Nitric oxide reversibly inhibits the epidermal growth factor receptor tyrosine kinase

Carmen ESTRADA*, Carmen GÓMEZ*, José MARTÍN-NIETO†, Trinidad DE FRUTOS†[1], Amparo JIMÉNEZ† and Antonio VILLALOBO†[2]

*Departamento de Fisiología, Facultad de Medicina, Universidad Autónoma de Madrid, Arzobispo Morcillo 4, 28029 Madrid, Spain, and †Instituto de Investigaciones Biomédicas, Consejo Superior de Investigaciones Científicas, Arturo Duperier 4, 28029 Madrid, Spain

Although it has been demonstrated that NO inhibits the proliferation of different cell types, the mechanisms of its antimitotic action are not well understood. In this work we have studied the possible interaction of NO with the epidermal growth factor receptor (EGFR), using transfected fibroblasts which overexpress the human EGFR. The NO donors *S*-nitroso-*N*-acetylpenicillamine (SNAP), 1,1-diethyl-2-hydroxy-2-nitrosohydrazine (DEA-NO) and *N*-{4-[1-(3-aminopropyl)-2-hydroxy-2-nitrosohydrazino]butyl}propane-1,3-diamine (DETA-NO) inhibited DNA synthesis of fibroblasts growing in the presence of fetal calf serum, epidermal growth factor (EGF) or EGF plus insulin, as assessed by [*methyl*-^{3}H]thymidine incorporation. Neither 8-bromo-cGMP nor the cGMP-phosphodiesterase inhibitor zaprinast mimicked this effect, suggesting that NO is unlikely to inhibit cell proliferation via a cGMP-dependent pathway. SNAP, DEA-NO and DETA-NO also inhibited the transphosphorylation of the EGFR and its tyrosine kinase activity toward the exogenous substrate poly-L-(Glu-Tyr), as measured in permeabilized cells using [γ-^{32}P]ATP as phosphate donor. In contrast, 3-[morpholinosydnonimine hydrochloride] (SIN-1), a peroxynitrite-forming compound, did not significantly inhibit either DNA synthesis or the EGFR tyrosine kinase activity. The inhibitory action of DEA-NO on the EGFR tyrosine kinase was prevented by haemoglobin, an NO scavenger, but not by superoxide dismutase, and was reversed by dithiothreitol. The binding of EGF to its receptor was unaffected by DEA-NO. The inhibitory action of DEA-NO on the EGF-dependent transphosphorylation of the receptor was also demonstrated in intact cells by immunoblot analysis using an anti-phosphotyrosine antibody. Taken together, these results suggest that NO, but not peroxynitrite, inhibits in a reversible manner the EGFR tyrosine kinase activity by S-nitrosylation of the receptor.

INTRODUCTION

NO is synthesized from L-arginine in different cell types and regulates a diversity of physiological functions [1–6]. Three distinct NO synthase (NOS) isoenzymes have been identified in mammals: eNOS and nNOS, constitutively expressed in endothelial cells and neurons respectively generate small amounts of NO, which acts as an intercellular messenger; iNOS, on the other hand, can be induced by pro-inflammatory cytokines in macrophages and in many other cell types, resulting in a sustained high output of NO, which leads to tumour cell and microorganism cytotoxicity, and also to functional cell modifications through its interaction with different proteins [7,8].

NO has been shown to arrest cell proliferation. This effect can be elicited either by exogenous NO, added as a gas or released from NO donors [9–13], or by endogenous NO on induction of iNOS in the target cells [14] or in their neighbours [15]. It has been proposed that activation of guanylate cyclase drives the anti-proliferative action of NO in smooth-muscle, cerebellar glial, and vascular endothelial cells [9,11–13]. However, the increase in intracellular cGMP concentration does not mimick the anti-mitotic effect of NO in fibroblasts [10], suggesting that other mechanisms are also involved. In this context, the inhibition of the enzyme ribonucleotide reductase by NO generated on induction of iNOS has been well documented in adenocarcinoma cells [14,16].

The first step in the intricate pathways leading the cell into mitosis is the activation of membrane-bound growth factor receptors by their specific ligands. The epidermal growth factor (EGF) receptor (EGFR), a member of the tyrosine kinase receptor superfamily [17–19], exhibits two prominent cysteine-rich domains in its extracellular region and is widely distributed in different cell types, including fibroblasts. NO has been shown to regulate the function of some enzymes and cell-signalling proteins by S-nitrosylation of critical cysteine residues [20–27]. Therefore the EGFR constitutes a possible target for NO effects on cell proliferation.

We have tested the hypothesis that NO may prevent cell proliferation by directly inhibiting the EGFR tyrosine kinase activity, using a fibroblast cell line transfected with a human EGFR cDNA. The advantages of using these cells are, first, that NO can be expected to inhibit their growth through cGMP-independent pathways as occurs in other fibroblasts [10] and, second, that they overexpress the EGFR, making it easier to

Abbreviations used: DAB, 3,3′-diaminobenzidine; DEA-NO, 1,1-diethyl-2-hydroxy-2-nitrosohydrazine; DETA-NO, *N*-{4-[1-(3-aminopropyl)-2-hydroxy-2-nitrosohydrazino]butyl}propane-1,3-diamine; DMEM, Dulbecco's modified Eagle's medium; DTT, dithiothreitol; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; FCS, fetal calf serum; NOS, NO synthase; SIN-1, 3-[morpholinosydnonimine hydrochloride]; SNAP, *S*-nitroso-*N*-acetylpenicillamine; SNP, sodium nitroprusside; SOD, superoxide dismutase; SPER-NO, 2,2′-(hydroxynitrosohydrazino)bis-ethanamine; zaprinast, 2-*O*-propoxyphenyl-8-azapurin-6-one.

[1] Present address: Laboratorio de Nefrología e Hipertensión, Fundación Jiménez Díaz, Avda. Reyes Católicos 2, 28040 Madrid, Spain.

[2] To whom correspondence should be addressed.

detect any functional alteration of this receptor. We present here evidence that NO directly acts on the EGFR, reversibly reacting with sensitive thiol group(s) and producing inhibition of both its transphosphorylation and its tyrosine kinase activity toward exogenous substrates without affecting binding of its ligand.

MATERIALS AND METHODS

Chemicals

The following products were used: [γ-^{32}P]ATP (triethylammonium salt) (3000–5000 Ci/mmol) from Amersham or ICN Pharmaceuticals; [^{125}I]EGF (150–200 μCi/μg) and [*methyl*-^{3}H]thymidine (40–60 Ci/mmol) from New England Nuclear; *S*-nitroso-*N*-acetylpenicillamine (SNAP) *N*-{4-[-1-(3-aminopropyl)-2-hydroxy-2-nitrosohydrazino]butyl}propane-1,3-diamine (DETA-NO) and 2,2′-(hydroxynitrosohydrazino)bis-ethanamine (SPER-NO) from Research Biochemicals International; 1,1-diethyl-2-hydroxy-2-nitrosohydrazine (DEA-NO) from Research Biochemicals International or Alexis Corporation; 3-[morpholinosydnonimine hydrochloride] (SIN-1) from Alexis Corporation; EGF (from mouse submaxillary glands) from Upstate Biotechnology or Sigma; Triton X-100, PMSF, 3,3′-diaminobenzidine (DAB), aprotinin, leupeptin, pepstatin A, Fast Green FCF, haemoglobin, 8-bromo-cGMP and *p*-nitrophenyl phosphate from Sigma; glutaraldehyde, H_2O_2 and Na_3VO_4 from Merck; Tween 20 from Bio-Rad; recombinant anti-phosphotyrosine monoclonal antibody (RC20) conjugated to horseradish peroxidase from Transduction Laboratories; PVDF membranes of 0.45 μm pore size (Immobilon-P®) from Millipore; culture medium and fetal calf serum (FCS) from Gibco and 2-*O*-propoxyphenyl-8-azapurin-6-one (zaprinast) from Rhone Poulenc Rorer. All other chemicals were of analytical grade.

Cell cultures

The EGFR-T17 fibroblast cell line used in this work derives from NIH 3T3 murine fibroblasts stably transfected with a human EGFR cDNA, and overexpresses this receptor [28]. The cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) FCS, 2 mM L-glutamine and 40 μg/ml gentamicin, in a humidified atmosphere of 5% (v/v) CO_2 in air at 37 °C. Cells were counted, after detachment from the culture dishes, using a Neubauer chamber.

[*methyl*-^{3}H]Thymidine incorporation

Incorporation of [*methyl*-^{3}H]thymidine into DNA was performed in confluent cultures essentially as previously described [29]. Cells grown in 24-well culture plates were washed twice with Dulbecco's phosphate-buffered saline (DPBS; 137 mM NaCl, 2.7 mM KCl, 0.9 mM $CaCl_2$, 0.5 mM $MgCl_2$ and 9.5 mM sodium/potassium phosphate, pH 7.4) and maintained for 24 h in DMEM with 1% (w/v) BSA in the absence of FCS. After two washes with DPBS, the cells were incubated for 24 h in 0.5 ml of DMEM supplemented with 1% (w/v) BSA, 10 nM (0.25 μCi) [*methyl*-^{3}H]thymidine and the growth factors or serum to be studied. The cells were washed twice with DPBS, treated with ice-cold 10% (w/v) trichloroacetic acid for 10 min, solubilized with 0.2 M NaOH for 24 h, and neutralized with 0.2 M HCl. The radioactivity incorporated into the acid-insoluble material was measured using a scintillation counter.

Determination of cell lysis

Cell lysis was tested by measuring the activity of the intracellular enzyme lactate dehydrogenase in the culture medium, using pyruvate as substrate and after the oxidation of NADH. Results were expressed as the cell lesion index as previously described [30].

Preparation of haemoglobin

To avoid the presence of methaemoglobin (oxidized haemoglobin), a solution of 1 mM haemoglobin was reduced with sodium dithionite as described [31]. The preparation was divided into aliquots, stored under argon at −70 °C and used within 48 h.

Phosphorylation assays in permeabilized fibroblasts

Cells grown to confluence were gently detached from the culture flasks using a plastic cell scraper, suspended in DPBS, and collected by centrifugation in a swinging-bucket rotor at 190 $\boldsymbol{g}_{max}$ for 5 min at room temperature. Thereafter the cells were permeabilized at 4 °C for 15 min in a medium containing 24 mM Hepes (sodium salt), pH 7.4, 9.5 mM $MgCl_2$, 0.8 mM EGTA, 16 μM leupeptin, 1.6 mM PMSF, 0.3 mM Na_3VO_4 and 0.15% (w/v) Triton X-100, and used for phosphorylation experiments. Standard phosphorylation assays were performed for 1 min at 37 °C in the absence and presence of NO donors in a total volume of 100 μl of a medium containing 7.5 mM Hepes (sodium salt), pH 7.4, 3 mM $MgCl_2$, 1 μM EGF (when added), 100 μg/ml poly-L-(Glu-Tyr), 100 μM Na_3VO_4, 0.5 mM PMSF, 5 μM leupeptin, 10–20 μl (1×10^4–5×10^4) permeabilized EGFR-T17 fibroblasts and 10 μM (2 μCi) [γ-^{32}P]ATP. The reaction was initiated by the addition of radiolabelled ATP and stopped with ice-cold 10% (w/v) trichloroacetic acid. The supernatant was discarded after centrifugation at 14900 $\boldsymbol{g}_{max}$ and the pellet was processed for electrophoresis and autoradiography as described below. Alternatively, intact cells were gently detached from the culture flasks, resuspended in buffer containing 137 mM NaCl, 5 mM KCl, 1 mM Na_2HPO_4, 20 mM Tris/HCl, pH 7.4, and 5 mM glucose, and incubated at 37 °C for 5 min in the absence and presence of DEA-NO. The cells were centrifuged to remove the NO donor, permeabilized as described above and used for phosphorylation assays.

Phosphorylation assays in intact cells

Cells grown to subconfluence in dishes of 3.3 cm diameter were deprived of FCS overnight, washed twice with 2 ml of DPBS buffer and incubated at 37 °C for 30–60 min in 1 ml of serum-free DMEM in the absence and presence of the NO donor. Thereafter 10 nM EGF was added and the cells were incubated for 10 min in the same conditions. Controls in the absence of EGF were also included. Afterwards, the medium was removed and the cells were lysed with 0.5 ml of an ice-cold buffer containing 50 mM Hepes (sodium salt), pH 7.4, 100 mM NaCl, 1% (w/v) Triton X-100, 5% (w/v) glycerol, 50 mM NaF, 1 mM Na_3VO_4, 10 mM *p*-nitrophenyl phosphate, 10 mM EDTA, 1 mM PMSF, 25 μM leupeptin, 1.5 μM aprotinin and 3 μM pepstatin A. The cell lysate (200 μl) was precipitated with 10% (w/v) ice-cold trichloroacetic acid and processed by slab-gel electrophoresis as described below. The proteins were electrotransferred to a PVDF membrane for 2 h at 300 mA, fixed with 0.2% (v/v) glutaraldehyde in TBS buffer (25 mM Tris/HCl, pH 8, 150 mM NaCl and 2.7 mM KCl), and temporarily stained with the dye Fast Green to verify that all the tracks contained the same amount of protein. Thereafter the PVDF membrane was blocked with 5% (w/v) BSA for 5 h at room temperature and washed with 0.1% (w/v) Tween 20 in TBS buffer. The phosphotyrosine-containing proteins were probed overnight with a

1:1000 dilution of the RC20 antibody against phosphotyrosine conjugated to peroxidase, washed first with 0.1 % (w/v) Tween 20 in TBS and thereafter with 50 mM Tris/HCl, pH 7.5. Colour was developed with 1.7 mM DAB and 0.03 % (v/v) H_2O_2 in 25 ml of 50 mM Tris/HCl, pH 7.4. The integrated photodensitometric intensity of the phosphorylated EGFR band was measured using a computer-assisted scanner.

[^{125}I]EGF cross-linkage assays

The reactions were carried out in the absence and presence of NO donors in 33 μl of a medium containing 15 mM Hepes (sodium salt), pH 7.4, 6 mM $MgCl_2$, 0.5 mM EGTA, 0.2 mM Na_3VO_4, 1 mM PMSF, 10 μM leupeptin, 2 nM (0.1 μCi) [^{125}I]EGF, increasing concentrations of non-radiolabelled EGF up to 1 μM, and 10 μl of (2×10^4–2.5×10^4) EGFR-T17 fibroblasts. After incubation for 1 h at room temperature, 1 mM bis-(*N*-hydroxysuccinimide ester) suberate was added and the mixture was maintained on ice for 15 min. Cross-linkage was stopped by the addition of 120 mM Tris/HCl, pH 7.4, and the samples were processed for electrophoresis and autoradiography as indicated below.

Other analytical procedures

Slab-gel electrophoresis was performed as described by Laemmli [32] at 12 mA overnight in linear 5–20 % (w/v) polyacrylamide gradient gels in the presence of 0.1 % (w/v) SDS at pH 8.3. Gels were stained with Coomassie Billiant Blue R-250, and dried under vacuum at 70 °C on Whatman 3MM Chr filter paper. X-ray films were exposed at −20 °C for 2–7 days to obtain autoradiographs. Labelled proteins were quantified by scanning the films in a photodensitometer. The intensities of the radiolabelled bands in the autoradiographs were linearly proportional to the amount of ^{32}P or ^{125}I in the bands within the exposure times used. Protein concentration was determined by the method of Bradford [33] using bovine γ-globulin as standard.

RESULTS

NO donors inhibit DNA synthesis by a cGMP-independent mechanism

A time course study of the rate of [*methyl*-^{3}H]thymidine incorporation by EGFR-T17 fibroblasts in the absence or presence of insulin, EGF, EGF plus insulin or FCS showed that maximum levels of DNA synthesis were attained between 16 and 24 h after the addition of the growth factors (results not shown). Taking the DNA synthesis obtained in the presence of FCS as 100 %, a typical experiment shows that mean ± S.E.M. values were 90 ± 7 % ($n = 4$) and 76 ± 6 % ($n = 4$) for EGF and EGF plus insulin respectively. Lower responses were obtained in the presence of insulin alone (59 ± 4 %, $n = 4$) or in the absence of any exogenously added growth factor (20 ± 2 %, $n = 4$).

To test the effect of NO on [*methyl*-^{3}H]thymidine incorporation, several NO donors with different mechanisms of action were used. SNAP (Figure 1A), DEA-NO (Figure 1B) and SNP (Figure 1C) inhibited DNA synthesis in a concentration-dependent manner when cells were growing in the presence of FCS, EGF or EGF plus insulin. In contrast, SIN-1 (Figure 1D), a peroxynitrite ($ONOO^-$)-forming agent [34], did not significantly affect DNA synthesis. DETA-NO, another NONOate similar to DEA-NO, also inhibited [*methyl*-^{3}H]thymidine incorporation in a concentration-dependent manner in the three growth conditions mentioned above (results not shown). Apparent inhibition constants (K_i values) for the four active NO donors were in the range 0.3–0.4 mM for SNAP, 3–5 mM for DEA-NO, 0.3–0.4 mM for DETA-NO and 0.3 mM for SNP. The inhibitory action of SNAP, DEA-NO and DETA-NO was slightly more effective when the fibroblasts were growing in the presence of EGF than when growing in the presence of FCS. This was also the case when DEA-NO was tested in the absence of added growth factors (results not shown). These latter findings are in agreement with a possible direct effect of NO on the EGFR, since the basal DNA synthesis observed in the absence of added growth factors should be due in part to signalling mediated by the overexpressed EGFR in the absence of ligand. This is consistent with the observation that the mean ± S.E.M. rate of [*methyl*-^{3}H]thymidine incorporation in EGFR-T17 fibroblasts in the absence of added growth factors was 11 ± 1 % ($n = 28$) of the value measured in the presence of FCS, as compared with 5 ± 1 % ($n = 24$) and 6 ± 1 % ($n = 24$) measured in non-transfected NIH-3T3 and Swiss-3T3 fibroblasts respectively.

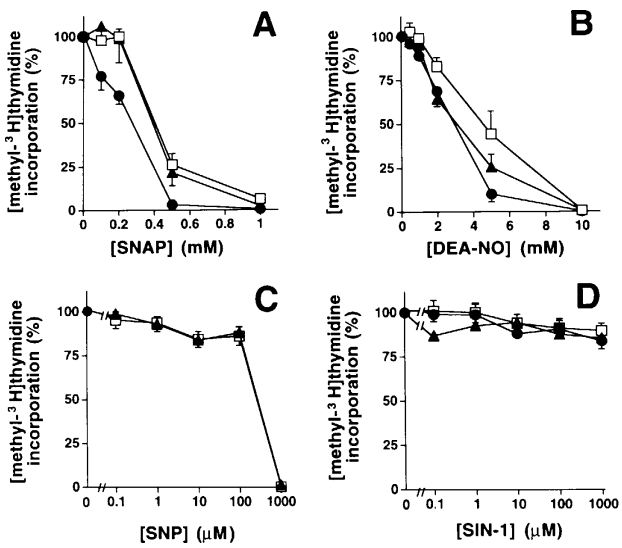

Figure 1 NO donors inhibit DNA synthesis in EGFR-T17 fibroblasts

Incorporation of [*methyl*-^{3}H]thymidine in cells growing for 24 h in the presence of 10 % (v/v) FCS (□), 10 nM EGF (●) or 10 nM EGF plus 1.7 μM insulin (▲), in the presence of the indicated concentrations of SNAP (**A**), DEA-NO (**B**), SNP (**C**) and SIN-1 (**D**). Data are expressed as the percentage of the value measured in the absence of NO donors in each experimental condition and are presented as the mean ± S.E.M. from 4 to 12 determinations.

In order to exclude the possibility that cell lysis occurred during these treatments, we treated the cells with different concentrations of the NO donors for 24 h and measured the release of lactate dehydrogenase. SNAP (1 mM), DEA-NO (5 mM) and SIN-1 (1 mM) did not provoke significant release of the cytosolic marker enzyme, with mean ± S.E.M. cell lesion indices of 7 ± 8 % ($n = 6$), 9 ± 4 % ($n = 6$) and 11 ± 6 % ($n = 8$) respectively. In contrast, 10 mM DEA-NO and 1 mM SNP had an obvious deleterious effect on the integrity of the cells, yielding mean ± S.E.M. cell lesion indices of 57 ± 8 % ($n = 6$) and 71 ± 3 % ($n = 8$) respectively. Therefore we excluded SNP altogether from further studies, and DEA-NO was used at concentrations no higher than 5 mM in the rest of the experiments performed with intact cells. Furthermore we verified that most cells remained attached to the culture dishes when non-lytic concentrations of the NO donors were used. Moreover, cells were able to recover the capacities of both [*methyl*-^{3}H]thymidine incorporation and reaching confluence after the NO donors were

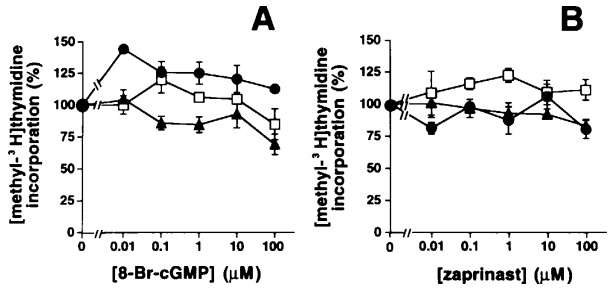

Figure 2 8-Br-cGMP and zaprinast do not mimic the effect of NO donors

Incorporation of [*methyl*-^{3}H]thymidine in cells growing for 24 h in the presence of 10% (v/v) FCS (□), 10 nM EGF (●) or 10 nM EGF plus 1.7 μM insulin (▲), and in the presence of the indicated concentrations of 8-Br-cGMP (**A**) or zaprinast (**B**). Data obtained from a typical experiment performed in quadruplicate are expressed as in Figure 1.

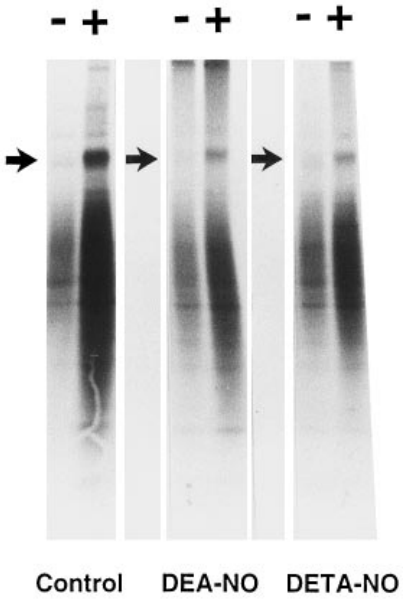

Figure 3 DEA-NO and DETA-NO inhibit the transphosphorylation of the EGFR and the phosphorylation of poly-L-(Glu-Tyr)

Phosphorylation assays were performed in permeabilized cells in the absence (Control) and presence of either 1 mM DEA-NO or 1 mM DETA-NO, as indicated, and in the absence (−) and presence (+) of 1 μM EGF. Arrows point to the phosphorylated 170 kDa EGFR. The dark smears along the tracks correspond to phosphorylated poly-L-(Glu-Tyr).

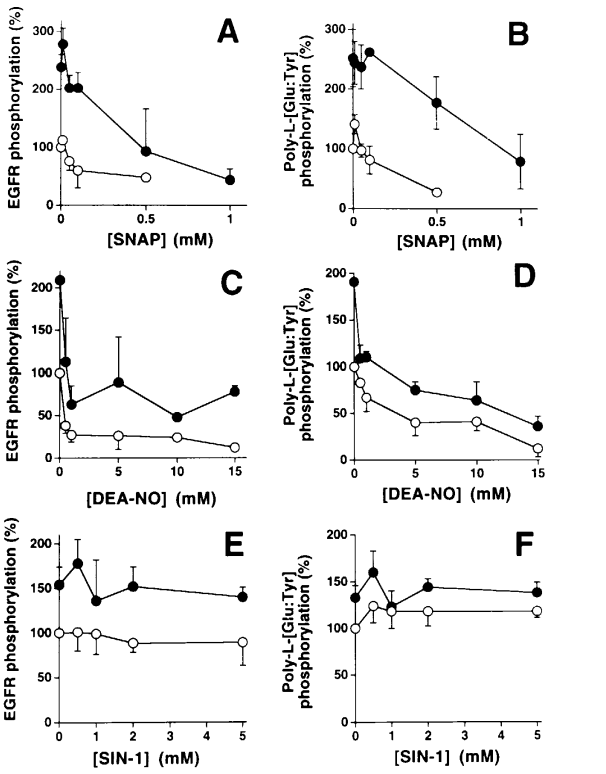

Figure 4 Effect of different concentrations of NO donors on the EGFR tyrosine kinase activity

Phosphorylation of the EGFR (**A**, **C** and **E**) and poly-L-(Glu-Tyr) (**B**, **D** and **F**) in permeabilized cells, in the presence of the indicated concentrations of SNAP (**A** and **B**), DEA-NO (**C** and **D**) or SIN-1 (**E** and **F**) and in the absence (○) and presence (●) of 1 μM EGF. Data are expressed as the percentage of the values measured in the absence of both EGF and NO donors, and are presented as the mean ± S.D. from two to four experiments.

removed and the cells were reseeded in fresh medium in the presence of FCS (results not shown).

A major pathway for NO action involves the activation of guanylate cyclase, which produces a subsequent increase in the concentration of intracellular cGMP [1–6]. Therefore we performed experiments to establish whether cGMP plays any role in the overall process leading to the inhibition of cell proliferation by NO donors in EGFR-T17 fibroblasts. As shown in Fig. 2(A), 8-Br-cGMP, a cell-membrane-permeant analogue of cGMP, did not exert any inhibitory effect of DNA synthesis induced by FCS or EGF, although a slight inhibition (25%) was observed when cells were growing in the presence of EGF plus insulin. In contrast, a slight activation was observed in the presence of EGF alone. Furthermore Figure 2(B) shows that zaprinast, a cGMP-phosphodiesterase inhibitor, also failed to significantly inhibit DNA synthesis induced by FCS, EGF or EGF plus insulin. These results suggest that cGMP-dependent protein kinases and/or other cGMP-response elements are unlikely to be essential for the arrest of the proliferative response induced by NO donors in EGFR-T17 fibroblasts, as previously shown in non-transfected fibroblasts [10].

NO donors inhibit the EGFR tyrosine kinase activity

To test the effect of NO on the EGFR tyrosine kinase activity, we used permeabilized cells to perform *in situ* assays of both the transphosphorylation of the receptor and the phosphorylation of the synthetic substrate poly-L-(Glu-Tyr), using [γ-^{32}P]ATP as the phosphate donor. Figure 3 shows the phosphorylation of both the 170 kDa EGFR and poly-L-(Glu-Tyr) in the absence and presence of EGF (control). As we have reported previously [35], the indicated 170 kDa phosphopolypeptide was indeed the EGFR, as demonstrated by immunoprecipitation using a specific antibody. We have also demonstrated that EGF strongly stimulates the phosphorylation of tyrosine residues in the EGFR without significantly affecting the phosphorylation levels of serine/threonine residues [36], which indicates that the observed EGF-dependent phosphorylation of the EGFR (the extent of phosphorylation in the presence minus that in the absence of EGF) was due to its transphosphorylation. Figure 3 also shows that the presence of NO donors, either DEA-NO or DETA-NO, significantly inhibited the transphosphorylation of the EGFR

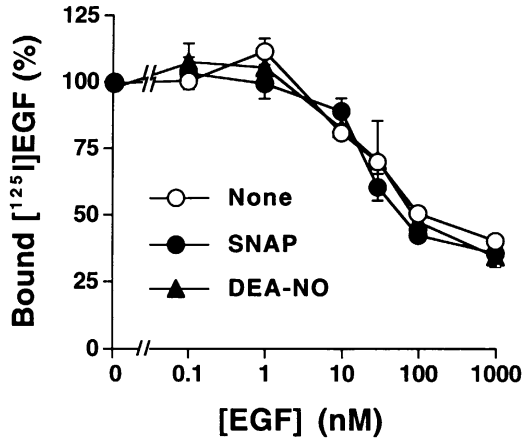

Figure 5 SNAP and DEA-NO do not inhibit the binding of EGF to its receptor

Binding of [^{125}I]EGF to its receptor was measured in the absence of NO donors (○) and in the presence of 1 mM SNAP (●) or 1 mM DEA-NO (▲) at the indicated concentrations of non-radiolabelled EGF. The plot represents the mean ± S.E.M. levels of bound [^{125}I]EGF expressed as percentage values from three experiments. 100% represents the value measured in the absence of non-radiolabelled EGF.

and the phosphorylation of poly-L-(Glu-Tyr), particularly in the presence of EGF. The inhibitory effects on both phosphorylation processes were also observed with other NO donors, such as SPER-NO (results not shown) and SNAP (see below).

Using the phosphorylation assay described above, we tested the effects of different concentrations of SNAP, DEA-NO and SIN-1 on both the transphosphorylation of the EGFR and its tyrosine kinase activity toward poly-L-(Glu-Tyr). SNAP inhibited in a concentration-dependent manner both the transphosphorylation of the receptor (Figure 4A) and the phosphorylation of poly-L-(Glu-Tyr) (Figure 4B) in the absence and presence of EGF. In the presence of the ligand, the K_i values for SNAP were approx. 0.3 mM for the transphosphorylation of the receptor and 0.7 mM for its tyrosine kinase activity toward the exogenous substrate. DEA-NO also inhibited in a concentration-dependent manner both the transphosphorylation of the EGFR (Figure 4C) and the phosphorylation of poly-L-(Glu-Tyr) (Figure 4D) in the absence and presence of EGF. We would like to mention that most of the inhibitory effects of DEA-NO on the EGFR tyrosine kinase were observed at non-cytolytic concentrations of this agent (5 mM or lower). In the presence of EGF, K_i values for DEA-NO were approx. 1 mM and 2 mM for the transphosphorylation of the EGFR and the phosphorylation of poly-L-(Glu-Tyr) respectively. On the other hand, SIN-1 exerted no inhibitory effect on either the transphosphorylation of the EGFR (Figure 4E) or the phosphorylation of poly-L-(Glu-Tyr) (Figure 4F), in the absence or presence of EGF.

In order to test whether NO inhibits the binding of EGF to its receptor, we performed [^{125}I]EGF-binding experiments in the absence and presence of NO donors. As shown in Figure 5, neither SNAP nor DEA-NO affected the binding of [^{125}I]EGF to its receptor. We calculated a dissociation constant (K_d) of EGF for its receptor of approx. 20 nM in the absence and presence of either NO donor.

NO, but not $ONOO^-$, inhibits the EGFR tyrosine kinase activity

To ascertain whether the inhibitory action of DEA-NO was due to the presence of NO in the medium, we used haemoglobin as a scavenger of this compound. Table 1 shows that DEA-NO in the absence of haemoglobin inhibited the transphosphorylation of the EGFR and the phosphorylation of poly-L-(Glu-Tyr), more significantly in the presence than in the absence of EGF. In contrast, the presence of haemoglobin completely prevented the inhibitory action of DEA-NO on the transphosphorylation of the EGFR in the presence of EGF. Haemoglobin was somewhat less effective in preventing the inhibitory action of DEA-NO on the phosphorylation of poly-L-(Gly-Tyr).

Table 1 Haemoglobin prevents the inhibitory action of DEA-NO

Phosphorylation of the EGFR and poly-L-(Glu-Tyr) was assayed in permeabilized cells, in the absence and presence of 5 mM DEA-NO, and in the absence and presence of 1 μM EGF as indicated. The assays were performed in the absence and presence of 5 μM haemoglobin. Data are expressed as the percentage of the values measured in the absence of both EGF and NO donor in the absence or presence of haemoglobin and represent the mean ± S.E.M. from four experiments.

	Phosphorylation (%)			
	EGFR		Poly-L-(Glu-Tyr)	
Addition	− EGF	+ EGF	− EGF	+ EGF
None	100	215 ± 52	100	187 ± 41
DEA-NO	52 ± 9	86 ± 31	83 ± 24	85 ± 30
Haemoglobin	100	181 ± 68	100	192 ± 25
DEA-NO + haemoglobin	48 ± 8	187 ± 64	72 ± 10	129 ± 17

Table 2 Inhibitory action of DEA-NO in the presence of SOD

Phosphorylation of the EGFR and poly-L-(Glu-Tyr) was assayed in permeabilized cells, in the absence and presence of 5 mM DEA-NO and/or 15 units/ml SOD, both in the absence and presence of 1 μM EGF, as indicated. Data are expressed as the percentage of the values measured in the absence of both EGF and NO donor and represent the mean ± S.E.M. or the mean ± range for duplicate values, from the number of experiments indicated in parentheses.

	Phosphorylation (%)			
	EGFR		Poly-L-(Glu-Tyr)	
Addition	− EGF	+ EGF	− EGF	+ EGF
None	100	246 ± 59 (3)	100	211 ± 68 (2)
DEA-NO	56 ± 13 (3)	103 ± 37 (3)	81 ± 38 (2)	96 ± 34 (2)
SOD	80 ± 19 (3)	287 ± 73 (3)	69 ± 21 (3)	218 ± 61 (2)
DEA-NO + SOD	29 ± 8 (3)	134 ± 27 (3)	40 ± 11 (3)	45 ± 5 (2)

To exclude the possibility that the observed inhibition of the EGFR tyrosine kinase activity by DEA-NO could be due to the presence of $ONOO^-$, a product of the reaction of NO with $O_2^{-\bullet}$, we performed similar phosphorylation experiments in the presence of superoxide dismutase (SOD). This enzyme should prevent the accumulation of $O_2^{-\bullet}$ required for the formation of $ONOO^-$. Table 2 shows that DEA-NO inhibited both the transphosphorylation of the EGFR and the phosphorylation of poly-L-(Glu-Tyr) in the absence and presence of SOD and both in the absence and presence of EGF. Notably, the inhibitory action of DEA-NO on the phosphorylation of poly-L-(Glu-Tyr) was even more effective in the presence of SOD.

Inhibitory action of NO is reversible

To establish whether NO inhibits the EGFR tyrosine kinase activity by modifying thiol groups on the receptor, we attempted

Table 3 DTT partially reverses the inhibition produced by DEA-NO

Whole cells were incubated in the absence and presence of 5 mM DEA-NO. Thereafter the NO donor was removed by centrifugation, the cells were permeabilized and phosphorylation of the EGFR and poly-L-(Glu-Tyr) was assayed in the absence or presence of 1 μM EGF, after incubation for 1 min at 37 °C in the absence or presence of 1 mM DTT. Data are expressed as the percentage of the values measured in the absence of both EGF and NO donor and represent the mean ± S.E.M. from three experiments.

	Phosphorylation (%)			
	EGFR		Poly-L-(Glu-Tyr)	
Addition	− EGF	+ EGF	− EGF	+ EGF
None	100	189 ± 26	100	135 ± 19
DEA-NO	38 ± 7	62 ± 13	30 ± 5	40 ± 4
DTT	103 ± 9	183 ± 33	106 ± 3	130 ± 14
DEA-NO + DTT	62 ± 7	159 ± 21	43 ± 4	70 ± 7

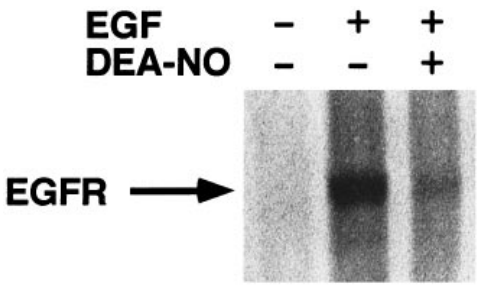

Figure 6 NO inhibits the transphosphorylation of the EGFR in intact cells

Serum-deprived cells were incubated in DMEM in the absence and presence of 5 mM DEA-NO (as indicated) for 30 min. Thereafter the cells were stimulated for 10 min with 10 nM EGF (as indicated), lysed and treated with ice-cold 10% (w/v) trichloroacetic acid. The lysates were processed by electrophoresis and the levels of tyrosine phosphorylation of the receptor were measured by immunoblot analysis as described in the Materials and methods section.

to reverse this inhibition using a reducing agent. Whole intact cells were incubated in the absence and presence of 5 mM DEA-NO for 5 min and, after centrifugation to remove most of the DEA-NO, phosphorylation assays were performed in permeabilized cells. Table 3 shows that DEA-NO pretreatment inhibited the EGF-induced transphosphorylation of the EGFR and the phosphorylation of poly-L-(Glu-Tyr). Addition of dithiothreitol (DTT) to the assay system did not modify the tyrosine kinase activity of the EGFR in untreated cells in either the absence or presence of EGF. DTT, however, re-activated the EGF-induced transphosphorylation of the EGFR and the phosphorylation of poly-L-(Glu-Tyr) in cells treated with DEA-NO. The re-activation of the transphosphorylation of the EGFR was more pronounced than the re-activation of the tyrosine kinase activity toward poly-L-(Glu-Tyr). Interestingly, removal of DEA-NO before the addition of DTT was an essential requirement to detect re-activation of the EGFR tyrosine kinase activity. When DEA-NO was present during the phosphorylation assays, the re-activation induced by DTT was not observed (results not shown).

NO inhibits the transphosphorylation of the EGFR in intact cells

To ascertain whether the inhibitory action of NO also occurs in intact cultured cells, we tested the effect of DEA-NO on the transphosphorylation of the EGFR in serum-deprived cells as measured by immunoblot analysis using an antibody against phosphotyrosine. Figure 6 shows that a short stimulation with EGF induces the transphosphorylation of the receptor in control non-treated cells. However, a strong inhibition of this transphosphorylation was observed in cells treated with 5 mM DEA-NO. The densitometric measurement of the intensity of the EGFR band in different immunoblots shows that the mean ± S.E.M. inhibition of the EGFR transphosphorylation was 69 ± 12% ($n = 4$) in the presence of DEA-NO.

DISCUSSION

Our results show that NO donors inhibit the EGFR tyrosine kinase activity in cultured fibroblasts, and that this phenomenon correlates well with their anti-proliferative effect in the same cells. NO exerted an anti-mitotic action, since both SNAP and the NONOates (DEA-NO and DETA-NO), which release NO by different mechanisms [37], inhibited DNA synthesis in a reversible manner and without inducing significant cell lysis. As previously shown in non-transfected fibroblasts [10], the anti-proliferative action of NO detected by us was not mediated by guanylate cyclase, because the enhancement of intracellular cGMP concentration did not significantly inhibit DNA synthesis. Also, the effect of NO donors was not the result of $ONOO^-$ formation, as can be concluded from the absence of any anti-mitotic effect of SIN-1. Although the inhibition of DNA synthesis was observed in all growth conditions tested (EGF, EGF plus insulin, FCS and absence of added growth factors), the sensitivity to NO donors was somewhat higher when either EGF was the only growth factor used or in the absence of exogenous growth factors. All these results were compatible with the hypothesis of a possible direct effect of NO on the EGFR molecule.

To test this possibility, the effects of NO donors on the EGFR tyrosine kinase activity were assayed. SNAP and DEA-NO, as well as two other NONOates, DETA-NO and SPER-NO, inhibited the EGF-induced phosphorylation of both the EGFR and the exogenous substrate poly-L-(Glu-Tyr), with K_i values similar to those observed in [*methyl*-^{3}H]thymidine incorporation experiments. Furthermore SIN-1 had no effect on the EGFR tyrosine kinase activity. The inhibition of the EGFR tyrosine kinase by NO donors observed in permeabilized fibroblasts exclude the participation of cGMP in the receptor inhibition, since soluble cytoplasmic components are highly diluted in the incubation medium on permeabilization, strongly suggesting a direct interaction of NO with the receptor protein. The possibility of NO inducing activation of a phosphotyrosine protein phosphatase, which would also result in a decreased phosphorylation of the EGFR and of poly-L-(Glu-Tyr), can be excluded because orthovanadate, a protein-phosphotyrosine phosphatase inhibitor [38], was always present in the phosphorylation assays. It is interesting to note that DEA-NO also inhibited the transphosphorylation of the EGFR in intact cells, supporting the physiological relevance of this process.

The active species inhibiting the EGFR tyrosine kinase activity was indeed NO, since the effects of DEA-NO were prevented by the NO scavenger haemoglobin. Haemoglobin protected in full the transphosphorylation of the EGFR, although it only partially prevented the inhibitory action of DEA-NO on the phosphorylation of poly-L-(Glu-Tyr). The reasons for this discrepancy are at present unclear.

NO rapidly reacts with $O_2^{-\bullet}$ in aqueous solutions, yielding $ONOO^-$, a highly reactive agent with cytotoxic effects [20]. Among other reactions, $ONOO^-$ is responsible for the nitration of tyrosine residues in proteins, which would result in decreased

phosphorylation [39,40]. However, our results suggest that the inhibition of the EGFR tyrosine kinase by NO does not involve $ONOO^-$ formation. First, as mentioned above, the inhibition of EGFR and poly-L-(Glu-Tyr) phosphorylation was not observed when SIN-1 was used as the source of NO. Secondly, the EGFR tyrosine kinase inhibition induced by DEA-NO also occurred in the presence of SOD, conditions under which the formation of any significant amount of $ONOO^-$ should be prevented.

A mechanism for the NO action on the EGFR could be the redox reaction of the gas with critical thiol group(s) of the receptor, leading to its S-nitrosylation. Nitrosylation of thiol groups has been shown to modulate the activity of certain enzymes [23,25,27] and membrane-associated proteins such as *N*-methyl-D-aspartate receptors [22], Ca^{2+}-dependent K^+ channels [24] or the G-protein Ras [26], and is at present considered to be a mechanism with broad regulatory implications [20]. DTT completely re-activated the EGF-dependent phosphorylation (the amount of phosphorylation in the presence minus that in the absence of EGF) of the EGFR and poly-L-(Glu-Tyr) when added to cells pretreated with an NO donor, suggesting that thiol groups in the EGFR were indeed involved in the inhibition process. The importance of active thiol groups for the EGFR tyrosine kinase is supported by the observation that the thiol group reagent *N*-ethylmaleimide strongly inhibits this activity (results not shown). However, the re-activation of the EGFR by DTT in the absence of EGF was not complete, particularly in the case of the exogenous substrate, suggesting that poly-L-(Glu-Tyr) might also be phosphorylated by other NO-sensitive tyrosine kinases not re-activated by the reducing agent. We propose that the EGFR is probably S-nitrosylated, resulting in conformational changes in the receptor molecule leading to inhibition of its tyrosine kinase activity, and without affecting the affinity or the maximum binding capacity for its ligand.

Although the concentrations of NO donors necessary to produce anti-proliferative effects in different cell types [9–11] or S-nitrosylation of different proteins [22,23,26,41–44] may appear too high to have physiological significance, considering the kinetics of NO release by these drugs [37] together with the low solubility and short half-life of NO itself [8], the resulting NO concentrations are probably close to those present in the tissues when NO is synthesized by iNOS [45]. Consequently, the regulation of the EGFR by NO might occur *in vivo* on induction of iNOS. This is supported by the fact that the anti-proliferative action of endogenous NO becomes apparent only when iNOS is expressed, either in cells treated with cytokines [14,15] or in engineered cells [46]. On the other hand, it has been demonstrated that EGF inhibits the expression of iNOS [47]. Therefore complex feedback mechanisms are likely to operate *in vivo* between EGF and endogenous NO to control cell proliferation.

Recently, NO has been reported to inhibit EGF-mediated phosphoinositide hydrolysis and the subsequent increase in cytosolic free Ca^{2+} in EGFR-T17 fibroblasts as well as other cells [48]. However, these actions do not seem to be derived from the direct inhibition of the EGFR tyrosine kinase activity demonstrated in this work, since they were prevented by a cGMP-dependent protein kinase I inhibitor [48]. Thus NO appears to inhibit several different components of the signal-transduction pathways initiated by EGFR activation.

The specific thiol group(s) of the EGFR sensitive to NO have not yet been identified. However, it is tempting to speculate that the two prominent cysteine-rich domains in its extracellular region may constitute antennas for NO. Other members of the tyrosine kinase superfamily of receptors also have cysteine-rich domains [19]. Therefore the study of the effects of NO on other growth factor receptors with tyrosine kinase activity should help to unravel the mechanisms of action of this regulator on the initial steps of cell proliferation signalling.

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