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Nitric oxide effects on cell growth: GMP-dependent stimulation of the AP-1 transcription complex and cyclic GMP-independent slowing of cell cycling

#Clara Sciorati, †Giuseppe Nisticò, #Jacopo Meldolesi & #,*,[1]Emilio Clementi

#Consiglio Nazionale delle Ricerche Cellular and Molecular Pharmacology Center, and B. Ceccarelli Center, DIBIT-H San Raffaele Scientific Institute, University of Milano, Milano, †Dept. Biology, Mondino Neurobiology Center, University of Roma "Tor Vergata", Roma and *Dept. of Pharmacology, Faculty of Pharmacy, CNR-IBAF University of Reggio Calabria, Catanzaro, Italy

1 The role of nitric oxide (NO) in the control of cell growth is controversial since both stimulation and (more often) inhibition have been demonstrated in various cell types. In order to reinvestigate the problem and identify the sites of NO action, we have employed murine NIH-3T3 fibroblasts overexpressing epidermal growth factor (EGF) receptors.

2 The effects of four structurally-unrelated NO donors: S-nitroso-N-acetyl penicillamine, S-nitroso-L-glutathione, 3-morpholinosydnonimine and isosorbide dinitrate (0.01–3 mM) on EGF (10 nM)-stimulated cell growth were estimated by both thymidine incorporation and the colorimetric MTT assay, while those of a messenger generated in response to NO, cyclic GMP, were revealed by the use of 8-Br cyclic GMP (0.01–3 mM) as well as of blockers of guanylyl cyclase and cyclic GMP-dependent kinase I.

3 Studies were focused on: (i) multiple signalling events, including receptor-induced tyrosine phosphorylations, phosphorylation of mitogen-activated protein kinase, activation of the AP-1 transcription complex and deoxyribonucleotide synthesis; (ii) the progression through the cell cycle, dissected out by the use of staurosporine (1 nM), lovastatin (10 μM), mimosine (200 μM), hydroxyurea (1 mM) and nocodazole (1.5 μM).

4 NO was found to have no effects on the phosphorylation events of the growth factor cascade. In contrast, later processes were modified by the messenger but with opposite effects.

5 A cyclic GMP-dependent stimulation of growth was shown to be sustained in part by the activation of the AP-1 transcription complex, while a predominant, cyclic GMP-independent inhibition was found to be mediated by both the negative regulation of ribonucleotide reductase and the marked slowing down of the cell cycle occurring at early and late G1 and during the S phase.

6 Although multiple and apparently conflicting, the effects of NO here described could work coordinately in a general programme of cell growth regulation. In particular, the cyclic GMP-dependent actions might function as rapid modulatory events, while the effects on cell cycle might operate collectively as a multi-switch process whenever growth inhibition is required.

Keywords: Nitric oxide; cyclic GMP; EGF-signalling; cell cycle

Introduction

Nitric oxide (NO) the short-lived, diffusible messenger produced by NO synthase isoenzymes in many cell types, exerts a variety of actions among which is modulation of cell growth. Inhibition, observed initially in vascular smooth muscle cells (see Newby *et al*., 1992 for review), was subsequently shown in other cell types including fibroblasts, hepatocytes, bone marrow, retinal and mesangial cells (Garg & Hassid, 1989; 1990; Miyazaki *et al*., 1992; Punjabi *et al*., 1992; Goureau *et al*., 1993). However, in endothelial and glial cells the effects obtained were not only inhibitory (Garg *et al*., 1992; Yang *et al*., 1994) but also stimulating (Konturek *et al*., 1993; Muñoz-Fernàndez & Fresno, 1993; Ziche *et al*., 1994), the latter apparently important for angiogenesis associated with tissue healing (Konturek *et al*., 1993). Finally, in primary cultures of rat aortic smooth muscle cells a selective amplification of FGF-2-induced mitogenesis was demonstrated. However, this was replaced by a switch to inhibition later in the course of culturing (Hassid *et al*., 1994).

As far as the mechanisms underlying the above actions of NO, no matter whether stimulating or inhibitory, they were shown to be mediated by increased guanosine 3′:5′-cyclic monophosphate (cyclic GMP) generation, probably operating via the activation of cyclic GMP-dependent protein kinase I (G kinase) (see Miyazaki *et al*., 1992; Garg *et al*., 1992; Yang *et al*., 1994; Ziche *et al*., 1994; Yu *et al*., 1997; but see also Garg & Hassid, 1990). However, in the case of smooth muscle cells the involvement of adenosine 3′:5′-cyclic monophosphate (cyclic AMP)-dependent kinases, cross-activated by the high levels of cyclic GMP, was also demonstrated (Cornwell *et al*., 1994). How these events evolve to regulate cellular growth remains unclear. A possibility that has been envisaged is a direct interaction with the pathways activated by growth factors. NO donors have indeed been shown to increase total tyrosine kinase phosphorylation (Peranovich *et al*., 1995) and mitogen-activated protein kinases (MAPK) dephosphorylation (Baker & Buss, 1996), but only when administered at concentrations 10–100 fold higher than those already active on cell growth. Additional steps where NO has been shown to exert an at least putative stimulating activity on growth are increased expression of immediate early genes and enhanced transcription from AP-1 responsive promoters (Peunova & Enikolopov, 1993; Pilz *et al*., 1995; Morris, 1995). Compared to stimulation, knowledge of the functional links between molecular targets of NO and inhibition of cell growth is more limited and concerns only two cell systems. In leukaemia cells the antiproliferative action has been shown to occur essentially via inhibition of the ribonucleotide reductase activity (reviewed in Gross & Wolin, 1995). In smooth muscle cells, on the other hand, the same effect appears to be dependent on multiple mechanisms, including the decrease of both protein synthesis and thymidine

[1] Author for correspondence at: Dip. Farmacologia, DIBIT, Scientific Institute San Raffaele, Via Olgettina 58, 20132 Milano, Italy.

kinase activity (Garg & Hassid, 1993; Kolpakov *et al*., 1995). In addition, G kinase-mediated inhibition of the *Ras*-dependent activation of Raf-1 appears as one of the mechanisms by which NO inhibits growth response to epidermal growth factor (EGF), a potent mitogen for these cells (Yu *et al*., 1997).

In the present paper, the effects of NO have been investigated in a cell model widely employed for growth studies, the murine NIH-3T3 fibroblasts overexpressing the human EGF receptor. Individual key steps of the EGF-stimulated signalling pathway, from the early tyrosine kinase events to the activation of transcription and progression through the cell cycle were investigated. The results obtained demonstrate that NO does affect not early but late steps of growth factor signalling, acting both at the transcriptional level and at various stages of the cell cycle. These effects were predominantly inhibitory the others being stimulating and the former were independent and the latter dependent on cyclic GMP generation.

Methods

Cell culture

NIH-3T3 EGFR-T17, a cell clone overexpressing the human EGF receptor (4×10^5 EGF receptors/cell) (Magni *et al*., 1991), was routinely grown at 37°C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% newborn calf serum, 100 u ml^{-1} penicillin, and 100 u ml^{-1} streptomycin under a humidified atmosphere with 5% CO_2 as described by Magni *et al*. (1991) and used within the tenth week from thawing.

[^{3}H]-thymidine incorporation

Cells were seeded on 24-well Costar plates at a density of 1×10^4 cells cm^{-2} and grown for 3 days. After washing with PBS, subconfluent monolayers were incubated for 48 h in 0.5 ml serum-free medium (DMEM containing 50 nM transferrin and 100 nM Na_2 SeO_3). Cells were then stimulated with EGF (10 nM) for 17 h at 37°C and pulsed with 4 μCi ml^{-1} [methyl-^{3}H]-thymidine for an additional 6 h. In the experiments in which the effects of the NO donors, 8-Br cyclic GMP, ODQ, hydroxyurea, haemoglobin (Hb), superoxide dismutase (SOD) and KT5823 were investigated, the drugs were added either together with the growth factor or at different time-intervals after mitogenic stimulation. When ribonucleotide reductase activity was analysed, mixtures of either deoxyadenosine, deoxyguanosine, deoxycytosine (final concentrations 400, 400 and 1 μM, respectively), or the corresponding ribonucleosides as control, were administered. The reactions were stopped on ice. The cells were washed twice with PBS and the soluble radioactivity was extracted with 0.5 ml of 10% trichloroacetic acid for 20 min on ice, followed by another wash with the same solution. Precipitates were solubilized in 0.6 ml of 0.2 N NaOH. The incorporated radioactivity was quantified by scintillation counting in a Beckman β-counter.

Cell proliferation assays

For the colorimetric assay with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (Denizot & Lang, 1986), cells were seeded on 12-well plates (2.4×10^3 cells cm^{-2}), grown for 3 days in complete medium, starved without serum for 48 h and then stimulated with 10 nM EGF, alone or combined with either 8-Br cyclic GMP (1 mM), SNAP (100 μM), ISDN (100 μM), SIN-1 (100 μM) or GSNO (100 μM). Every 24 h the medium was changed and fresh EGF, NO donors or 8-Br cyclic GMP were added. At the indicated time points cells were washed and 0.5 ml of MTT stock solution (2.4 mM in RPMI 1640 without phenol red, filtered) was added and the plates were incubated for 3 h at 37°C. At the end of the incubation the untransformed MTT was carefully removed and the dye crystals solubilized in 1 ml of 2-propanol. Absorbances were read immediately in a UVKON 941 spectrophotometer by use of a test wavelength of 570 nm and a reference wavelength of 690 nm.

For cell counts, serum-starved cells received EGF with or without different concentrations of NO donors and 8-Br cyclic GMP as above. Samples, collected by trypsinization every 24 h, were diluted 1/10 in PBS and their cell number was established in a Coulter Counter (ZM, Coulter Electronics, Hialeah, FL, U.S.A.), with a lower threshold of 8.1 μm (Magni *et al*., 1991). Assays were performed in triplicate.

Immunoprecipitation, SDS-PAGE and Western blotting

EGFR-T17 cell monolayers were grown to ~80% confluence and subsequently starved in serum-free medium as described above. For the detection of EGF receptor and phospholipase Cγ (PLCγ) tyrosine phosphorylation, monolayers were incubated with 10 nM EGF for 2 min at 37°C in the presence or absence of either SNAP (200 μM) or 8-Br cyclic GMP (1 mM). For the analysis of the MAPK activity, treatments with EGF, alone or in the presence of either SNAP (200 μM) or 8-Br cyclic GMP (1 mM), were for 5, 15, 30 and 120 min. After the treatments, cultures were immediately washed with ice-cold Ca^{2+}/Mg^{2+}-free PBS and then solubilized for 15 min at 4°C in a lysis buffer containing: 1% Triton X-100 (v/v), 10% glycerol, 100 mM NaCl, 1.5 mM $MgCl_2$, 10 mM Na_3VO_4, 4 mM phenylmethylsulphonylfluoride, 20 mM sodium pyrophosphate, 5 mM [ethylene-*bis*(oxyethylenenitrilo)] tetracetic acid (EGTA), 20 μM leupeptin, 0.007 u ml^{-1} aprotinin, 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES), pH 7.5. The lysates were clarified by centrifugation ($14000 \times g$) for 15 min at 4°C. Immunoprecipitation of PLCγ and EGF receptor was performed by use of the appropriate anti-PLCγ and anti-EGF receptor Abs, in the presence of protein G-Sepharose 4B beads (Pharmacia LKB, Uppsala, Sweden), after 4 h incubation at 4°C with 3 mg of cellular lysates (determined by the bicinchoninic acid assay: BCA protein assay reagent, Pierce, Milano, Italy). Immunoprecipitates obtained by centrifugation were washed four times with a buffer containing: 150 mM NaCl, 0.1% Triton X-100 (v/v), 10% glycerol, 1 mM Na_3VO_4, 20 mM HEPES, pH 7.5.

For sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), 60 μg proteins (MAPK experiments) or 9/10 of the total immunoprecipitates were boiled in 8% SDS, 50% glycerol, 5 mM dithiothreitol, 80 mM Tris/HCl, pH 6.8 (Laemmli buffer) before separation on 10% SDS-polyacrylamide gels. High-efficiency transfer of proteins onto nitrocellulose membranes was carried out at 200 mA for 18 h in a buffer containing 25 mM Tris, 192 mM glycine, 20% methanol, pH 8.3. After transfer both the gels and the blots were routinely stained with Ponceau red. For Western blotting analysis of the PLCγ and EGF receptor immunoprecipitates, the nitrocellulose sheets were processed at room temperature first for 1 h with PBS + 3% bovine serum albumin, then for 2 h with appropriate concentrations of an anti-phosphotyrosine mAb (UBI, Frankfurt, F.R.G.). After washing five times for 5 min with 150 mM NaCl, 50 mM Tris-HCl, 0.05% Tween-20, 5% powdered milk, pH 7.4, the blotted bands were enhanced with ^{125}I-labelled goat anti-mouse Ab. In the case of the immunoprecipitation experiments, in order to reveal the efficiency of the process, both pellets and supernatants were routinely analysed by Western blotting using the same Abs employed for the precipitation. Analysis of MAPK activation (revealed by the appearance in SDS-PAGE of slower migrating forms of the enzyme due to phosphorylation of specific threonine and tyrosine residues) was performed as described for PLCγ and EGF receptor experiments with a rabbit anti-MAPK pAb and ^{125}I-labelled protein A. The blots were washed 5 times for 10 min with the above buffer, dried and finally autoradiographed at −80°C for variable periods of time. Microdensitometry of the relevant bands of immunoblots was carried out with a Molecular Dynamics Imagequant apparatus.

Enzyme assays

EGFR-T17 cells, plated on 10 cm Petri dishes, were transfected in serum-free medium by the calcium phosphate technique with 35 μg of either the puPAwtCAT3 plasmid, containing a chloramphenicol acetyl transferase (CAT) reporter gene under the control of the human urokinase enhancer (Nerlov *et al*., 1992), or the control plasmid devoid of the enhancer. In both cases, 15 µg of the pCH110 plasmid, containing the *lac*Z gene under the control of the SV40 early promoter (Rørth *et al*., 1990), were co-transfected as an internal control for transfection efficiency. Eighteen hours after transfection the cells were extensively washed in PBS and then grown in DMEM for an additional 24 h with or without 10 nM EGF, in the presence or absence of either SNAP (200 μM), 8-Br cyclic GMP (1 mM) or ODQ (1 μM). Cells were harvested by trypsinization, washed and homogenized at 4°C by 40 strokes of a Teflon-glass homogenizer in a medium containing: 100 mM NaCl, 1 mM EDTA, 10 mM Tris/HCl, pH 8.0. The homogenates were centrifuged at 500 $\times$ *g* for 2 min to eliminate nuclei and cell debris, then samples were divided for CAT and β-galactosidase assays. CAT assay was performed as described by Nerlov *et al*. (1992). For β-galactosidase assay, 100 µg protein of cell homogenates were dissolved in 1 ml of a buffer containing: 39 mM NaH_2PO_4, 60 mM Na_2HPO_4, 3 mM $MgSO_4$, 2 mM ethylenediaminetetraacetic acid (EDTA), 0.2 mM $MnCl_2$, 0.03% deoxycholate, 1.3 μM o-nitrophenyl-β-D-galactopyranoside, then 100 mM β-mercaptoethanol in the same buffer was added at 37°C. The reaction was stopped with 0.5 ml of 1 M Na_2CO_3 and the increase in OD_{420} min^{-1} used as a measure of enzyme activity. Final ratios of reporter enzyme levels were calculated after normalization to protein concentration and β-galactosidase activity.

Cell cycle analysis by flow cytometry

To obtain cell synchronization in the G0 phase, EGFR-T17 cells, plated in 6 cm Petri dishes at a density of 5×10^3 cells cm^{-2} and grown for 3 days, were washed twice with PBS and starved for 48 h in serum-free DMEM. The cells were then stimulated with 10 nM EGF to induce synchronous entry into the cell cycle. For synchronization in early or late G1 phase the cells, after EGF restimulation, were cultured for an additional 20 h in the presence of staurosporine (1 nM), lovastatin (10 μM) or mimosine (200 μM), respectively. Hydroxyurea (1 mM) was used under the same conditions to synchronize the cells in S phase. Growth arrest in G2/M phase was obtained by 6 h treatment with 1.5 μM nocodazole, administered 20 h after EGF. At the end of these treatments the cells were released from blockade by 3 washes with PBS, and then bathed in fresh, EGF-containing medium, with or without SNAP (200 μM) or 8-Br cyclic GMP (1 mM). In the experiments with lovastatin, 500 μM mevalonate was added after washing. Samples were collected every 2 h and processed as described (Vindelov & Christensen, 1990). Harvested cells were resuspended in 0.1% sodium citrate containing 75 μM propidium iodide at a density of 1×10^6 cells ml^{-1} and treated for 30 min at 4°C in the dark with 2.5 u ml^{-1} RNase A and 0.012% NP-40. Finally the cells were filtered through a Filcons filter (30 μm) to remove aggregates and analysed for DNA content by quantitating the red fluorescence in a FACSCAN apparatus (Becton & Dickson). The percentage of cells in G0/G1, S or G2/M phases of cell cycle were determined by analysis of the results by use of the CELLFIT computer programme (Becton & Dickson).

Methods

Materials and drugs

Culture sera and media were purchased from GIBCO (Basel, Switzerland); EGF, S-nitroso-N-acetylpenicillamine (SNAP), KT5823 (8R,9S,11S)-(−)-2N-methyl-9-methoxy-9-methoxcarbonyl-8-methyl-2,3,9,10 tetrahydro-8,11-epoxy-1H.8H. 11H-2,7b, 11e-triazadibenzo[a,g]cycloocte[cde]trinolei-1-one), propidium iodide, RNase A and S-nitroso-L-glutathione (GSNO) from Calbiochem (San Diego, CA, U.S.A.). [1H-[1,2,4] oxadiazole[4,3-a]quinoxalin-1-one] (ODQ) was purchased from Alexis (Läufelfingen, Switzerland). Mimosine was obtained from Biomol (Hamburg, F.R.G.); 3-morpholinosydnonimine (SIN-1) from Cayman (Miami, FL, U.S.A.). Deoxyribonucleosides and ribonucleosides were from Aldrich (Steinheim, F.R.G.); [methyl-^{3}H]-thymidine, [^{14}C]-chloramphenicol, ^{125}I-labelled goat anti-mouse antibody (Ab), ^{125}I-labelled protein A from Amersham Corporation (Buckinghamshire, U.K.). The anti-phospholipase Cγ (PLCγ) monoclonal Ab (mAb) was purchased from Transduction Laboratories (Lexington, KY, U.S.A.); the anti-MAPK polyclonal Ab (pAb) from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). The anti-EGF receptor 2913 pAb was a kind gift of Laura Beguinot (Milano Italy). The puPAwtCAT3 and pCH110 plasmids, as well as lovastatin, were kind gifts of Dario De Cesare and Francesco Blasi (Milano, Italy). 8-Br cyclic GMP, isosorbide dinitrate (ISDN), nocodazole and the remaining chemicals were from Sigma (Milano, Italy). Drugs were all dissolved in phosphate buffered saline (PBS), except for SNAP, ISDN, nocodazole, staurosporine and KT5823, for which dimethylsulphoxide (DMSO) was employed and mimosine which was dissolved in 500 mM $NaHCO_3$. In all the experiments appropriate controls with solvents alone were performed in parallel.

Statistical analysis

The results are expressed as means $\pm$ s.e.mean; *n* represents the number of different experiments. In the case of thymidine incorporation, cell proliferation and CAT assays results shown are averages of 6 experiments performed in triplicate. Western blots and flow cytometry data are representative of 4 experiments. Statistical analysis was performed by Student's *t* test for unpaired data (two-tail). A value of *P* less than 0.05 was considered to be statistically significant.

Results

Effects of NO and cyclic GMP on EGF-induced cell growth

To analyse in detail the effects of both NO and simple activation of the cyclic GMP pathway on DNA synthesis and proliferation of EGFR-T17 fibroblasts, four structurally unrelated NO donors, SNAP, ISDN, SIN-1 and GSNO, as well as the cyclic GMP analogue, 8-Br cyclic GMP, were investigated in parallel. Subconfluent monolayers, synchronized in the G0 phase by serum starvation, were first stimulated for 17 h with 10 nM EGF (a concentration known to be highly mitogenic in these cells, see Magni *et al*., 1991) administered in the presence or absence of increasing concentrations of either NO donors or 8-Br cyclic GMP, and then pulse-labelled for 6 h with [^{3}H]-thymidine. The results obtained are shown in Figure 1a. All four NO donors were able to inhibit thymidine incorporation in dose-dependent fashions, although with varying potencies. In contrast, 8-Br cyclic GMP treatment resulted in a slight, yet appreciable potentiation of DNA synthesis. Likewise, all NO donors were able to inhibit long term cell growth while 8-Br cyclic GMP exerted the opposite effect, as demonstrated by both the colorimetric MTT assay and the cell count experiments (Figure 1b and not shown).

To investigate whether the above inhibition by NO donors was indeed mediated through the generation of NO or due to additional actions of the compounds employed, DNA synthesis experiments were carried out in the presence of Hb, which binds and inactivates the gaseous messenger (Gross & Wolin, 1995). As shown in Figure 1c SNAP and SIN-1 completely lost their inhibitory effects on [^{3}H]-thymidine incorporation when

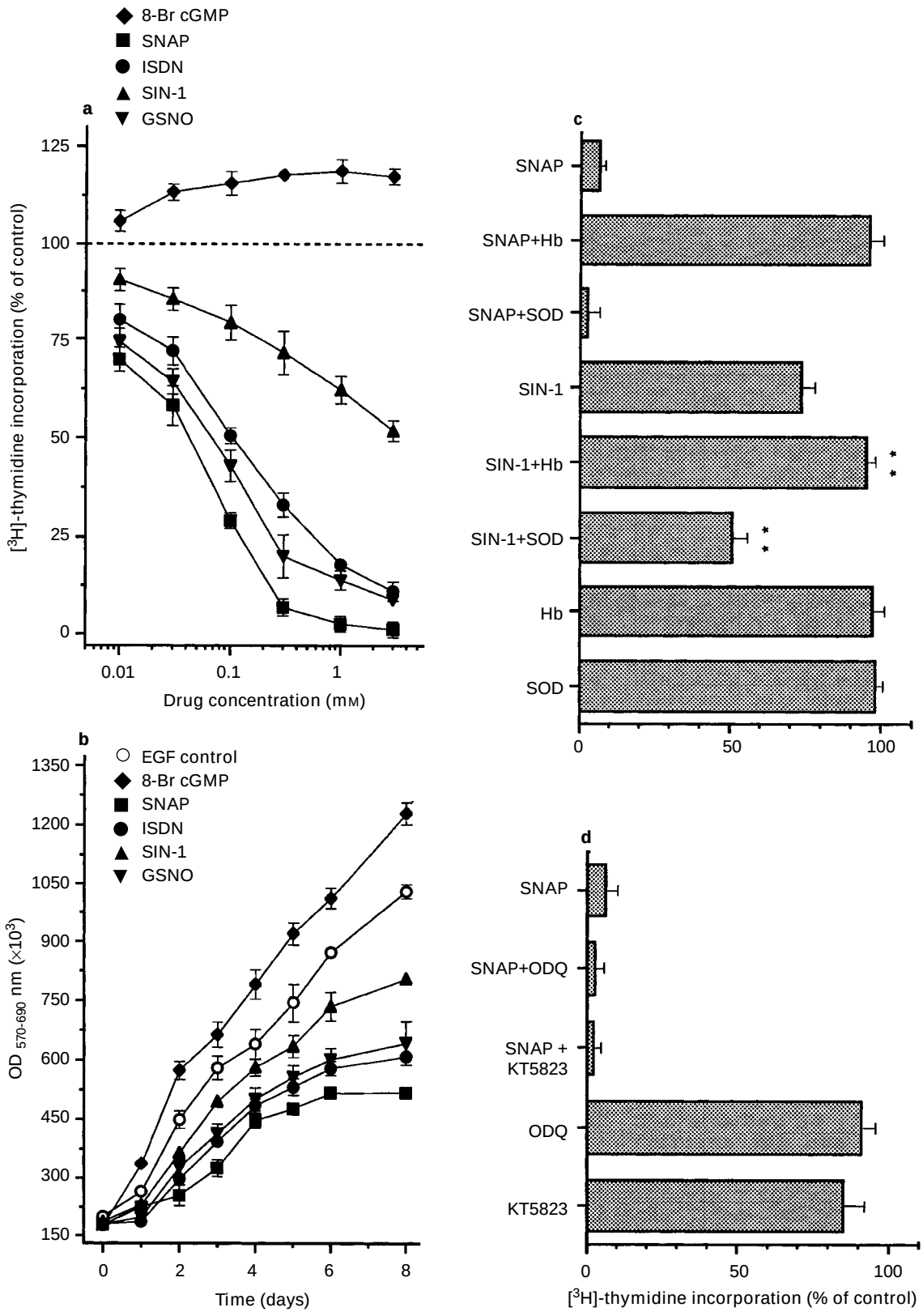

Figure 1 Effects of NO donors, 8-Br cyclic GMP, ODQ and KT5823 on EGF-stimulated growth of EGFR-T17 fibroblasts as revealed by the thymidine incorporation (a, c and d) and the colorimetric MTT growth assay (b). (a, c and d) Cell monolayers were synchronized for 48 h in serum-free medium and then exposed for 17 h to EGF (10 nM) in the presence of either 8-Br cyclic GMP, SNAP, ISDN, SIN-1 or GSNO, administered, before a 6 h pulse with [methyl-^{3}H]-thymidine, alone or together with Hb, SOD, ODQ or KT5823 as indicated. Where not specified (c, d) the NO donors were employed at 200 μM, Hb at 50 μM, SOD at 200 u ml^{-1}, ODQ at 1 μM and KT5823 at 3 μM. Results are expressed as % of control, i.e. of cells stimulated with EGF and treated only with the solvents used for the various drugs (see Methods). $^{**}P<0.01$ versus cells treated with SIN-1 (b) The cell monolayers were synchronized as in (a), (c) and (d), but the treatments with EGF either alone (control) or together with 1 mM 8-Br cyclic GMP, 100 μM SNAP, 100 μM ISDN, 100 μM SIN-1 or 100 μM GSNO, were protracted for the times shown on the abscissa scale with medium replacement every 24 h. Results shown are averages of 6 experiments, each run in triplicate; vertical lines show s.e.mean.

administered in a medium supplemented with Hb (50 μM), and the same was found when using the other NO-generating drugs, ISDN and GSNO (not shown). Another mechanism known to inactivate the effect of NO is its reaction with other radicals. In particular, NO can react with superoxide anions, produced within cells by various metabolic routes, thus generating peroxynitrites (Gross & Wolin, 1995). In order to investigate this problem, DNA synthesis was analysed in the presence of SOD (200 u ml^{-1}), an enzyme that removes superoxide anions thereby preventing their NO scavenging action (Gross & Wolin, 1995). Under these conditions, the inhibitory effects of both SNAP and SIN-1 on DNA synthesis were enhanced (Figure 1c) and the same occurred with ISDN and GSNO (not shown). The SOD potentiating effect was more pronounced with SIN-1, a result not unexpected for the drug is known to generate simultaneously not only NO but also superoxide anions (Hogg *et al*., 1992). Taken together, the results of these experiments clearly demonstrate that the inhibitory effects of NO donors are indeed mediated by the released messenger. This action of NO appears to be independent of cyclic GMP generation, since (i) the effects of 8-Br cyclic GMP were opposite to those of NO donors and (ii) both the selective inhibitor of the soluble guanylyl cyclase, ODQ (1 μM) (Garthwaite *et al*., 1995) and the specific G kinase inhibitor, KT5823 (3 μM), a drug previously shown to block the cyclic GMP-dependent effects in EGFR-T17 cells (Clementi *et al*, 1995), failed to affect the inhibition of DNA synthesis induced by NO donor administration (Figure 1d).

Effects of NO and cyclic GMP on EGF-activated cell signalling

From the experiments described above the ultimate effects of NO and cyclic GMP appear well defined. However, their mechanisms of action remain unclear. A detailed investigation of the signal transduction events induced by EGF when administered with or without NO donors or 8-Br cyclic GMP, from receptor autophosphorylation to AP-1 transcription complex activity, was thus carried out. Serum-starved EGFR-T17 cells, preincubated in the presence or absence of SNAP (200 μM) or 8-Br cyclic GMP (1 mM) for 15 min at 37°C, were treated with or without EGF (10 nM) for 2 min, then lysed and immunoprecipitated with Abs specific for either the EGF receptor or PLCγ. In both resting and EGF-stimulated cells, phosphorylation of the EGF receptor itself and PLCγ, revealed by Western blotting with an anti- phosphotyrosine mAb, was unaffected by the SNAP and 8-Br cGMP treatments (Figure 2 and not shown). Likewise, SNAP and 8-Br cyclic GMP failed to modify the time-dependent shift in mobility of MAPK, the well known result of the EGF-induced phosphorylation, and thus activation, of the enzyme (Figure 2 and not shown; drug concentrations as above). The effects of NO and cyclic GMP on transcription from AP-1-responsive promoters were next investigated. To this end, EGFR-T17 cells were transiently transfected with a construct including the human urokinase enhancer, that is known to contain two AP-1 sites

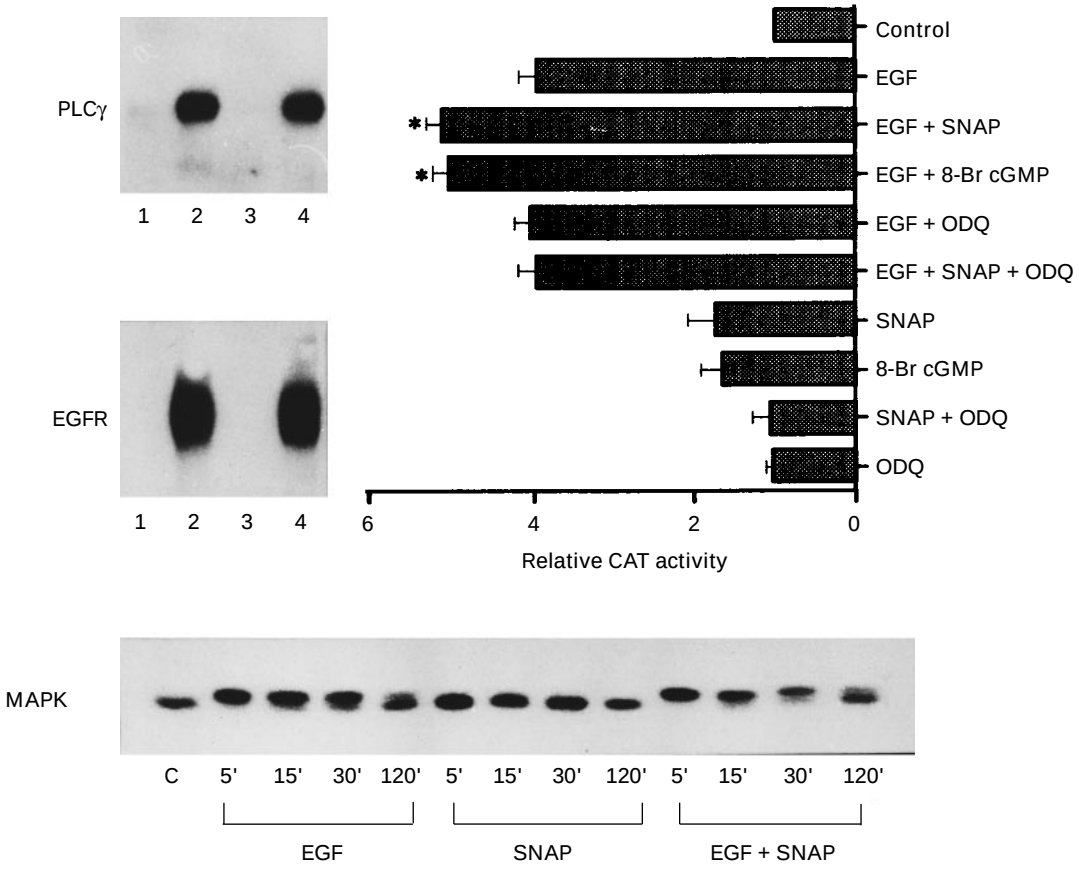

Figure 2 Effects of SNAP, 8-Br cyclic GMP, and ODQ on the control and EGF-induced tyrosine phosphorylation of EGF receptor and PLCγ, phosphorylation of MAPK, and AP-1-controlled CAT activity in EGFR-T17 fibroblasts. For tyrosine phosphorylation cell monolayers were treated with EGF (10 nM) with or without SNAP (200 μM) for 2 min at 37°C; for MAPK phosphorylation for the times indicated on the abscissa scale. In the case of EGF receptor and PLCγ, results shown were obtained with immunoprecipitates of cells treated with EGF (lanes 2 and 4) with (lanes 1 and 2) or without (lanes 3 and 4) SNAP. Results shown are representative of 4 experiments. CAT activity was assayed in monolayers of cells transfected by 18 h exposure to the CAT reporter gene under the control of the human urokinase enhancer, followed by 24 h culture with or without EGF (10 nM), SNAP (200 μM), 8-Br cyclic GMP (1 mM) or ODQ (1 μM). Results shown are averages $\pm$ s.e.mean obtained in 6 experiments run in triplicate. $^*P<0.05$ versus cells treated with EGF alone EGF plus ODQ, and EGF plus ODQ and SNAP.

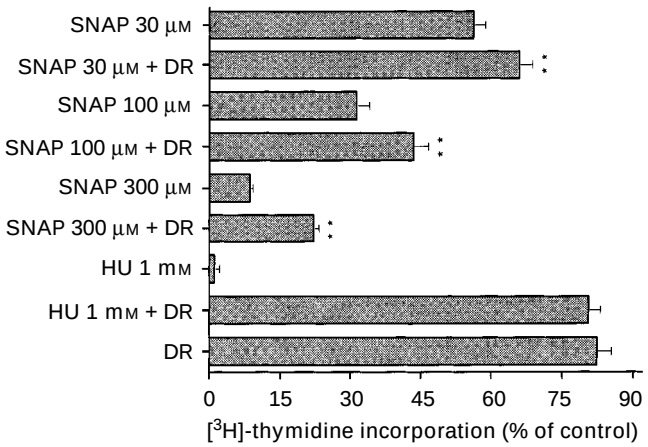

Figure 3 Role of deoxyribonucleotide synthesis in the blockade of [methyl-^{3}H]-thymidine incorporation by SNAP and hydroxyurea. EGFR-T17 monolayers, synchronized by starvation, were treated with EGF (10 nM) alone (control) or together with the indicated concentrations of SNAP and hydroxyurea (HU). DR indicates a mixture of deoxyribonucleosides as specified in the Methods section. Results shown are averages ± s.e.mean from 6 experiments run in triplicate. $^{**}P<0.01$ versus cells treated with SNAP.

inducible by EGF (Rørth *et al*., 1990; Nerlov *et al*., 1992), followed by a CAT reporter gene. Both the SNAP and the 8-Br cyclic GMP treatments were found to induce slight, but statistically significant stimulations of CAT activity over control and EGF-induced values, which were completely blocked by pretreatment of the cells with ODQ (1 μM; Figure 2). These results are therefore consistent with previous observations of NO-induced, cyclic GMP-dependent increase in transcription from promoters containing AP-1-binding sites (Pilz *et al*., 1995; Gudi *et al*., 1996).

Effects of NO on deoxyribonucleotide synthesis

The results illustrated in the preceding section may explain, at least in part, the slight potentiation of cell growth observed with 8-Br cyclic GMP. In addition, they reveal that the molecular level(s) at which NO exerts its cyclic GMP-independent inhibitory function are located downstream with respect to the classical cascade of EGF-activated signals. Among the known sites of action of the messenger, a possible candidate for cell growth control appeared to be ribonucleotide reductase, the rate limiting enzyme for deoxyribonucleotide synthesis that, in various cell systems, has been shown to be inhibited in a cyclic GMP-independent way (reviewed in Gross & Wolin, 1995). In order to investigate this possibility, serum-starved monolayers,

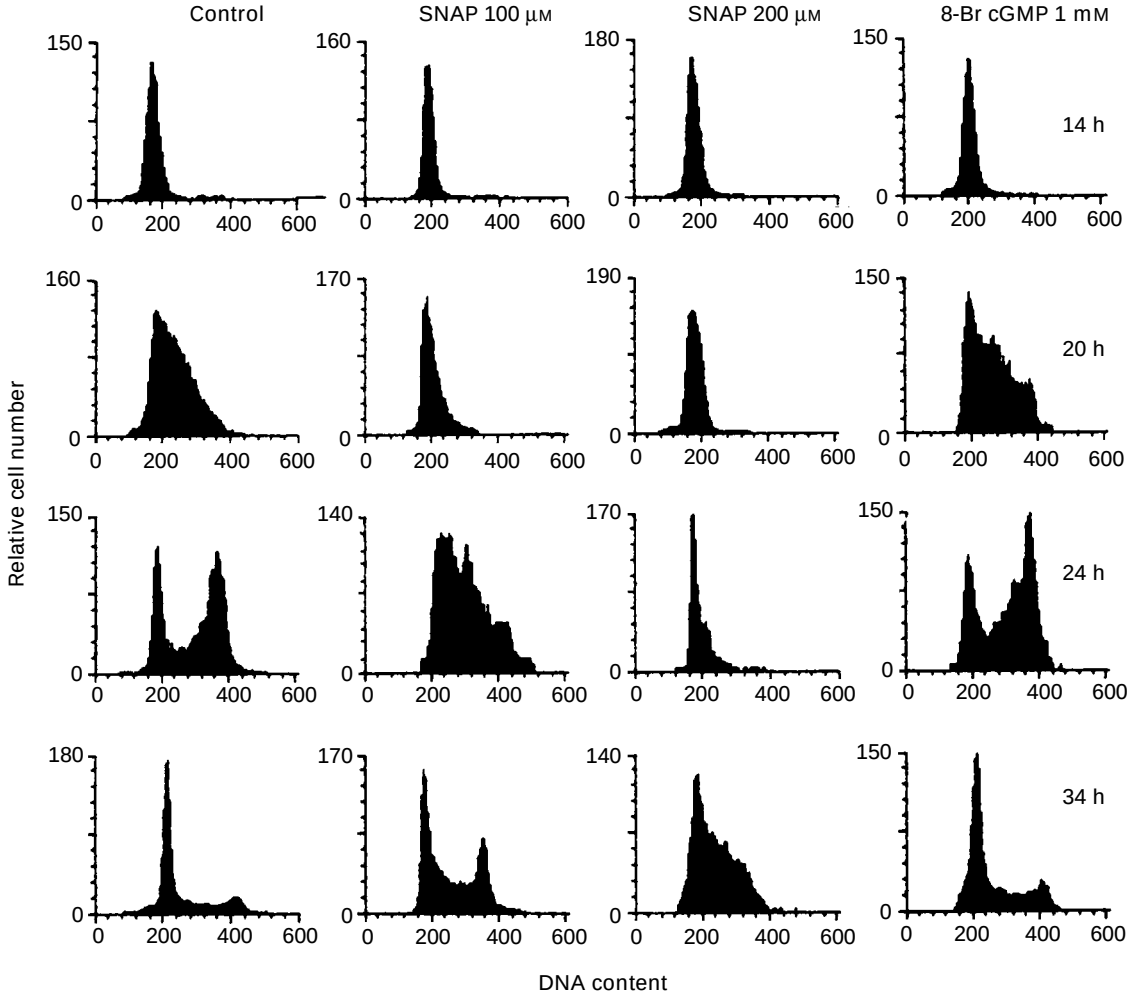

Figure 4 Effects of NO and 8-Br cyclic GMP on the cell cycle of EGFR-T17 cells. Cell monolayers, synchronized by starvation, were treated with EGF (10 nM), alone (controls) or together with SNAP or 8-Br cyclic GMP (indicated at the top), and analysed for DNA content at the time points specified to the right. Results shown here and in Figures 5 and 6 are representative of 4 experiments.

stimulated with EGF (10 nM) in the presence or absence of various concentrations of SNAP, were pulsed with [^{3}H]-thymidine, added alone or together with a mixture of deoxyribonucleosides to bypass any possible block at the level of the enzyme. In control experiments the effect of hydroxyurea (1 mM), a well-known blocker of ribonucleotide reductase, was also tested, with or without deoxyribonucleosides added. As shown in Figure 3, while deoxyribonucleosides almost completely abolished the inhibitory effect of hydroxyurea on DNA synthesis, their attenuation of the SNAP inhibition was only partial, at all concentrations of the NO donor tested. However, the effects observed appeared specific, because, similar to those of hydroxyurea, the inhibitory actions of SNAP were unaffected when a mixture of ribonucleosides was employed instead of deoxyribonucleosides (not shown).

Effects of NO and cyclic GMP on cell cycle

The above results suggest that inhibition of ribonucleotide reductase is not the only effect of NO responsible for inhibition of cell growth. In order to identify other sites of action, the effects of NO on the cell cycle were investigated in detail. Serum-starved, G0-synchronized monolayers were challenged with 10 nM EGF in the presence or absence of various concentrations of SNAP, and aliquots of cells, collected at different times after stimulation, were examined by flow cytometric analysis. As shown in Figure 4, at 20 h after EGF administration the fraction of control cells in the S phase was $31.2 \pm 3.60\%$ ($n=4$). With 8-Br cyclic GMP (1 mM) more cells were in the S phase ($54.7 \pm 4.11\%$ cells after 20 h, $n=4$), documenting a slight acceleration of cell cycle, consistent with the modest increase in cell growth described in Figure 1b. In contrast, essentially all the SNAP-treated cells were still in G0/G1 at the 20 h time point. These cells entered S phase 4–14 h later, depending on the concentration of the NO donor employed (100 and 200 μM, respectively) (Figure 4).

To reveal at which phase the inhibitory action of NO was exerted, the cell cycle was dissected out by the use of various pharmacological agents: the PKC blocker, staurosporine, known to arrest fibroblasts 2.5 h after the beginning of G1 (Gadbois *et al*., 1992); the fungal metabolite, lovastatin, and the plant amino acid, mimosine, which arrest cell cycle at later steps during G1, due especially to their interference with the activity of cyclin-dependent kinases (Lalande, 1990; Yoshida *et al*,. 1992 Hengst *et al*., 1994); hydroxyurea and the microtubule disassembler, nocodazole, that arrest cells in the S and G2/M phases, respectively (Moore & Hurlbert, 1985; Jordan *et al*., 1992). Recently mimosine was shown to inhibit also enzymes active in the S phase of the cycle, i.e. ribonucleotide reductase (Gilbert *et al*., 1995; Alpan & Pardee, 1996) and serine methyltransferase (Lin *et al*., 1996), two effects which do not appear to be of major importance in our experimental conditions (see below).

The results obtained with the various drugs are shown in Figures 5 and 6. Treatments of cell monolayers growing in EGF-containing, serum-free medium were with either staurosporine (1 nM), lovastatin (10 μM), mimosine (200 μM) or hydroxyurea (1 mM) for 20 h, or with nocodazole (1.5 μM) for 6 h. Cells were released from the blockade by careful drug removal (see Methods), then bathed in fresh medium containing EGF, with or without SNAP (200 μM). Samples were collected at various times and analysed by flow cytometry. As shown in Figure 5, of the cells first treated with staurosporine and then grown in the medium containing EGF alone, $71.6 \pm 6.43\%$ ($n=4$) were found in S phase 18 h after washing, while those treated also with SNAP were still arrested. Of the latter cells, only $25.9 \pm 3.12\%$ ($n=4$) were in S phase after 32 h,

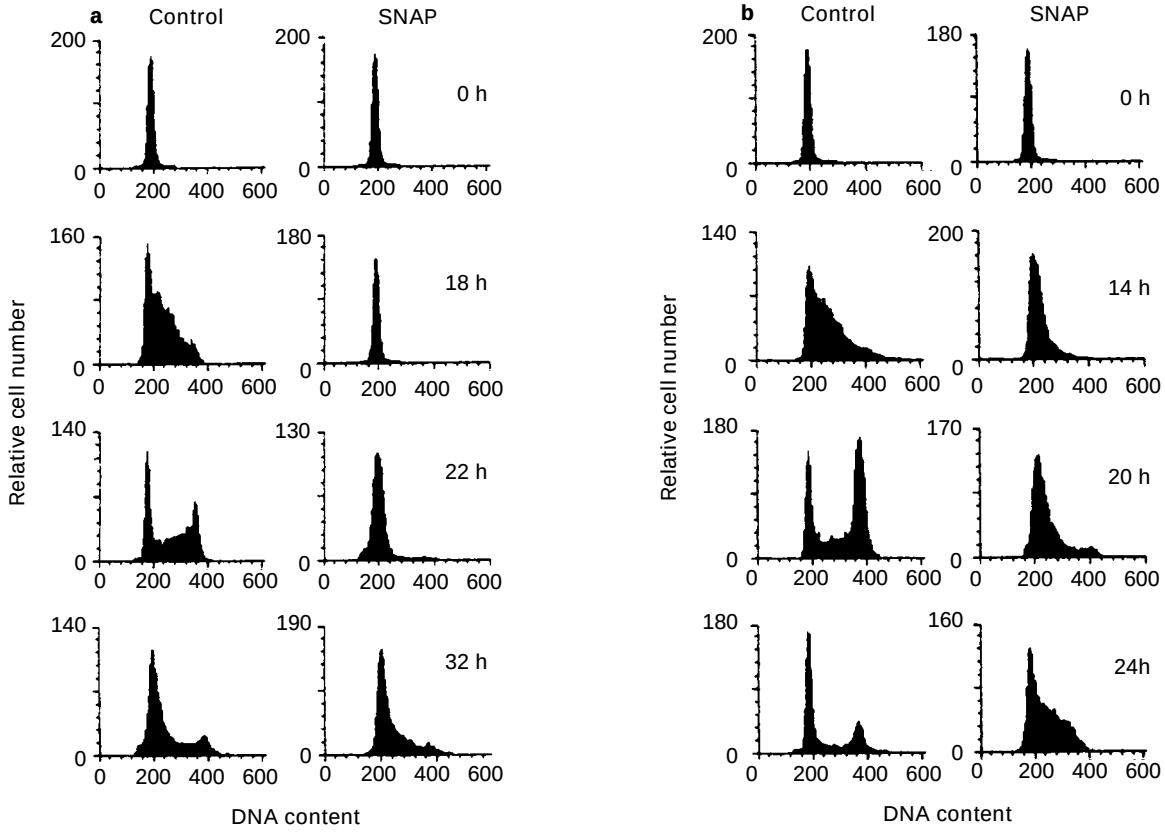

Figure 5 Cell cycle effects of NO at early and late steps of the G1 phase. EGFR-T17 monolayers, synchronized by starvation, were treated with EGF (10 nM) for 20 h in the presence or absence of either (a) staurosporine (1 nM) or (b) mimosine (200 μM). After washing they were cultured with EFG alone (control) or together with SNAP (200 μM) for the times indicated to the right of each panel.

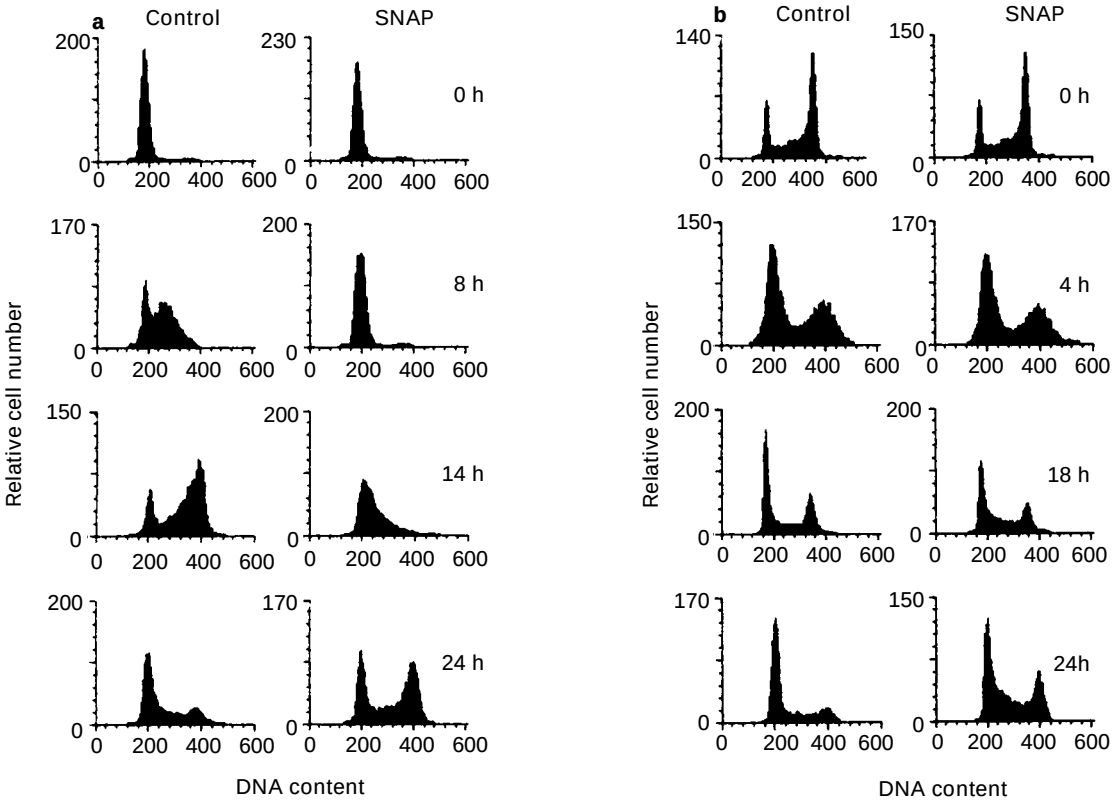

Figure 6 Cell cycle effects of NO at the S and G2/M phases. Experiments were performed as in Figure 5. However, the treatment with EGF (10 nM) and (a) hydroxyurea (1 mM) was for 20 h while that with (b) nocodazole (1.5 μM) was for 6 h. After washing the cells were cultured with EFG alone (control) or together with SNAP (200 μM) for the times indicated to the right of each panel.

with a delay similar to that observed when the NO donor was applied to serum-starved, G0-arrested cells. Mimosine treatment synchronized cells in G1 (figure 5, upper panels). However, its action occurred at a later step with respect to staurosporine, since the time necessary for cell re-entry in the S phase after drug removal was shorter. Of importance, also the delay of SNAP-treated cells versus controls was reduced with mimosine with respect to that observed with staurosporine. In particular, an S phase value approaching that of controls at 14 h ($77.0 \pm 6.83\%$, $n=4$) was observed in SNAP-treated cells at 24 h ($56.2 \pm 4.89\%$, $n=4$). With lovastatin, both the time necessary for cell re-entry in the S phase and the delay induced by SNAP treatment were similar to those observed with mimosine (not shown). More rapid was the progression through the cycle in cells treated with hydroxyurea, where $69.6 \pm 6.71\%$ ($n=4$) of cells were already in S phase 8 h after removal of the drug (Figure 6). Also in this case, SNAP treatment resulted in a delay in cell cycle progression ($58.2 \pm 5.17\%$ of cells in S phase 14 h after hydroxyurea removal, $n=4$), which was clearly shorter if compared with that observed with staurosporine and mimosine. A completely different picture emerged when cells were blocked in G2/M with nocodazole. In this case no difference was observed between control and SNAP-treated cells (Figure 6). However, when a new cycle began, SNAP-treated cells were delayed in G1/S (bottom panels), as expected from the inhibitory effect exerted by NO at these levels (see above).

Additional evidence for the inhibitory role of NO on the cell cycle was obtained by experiments in which the effects of addition/subtraction of NO to serum-starved, G0-arrested cells at various times after EGF administration were investigated (Figure 7). As can be seen, when administered before the entry into the S phase of the cycle (in these cells about 18 h after EGF stimulation, C. Sciorati, unpublished observations), SNAP (200 μM) was able to reduce dramatically [^{3}H]-thymidine incorporation (Figure 7, solid circles). Such an overall effect appears to result from the summation of individual events occurring at three major steps i.e. at $\sim$2, 8–9 and 18 h after EGF administration, in agreement with the results obtained with staurosporine- and lovastatin/mimosine-synchronized cells. When, by contrast, SNAP was administered after cell entry into the S phase, its inhibitory effect was clearly reduced, as expected from the results of the experiments performed with hydroxyurea-synchronized cells. Experiments run in parallel, in which SNAP was administered together with EGF and the NO produced was trapped at various times by Hb (50 μM) addition, gave results consistent with those described above. The inhibitory effect of NO on [^{3}H]-thymidine incorporation in fact dropped, remaining visible only if the messenger was trapped not immediately but after at least 3 h of incubation (Figure 7, open circles). The lack of reversibility of the NO effect that emerged in these experiments with Hb was only apparent, due to the delayed entry into S phase induced by the SNAP treatment (32 instead of 18 h after EGF administration, see Figure 5, i.e. far away from the 17–23 h time-window employed for [^{3}H]-thymidine incorporation). When in fact the time-window was prolonged, the inhibition was found to be reversible (not shown), as expected from the FACS analysis results. Taken together, these results indicate that the inhibitory, cyclic GMP-independent effect of NO on cell growth is a complex process occurring at multiple steps during G1 and S phases of cell cycle. The only phase apparently not affected by NO appears G2/M transition.

Discussion

Until now the information about the role of NO in the control of cell growth has remained largely phenomenological and also conflicting since both stimulant and inhibitory effects have been obtained. A striking example is the vascular system, where NO produced by endothelia is known to

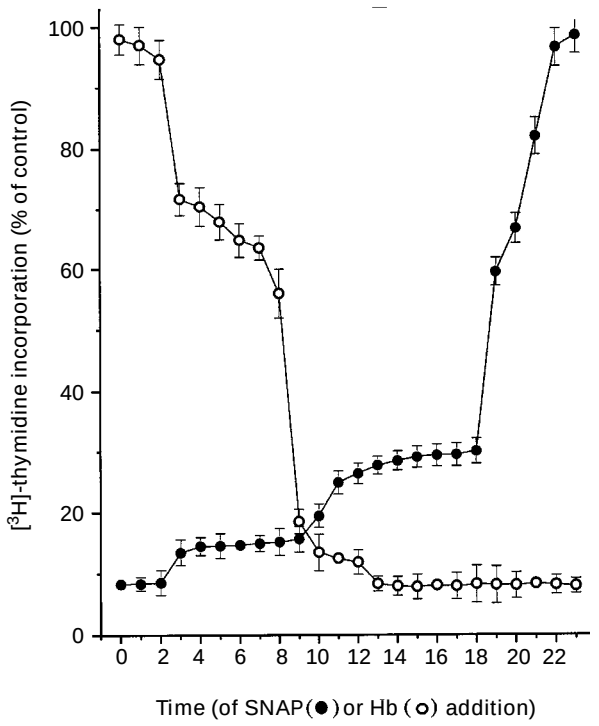

Figure 7 Effects of thymidine incorporation of NO addition/trapping at various times during the cell cycle. Parallel cell monolayer preparations (a total of 144) were synchronized for 48 h in serum-free medium and then exposed (time 0) to EGF (10 nM). Of these 72 (open circles) received SNAP (200 μM) and were then divided in 24 groups of 3 monolayers each, which received Hb (50 μM) one group every h, from time 0 to the 23rd h. The other half (solid circles) received SNAP alone, administered according to the schedule of Hb. The 6 h pulse with [methyl-^{3}H]-thymidine was from the 17th to the 23rd h, as described in Figure 1. Results shown are averages from 6 experiments run in triplicate (vertical lines show s.e.mean) and are expressed as % of control, i.e. of cells stimulated with EGF and treated only with the solvents used for SNAP and Hb (see Methods).

induce two distinct effects: quiescence of smooth muscle cells, an effect synergic with the inhibition of platelet and monocyte adhesion/activation in the overall control of peripheral vascular resistance (De Mey *et al*., 1991); and stimulation of endothelial cell proliferation, based on which the messenger is envisaged as an autocrine mediator for neovascularization (Ziche *et al*., 1994). Such a dual NO-induced effect is believed to be particularly relevant in the control of solid tumours, where pro- or anti-growth effects depend on the balance between the angiogenic activity of the messenger and its cytostatic effect on tumour cells (Jenkins *et al*., 1995). Largely unknown have remained the mechanisms that mediate intracellularly the NO signal to cell growth. Except for the cyclic GMP-independent inhibition of ribonucleotide reductase (Gross & Wolin, 1995), the attenuation of *Ras*/Raf coupling (Yu *et al*., 1997) and the inhibition of protein synthesis and thymidine kinase (Kolpakov *et al*., 1995; Garg & Hassid, 1993), still partially explained are the links with growth control for the other effects of the messenger including stimulation of immediate early gene expression (Peunova & Enikolopov, 1993; Pilz *et al*., 1995; Morris, 1995; Gudi *et al*., 1996), MAPK dephosphorylation and increased EGF receptor phosphorylation (Peranovich *et al*., 1995; Baker & Buss, 1996).

The aim of the present investigation was to identify both the nature and the mechanism by which NO intervenes in growth-related events, as revealed by a single, yet widely employed experimental model, i.e. the EGF treatment of mouse NIH-3T3 fibroblasts overexpressing the specific receptor. At least two well known properties had recommended the use of these cells: their clear signalling and growth responses induced by EGF (see e.g. Magni *et al*., 1991; Clementi *et al*., 1995) and their sensitivity to NO, with rapid increase of their cytosolic cyclic GMP levels (Clementi *et al*., 1995).

The results we have obtained reveal a series of important new aspects of NO action. First, the effects on phosphorylation of the EGF receptor and MAPK, previously described by others in NIH-3T3 (Peranovich *et al*., 1995; Baker & Buss, 1996), but only with high (mM) concentrations of NO donors or 8-Br cyclic GMP, failed to appear in our cells at concentrations of either these drugs sufficient to induce marked effects on growth. Our results raise serious questions about the physiological significance of these previously demonstrated effects. Second, the two conflicting actions of NO, i.e. stimulation and inhibition of cell growth, previously described at different culture stages (Hassid *et al*., 1994) or in different cell systems (Konturek *et al*., 1993; Muñoz-Fernàndez & Fresno, 1993; Ziche *et al*., 1994; Jenkins *et al*., 1995), were found in contrast to take place simultaneously in our cells. Third, and more importantly, the intracellular events supporting the conflicting actions of NO in EGFR-T17 cells were found (i) to be multiple in both cases and (ii) to depend on different signalling pathways. The slight stimulation was in fact mediated via cyclic GMP generation; the strong inhibition via cyclic GMP-independent pathway(s). A role of AP-1 specific transcription in the stimulation by NO of EGF-induced growth had already been described in several cell types (Angel & Karin, 1991), although not yet in NIH-3T3 cells. The possibility therefore exists that the stimulation of growth mediated by cyclic GMP is due, at least in part, to the activation of these transcriptional events. In our experimental conditions NO/cyclic GMP effects were of apparently modest entity. However, they are significant, if one takes into account that, when exposed to a potent mitogen such as EGF, the AP-1-sensitive promoter employed in our study induced only a 4–5 fold increase in transcription above the basal level (see Figure 3 and Rørth *et al*., 1990). Other cyclic GMP-dependent actions of NO on EGF-mediated signalling, already described in the EGFR-T17 cell system, might also play an important role. Of particular interest in this respect is the increase of the EGF-stimulated Ca^{2+} influx (Clementi *et al*., 1995), a process known to have pro-mitogenic effects in these as in many other cell types (reviewed in Clementi & Meldolesi, 1996).

As far as the NO inhibitory effects, they appear to be exerted at the level of the cell cycle, i.e. downstream of early gene expression. At this level the only mechanism already known was the blockade of ribonucleotide reductase, which in our cells was found to account for only a small fraction of the overall inhibition. Additional, considerable contributions were revealed by the consistent results obtained in two distinct types of experiments: addition/trapping of NO at various time-points after mitogenic stimulation; and pharmacological synchronization of the cells at specific steps, i.e. at early and late G1, and S phases, respectively. At each of these steps the cyclic GMP-independent events induced by NO resulted in marked delays of cycling. In contrast, the G2/M step was unaffected by NO, a negative observation that confirms the specificity of the inhibition found at the other steps of the cell cycle. We conclude therefore that the NO action on the cell cycle is not widespread but rather concentrated at discrete, yet multiple steps. The signal transduction pathways responsible for the cell cycle effects of NO remain at present undefined. Since the most effective among the NO donors employed were SNAP and GSNO, both S-nitroso compounds, a possible mechanism could be S-nitrosylation of proteins. Additional pathways, such as protein ADP-ribosylation, could also be involved (Gross & Wolin, 1995). Whatever the mechanism(s), it is worth noting that the results presented here have been obtained with NO donors and 8-Br cyclic GMP employed in a wide range of concentrations. Thus, although in living cells the endogenous NO levels may not reach those generated by the highest concentrations of the drugs employed, the physiological significance of our conclusions remains valid.

The unusual variability of the NO effects in the regulation of cell growth emerging from the literature appears to correlate well with both the complexity of the signalling pathways activated by the gaseous messenger and with the varying mechanism of growth control present in different cell systems. For instance, inhibition of the EGF-induced proliferation was recently shown in vascular smooth muscle, but via a pathway completely different from the one active in our cells, i.e. activation of G kinase, with ensuing inhibition of Raf-1 and then of MAPK activities (Yu *et al*., 1997). The heterogeneity and cell specificity of NO functions do not concern cell growth only. Indeed, in different cells, G kinase-dependent phosphorylation of inositol 1,4,5-trisphosphate receptor may result in either stimulation or inhibition of its activity (see e.g. Cavallini *et al*., 1996; Guihard *et al*., 1996). Similarly, the capacitative Ca^{2+} influx may be either stimulated or inhibited (see e.g. Xu *et al*., 1994; Nakamura *et al*., 1995).

The effects of NO on cell growth as they emerge from the data presented here although multiple and apparently conflicting, may be ultimately coordinate in a general cell physiology programme. In fact, the cyclic GMP-dependent actions might function as modulatory events, readily tuned because of both the short times required for their onset/offset and the fine modulation of the cytosolic cyclic GMP levels. The inhibitory, cyclic GMP-independent events, on the other hand, might function collectively as a multi-switch process to be activated whenever growth inhibition is required, e.g. as a safety response to pathological conditions, including tumour growth (Jenkins *et al*., 1995), or at crucial steps of cell life, such as differentiation toward a defined phenotype. The unique property of NO revealed by the present study, i.e. the ability to operate differentially and at multiple levels, taken together with its well known diffusibility, with ensuing actions not only in the generating but also in adjacent cells, may be particularly appropriate for regulating growth of multicellular structures and organs. At least in the case of the nervous system such a role is now being recognized. NO-induced growth arrest appears in fact critical for neuronal differentiation (Peunova & Enikolopov, 1995) and for the coordinate development of important brain areas, such as the visual cortex and olfactory bulb (Roskams *et al*., 1994; Wu *et al*., 1994).

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