductors such as tumour necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) (Muñoz-Fernandez *et al*. 1994; Ogura and Esumi 1996; Obregon *et al*. 1997), retinoic acid (Ghigo *et al*. 1998) or nerve growth factor (NGF) (Peunova and Enikolopov 1995; Phung *et al*. 1999), iNOS may also be expressed and cellular NO production can be increased. Under these conditions, NOS inhibitors lead to reversal of the differentiation process, whereas exogenous NO induces differentiation. We have not studied whether expression of NOS isoforms is also enhanced in NB69 cells when exposed to differentiating stimuli; however, participation of endogenous NO in their differentiation is not improbable, as these cells increase their catecholaminergic properties when treated with low concentrations of SNAP for times longer than those used in the present study (Rodriguez-Martin *et al*. 2000).

Neuroblasts of the rodent subventricular zone (SVZ), a brain region in which neurogenesis is maintained after birth and throughout adulthood (Lois and Alvarez-Buylla 1993), have a phenotypic profile similar to that of NB69 cells (nestin$^{low}$, Tuj-1$^{+}$, GFAP$^{-}$; Doetsch *et al.* 1997 and E. R. Matarredona, unpublished results), and are equally responsive to the proliferative action of EGF (Reynolds and Weiss 1992; Craig *et al*. 1996; Kuhn *et al*. 1997; Gritti *et al*. 1999). As recent findings from our laboratory have shown that SVZ neural precursors are immersed in an axonal network of nitrergic neurones (Moreno-López *et al*. 2000), and are therefore exposed to the action of NO *in vivo*, it is plausible that the mechanisms described in the present study are also operative in physiological neurogenesis. If this were the case, NB69 cells could be an interesting model for using to further investigate the molecular mechanisms of NO action on neural precursor proliferation and differentiation.

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