

Nitric oxide-induced epidermal growth factor-dependent phosphorylations in A431 tumour cells

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Nitric oxide (NO[•]) strongly inhibits the proliferation of human A431 tumour cells. It also inhibits tyrosine phosphorylation of a 170-kDa band corresponding to the epidermal growth factor receptor (EGFR) and induces the phosphorylation at tyrosine residue(s) of a 58-kDa protein which we have denoted NOIPP-58 (nitric oxide-induced 58-kDa phosphoprotein). The NO[•]-induced phosphorylation of NOIPP-58 is strictly dependent on the presence of EGF. Phosphorylation of NOIPP-58 and inhibition of the phosphorylation of the band corresponding to EGFR are both cGMP-independent processes. We also demonstrate that the p38 mitogen-activated protein kinase (p38MAPK) pathway is activated by NO[•] in the absence and presence of EGF, whereas the activity of the extracellular signal-regulated protein kinase 1/2 (ERK1/2) and the c-Jun N-terminal

kinase 1/2 (JNK1/2) pathways are not significantly affected or are slightly decreased, respectively, on addition of this agent. Moreover, we show that the p38MAPK inhibitor, SB202190, induces rapid vanadate/peroxovanadate-sensitive dephosphorylation of prephosphorylated EGFR and NOIPP-58. We propose that the dephosphorylation of both NOIPP-58 and EGFR are mediated by a p38MAPK-controlled phosphotyrosine-protein phosphatase (PYPP). Activation of the p38MAPK pathway during nitrosative stress probably prevents the operation of this PYPP, allowing NOIPP-58, and in part EGFR, to remain phosphorylated and therefore capable of generating signalling events.

Keywords: cell proliferation; p38MAPK; phosphotyrosine phosphatase; tyrosine kinase.

Nitric oxide (NO[•]), a highly reactive gas synthesized in mammalian cells from L-arginine by a family of related enzymes denoted NOS (nitric oxide synthase), is involved in

multiple physiological processes, such as control of the blood pressure, regulation of neuronal activities, and immune response [1]. In addition, NO[•] participates in the control of cell proliferation in a great variety of cell types [2–12].

The relevance of NO[•] in the control of cell proliferation *in vivo* has been demonstrated during development in *Drosophila*. Inhibition of NOS from embryonic imaginal discs produces hypertrophy of organs, and, conversely, the ectopic expression of NOS has a hypotrophic effect [7]. NO, however, has a complex mode of action, as it can exert opposite effects on cell proliferation. In this context, NO[•] has been reported to stimulate cell proliferation by cGMP-dependent mechanisms associated with activation of the AP-1 transcription complex [5,9] and, on the other hand, to inhibit cell proliferation by cGMP-dependent [2,4,6] and cGMP-independent [3,8–12] mechanisms. However, these apparently contradictory actions of NO[•] depend on, among other factors, the type of cells under study.

Activation of a cAMP-dependent protein kinase, but not a cGMP-dependent protein kinase, appears to be responsible in part for the NO[•]-mediated inhibition of cell proliferation mediated by the cGMP-dependent pathway in smooth muscle cells [6]. On the other hand, the concomitant inhibition of both the ribonucleotide reductase [9] and the intrinsic tyrosine kinase activity of epidermal growth factor receptor (EGFR) [10,12] by NO[•] may contribute to the inhibition of cell proliferation through the cGMP-independent pathway. The inhibition of the cell cycle that takes place in NO[•]-exposed cells has been reported to occur at either the early G₂ plus M phases [13] or the early and late G₁ phase [9,14]. Cell growth arrest at

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Abbreviations: DEA-NO, 1,1-diethyl-2-hydroxy-2-nitroso-hydrazine sodium; DETA-NO, 2,2'-(hydroxynitrosohydrazono)bis-ethanamine; DMEM, Dulbecco's modified Eagle's medium; ECL, enhanced chemiluminescence; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; ERK1/2, extracellular signal-regulated protein kinases 1 and 2; JNK1/2, c-Jun N-terminal kinases 1 and 2; MAPK, mitogen-activated protein kinase; MEK, MAP/ERK kinase; NOIPP-58, nitric oxide-induced 58 kDa phosphoprotein; NOS, nitric oxide synthase; ODQ, 1-*H*-[1,2,4]oxadiazolo[4,3- α]quinoxalin-1-one; PDI53035, 4-[(3-bromophenyl)amino]-6,7-dimethoxyquinazoline; P-ERK1/2, phosphorylated form of ERK1/2; P-JNK1/2, phosphorylated form of JNK1/2; P-p38MAPK, phosphorylated form of p38MAPK; PVDF, poly(vinylidene difluoride); PYPP, phosphotyrosine-protein phosphatase; SB202190, 4-(4-fluorophenyl)-2-(4-hydroxyphenyl)-5-(4-pyridyl)1*H*-imidazole; SPER-NO, *N*-(2-aminoethyl)-*N*-(2-hydroxy-2-nitrosohydrazino)-1,2-ethylenediamine.

Enzymes: Nitric oxide synthase (EC 1.14.13.39); phosphotyrosine-specific phosphatase (EC 3.1.3.48); protein-tyrosine kinase (EC 2.7.1.12); protein kinase (EC 2.7.1.37); ribonucleotide reductase (EC 1.17.4.1 and EC 1.17.4.2).

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the G₁ phase appears to be associated with the induction of p21^{Waf1/Cip1}, a cyclin-dependent kinase inhibitor [14].

The transduction of extracellular signals into cellular responses is mediated in many instances by an array of different mitogen-activated protein kinase (MAPK) pathways [15–18]. Among these kinases is the family of p38MAPKs [19–23], which are activated by dual tyrosine/threonine kinases responsive to pro-inflammatory cytokines and environmental stress [24]. However, there is increasing evidence that the p38MAPK pathways are involved in important physiological functions besides the stress response [18,22]. Of special interest is the fact that p38MAPK is activated by NO[•] ([25–28] and this work) and its derived metabolites [29,30]. This process appears to be mediated by a cGMP-dependent protein kinase [28].

In this paper, we demonstrate that NO[•] inhibits tyrosine phosphorylation of the 170-kDa band corresponding to EGFR and induces reversible phosphorylation at tyrosine residue(s) of a newly identified 58-kDa protein which we have named NOIPP-58 (nitric oxide-induced 58-kDa phosphoprotein) in the presence, but not in the absence, of EGF. Both of these processes are mediated by cGMP-independent mechanisms. We also show that the phosphorylation/dephosphorylation cycle of NOIPP-58 appears to be under the control of EGFR and a p38MAPK-regulated phosphotyrosine-protein phosphatase (PYPP). Moreover, this phosphatase also dephosphorylates EGFR with great efficiency. Therefore, activation of the p38MAPK pathway by nitrosative stress probably prevents operation of this PYPP, allowing NOIPP-58, and in part EGFR, to generate signalling events.

Experimental procedures

Reagents

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum and L-glutamine were obtained from Gibco, [*methyl*-³H]thymidine (46 Ci·mmol⁻¹) and enhanced chemiluminescence (ECL) reagents were from Amersham, and OptiPhase HiSafe 2 scintillation fluid was from Wallac, Turku, Finland. The nitric oxide donors 1,1-diethyl-2-hydroxy-2-nitrosohydrazine sodium (DEA-NO), 2,2'-(hydroxynitrosohydrazono)bis-ethanamine (DETA-NO) and *N*-(2-aminoethyl)-*N*-(2-hydroxy-2-nitrosohydrazino)-1,2-ethylenediamine (SPER-NO) were from Research Biochemicals International, St Louis, MO, USA. EGF (from male mouse submaxillary glands) and the antibody to nitrotyrosine were from Upstate Biotechnology, Lake Placid, NY, USA. The recombinant monoclonal antibody to phosphotyrosine (RC20) conjugated to horseradish peroxidase was from Transduction Laboratories, Heidelberg, Germany. Fast Green FCF, Trypan blue, catalase (from bovine liver), and peroxidase-conjugated anti-mouse IgGs (Fc-specific) were from Sigma. Polyclonal antibody to phospho-specific p38MAPK (developed in rabbit using a phosphopeptide corresponding to residues 172–186 of human p38MAPK), anti-(total p38MAPK) (developed in rabbit against residues 341–360 of the human protein), 4-[(3-bromophenyl)amino]-6,7-dimethoxyquinazoline (PD153035), and 4-(4-fluorophenyl)-2-(4-hydroxyphenyl)-5-(4-pyridyl)1*H*-imidazole (SB202190) were obtained from Calbiochem. Monoclonal

antibodies to phospho-specific extracellular signal-regulated protein kinases 1 and 2 (ERK1/2) (E-4) (developed in mouse against a segment of the human ERK1 protein that contains phosphorylated Tyr204) and to phospho-specific c-Jun N-terminal kinases 1 and 2 (JNK1/2) (G-7) (developed in mouse against a conserved segment of the human proteins containing phosphorylated Thr183 and Tyr185 residues) were obtained from Santa Cruz Biotechnology. Horseradish peroxidase-conjugated goat anti-rabbit IgGs were provided by Zymed, San Francisco, CA, USA. Poly(vinylidene difluoride) (PVDF) membranes were from Millipore, and PPI was obtained from Biomol, Plymouth Meeting, PA, USA. Gentamicin was obtained from Normon, Madrid, Spain, and Tween 20 was from Bio-Rad. AX X-ray films were purchased from Konica, and 1-*H*-[1,2,4]oxadiazolo-[4,3- α]quinoxalin-1-one (ODQ) was obtained from Tocris, London, UK.

Cell cultures

Human epidermoid carcinoma A431 cells, a cell line that overexpresses both the wild-type EGFR and aberrant extracellular forms of this receptor [31], and the different fibroblast cell lines used were grown in DMEM supplemented with 10% (v/v) fetal bovine serum, 2 mM L-glutamine and 40 μ g·mL⁻¹ gentamicin in a humidified atmosphere of 5% (v/v) CO₂ in air at 37 °C. Cells were counted using a Neubauer chamber after detachment from the culture dishes.

Cell viability

Living and dead cells were counted by the Trypan blue exclusion method after control and DEA-NO-treated cells had been detached from the culture dishes by trypsinization. The viability of A431 tumour cells was not affected by DEA-NO treatment in the conditions used in this study. Untreated cells and cells treated with 5 mM DEA-NO for 15 min had a viability of 85 ± 9% (*n* = 4) and 93 ± 3% (*n* = 3), respectively. We observed no significant cell detachment from the culture dishes on overnight treatment with 1 mM DEA-NO.

Incorporation of [*methyl*-³H]thymidine

Incorporation of [*methyl*-³H]thymidine into DNA was carried out in confluent cell cultures essentially as described [32], but in the absence of EGF to attain maximum proliferation, as this growth factor has an antimitogenic effect on A431 tumour cells [33]. Cells grown to confluence in 24-well culture dishes and deprived of fetal bovine serum overnight, were washed twice with 130 mM NaCl/2.7 mM KCl/11.5 mM sodium/potassium phosphate (pH 7.4) (NaCl/P_i) and incubated for 14–16 h in 0.5 mL DMEM supplemented with 1.2 μ M (2 μ Ci·mL⁻¹) [*methyl*-³H]thymidine in the absence and presence of 50 μ M ODQ and the concentrations of DEA-NO indicated in the legends of the figures. Thereafter, cells were 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.

Detection of phosphotyrosine-containing proteins

Cells grown to confluence in 6-well culture dishes were deprived of fetal bovine serum overnight, washed twice with NaCl/P_i, and incubated, unless indicated otherwise, at 37 °C for 15 min in 1.5 mL fetal bovine serum-free DMEM in the absence and presence of the concentrations of DEA-NO indicated in the legends of the figures. Thereafter, 10 nM EGF was added and the cells were incubated for 1–5 min under the same conditions. Controls in the absence of EGF were also performed. Ice-cold 10% (w/v) trichloroacetic acid was then added, and the fixed cells were scraped from the plates and processed by slab-gel electrophoresis using the method of Laemmli [34], at 12 mA in linear 5–20% (w/v) polyacrylamide gradient gels in the presence of 0.1% (w/v) SDS at pH 8.3. The proteins were then electrotransferred to a PVDF membrane for 2–3 h at 300 mA, fixed with 0.2% (v/v) glutaraldehyde in 25 mM Tris/HCl (pH 8)/150 mM NaCl/2.7 mM KCl (NaCl/Tris), and transiently stained with Fast Green FCF to ascertain the regularity of the transfer procedure. The PVDF membrane was blocked with 5% (w/v) BSA in NaCl/Tris for 2 h at room temperature and washed with 0.1% (w/v) Tween 20 in NaCl/Tris. The PVDF membrane was then probed overnight with a 1 : 5000 dilution of the RC20 antibody conjugated to horseradish peroxidase, and washed with 0.1% (w/v) Tween 20 in NaCl/Tris. The phosphotyrosine-containing proteins were visualized on development with the ECL reagents following instructions from the manufacturer and exposure of X-ray films for appropriate periods of time. The intensities of the phosphotyrosine-containing protein bands of interest were quantified with a computer-assisted scanning densitometer using the NIH Image 1.59 program. Corrections were made for the amount of protein present in the electrophoretic tracks as detected by Fast Green FCF staining followed by densitometric reading. To avoid any exposure time differences between gels loaded with samples corresponding to experiments performed in parallel, we exposed a single film to two different gels at the same time, or used a fix chronometer-measured exposure time for each film.

Detection of the active forms of different MAPKs

Cells grown and treated with DEA-NO and/or EGF as described were scraped from the culture dishes. The solubilized proteins were processed by SDS/PAGE and transferred to a PVDF membrane. After blocking of the membrane as described above, P-ERK1/2, P-JNK1/2 and P-p38MAPK, which represent the active forms of these kinases, were probed overnight using 1 : 1000–1 : 2000 dilutions of specific antibodies against the human phosphorylated proteins, washed three times with 0.1% (w/v) Tween 20 in NaCl/Tris, and thereafter incubated for 3 h with a 1 : 2000 dilution of appropriate secondary IgGs conjugated to horseradish peroxidase. Development was carried out by ECL, and band intensities were quantified as described above. To confirm identical loading in the electrophoretic wells, protein staining of the PVDF membrane with Fast Green FCF and densitometric reading with a computer-assisted scanning densitometer using the NIH Image 1.59 program was routinely performed.

Preparation of peroxovanadate

Peroxovanadate was prepared from orthovanadate essentially as described [35], with the following modifications. A solution of 10 mM sodium orthovanadate was incubated with 10 mM H₂O₂ for 30 min in 5 mL NaCl/P_i at room temperature. After completion of the synthesis, 17 U·mL⁻¹ catalase was added for 30 min to reduce any trace of unreacted H₂O₂ remaining in the sample. One unit of catalase transforms 1 μmol H₂O₂·min⁻¹ at pH 7 and 25 °C. The resulting peroxovanadate solution was used immediately without being stored.

ODQ bioassay

To determine the inhibitory action of the ODQ stocks used in this work, we assayed the effect of this compound on a well-known cGMP-dependent system using an acetylcholine-induced arterial relaxation bioassay as described [36]. We observed that 10 μM ODQ prevents 99% of the relaxation induced by 10 μM acetylcholine in noradrenaline-precontracted rat carotid arterial segments. From this we ascertained that the concentration of 50 μM ODQ used in the treatment of A431 tumour cells was sufficient to inhibit any endogenous guanylate cyclase activity.

Results

NO[•] inhibits cell proliferation by a cGMP-independent mechanism

We studied the effect of NO[•] on the proliferation of A431 tumour cells by measuring the incorporation of [*methyl*-³H]thymidine into DNA. Figure 1 shows that the NO[•] donor DEA-NO strongly inhibits this process in a

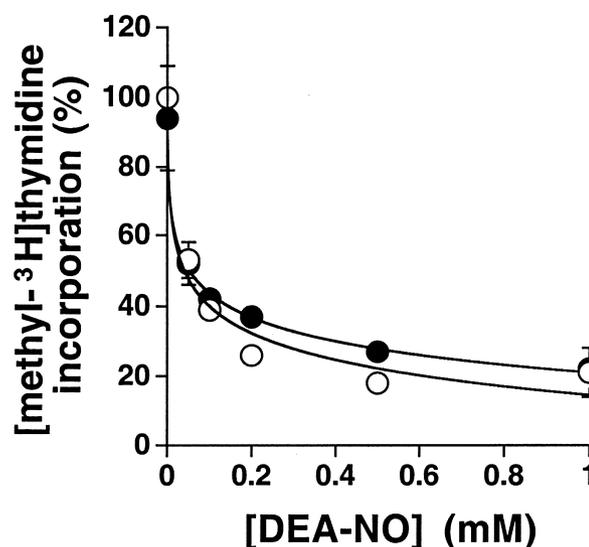


Fig. 1. NO[•] inhibits DNA synthesis by a cGMP-independent mechanism. Incorporation of [*methyl*-³H]thymidine into DNA was determined as described in Experimental procedures in confluent cells treated with the indicated concentrations of DEA-NO, in the absence (○) and presence (●) of 50 μM ODQ. Results are from quadruplicate samples from two separate experiments, and the error bars represent the SEM.

concentration-dependent manner in the absence (open symbols) and presence (filled symbols) of ODQ, a potent inhibitor of the soluble NO[•]-sensitive guanylate cyclase [37]. Thus, it appears that NO[•]-promoted inhibition of cell proliferation does not require the synthesis of cGMP. Moreover, the proliferation of A431 tumour cells appears to be far more sensitive to DEA-NO than other cell lines tested. Thus, we determined an apparent inhibition constant for DEA-NO ($K'_{i[\text{DEA-NO}]}$) in the proliferation process of 50 μM in A431 tumour cells (Fig. 1), compared with 3–5 mM in EGFR-T17 fibroblasts [10] and 0.75–2 mM in NB69 neuroblastoma cells [12].

NO[•]-induced EGF-dependent phosphorylations

The action of NO[•] on the EGF-dependent phosphorylation of proteins was assessed in whole cells treated with different NO[•] donors. Increasing concentrations of DEA-NO progressively inhibited tyrosine phosphorylation of the 170-kDa band corresponding to EGFR (Fig. 2A). Although we cannot exclude the possibility that additional proteins form part of this band, most of the observed phosphorylation probably occurred on the EGFR itself, as A431 tumour cells overexpress this receptor (10–50 times more receptors

per cell than most cell types) [31]. Moreover, PD153035, a potent and selective inhibitor of EGFR [38], completely prevented phosphorylation of the 170-kDa band. Therefore, for simplicity we shall refer to phosphorylation of EGFR from now on. Quantitative determinations showed that this process has a $K'_{i[\text{DEA-NO}]}$ of ≈ 1 –2 mM. In contrast, similar concentrations of DEA-NO induce, in the presence of EGF, phosphorylation at tyrosine residue(s) of a 58-kDa protein which we have named NOIPP-58 (Fig. 2B,C). The phosphorylation of NOIPP-58 has an apparent activation constant for DEA-NO ($K'_{a[\text{DEA-NO}]}$) of ≈ 2 mM. Phosphorylation of NOIPP-58 is not detected, however, in the presence of increasing concentrations of DEA-NO but in the absence of EGF (Fig. 2C). The inhibition of EGFR phosphorylation by PD153035 results in the parallel inhibition of NOIPP-58 phosphorylation (results not shown). Using other NO[•] donors of the NONOate family that have different efficiencies in releasing NO[•] [39], such as SPER-NO and DETA-NO, we found that the inhibition of EGFR phosphorylation was linear and inversely proportional to \log_{10} of the half-life of NO[•] release into the medium (results not shown). Figure 3 shows that phosphorylation of EGFR and NOIPP-58 have dissimilar kinetics. The phosphorylation of EGFR (circles) is progressively inhibited with increasing exposure to DEA-NO with a $t_{1/2}$ of ≈ 5 min. In contrast, the phosphorylation of NOIPP-58 (triangles) is a transient process reaching a maximum at ≈ 5 min followed by dephosphorylation with a $t_{1/2}$ of ≈ 10 min.

As the molecular mass of NOIPP-58 is close to that of the nonreceptor tyrosine kinase Src, we investigated whether the two molecules were identical. We excluded this possibility by demonstrating that the immunoblot signal from

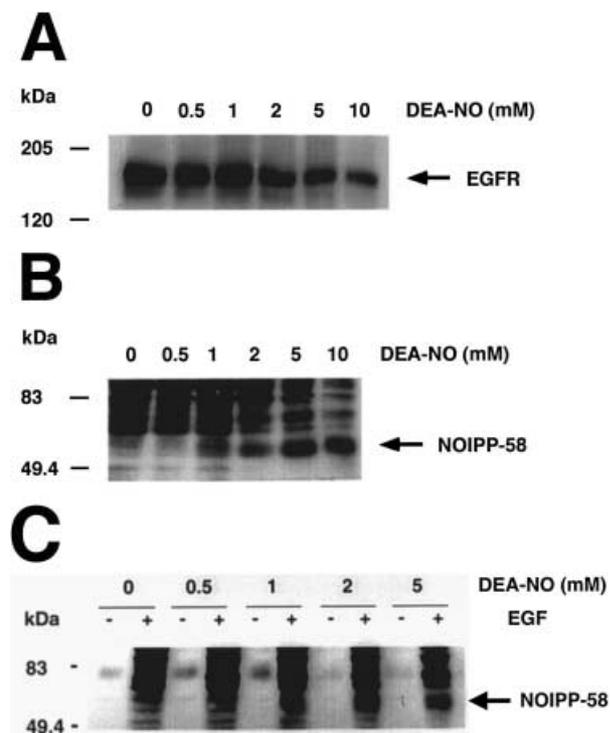


Fig. 2. NO[•] inhibits the phosphorylation of EGFR and promotes the phosphorylation of NOIPP-58 in an EGF-dependent manner. Cells were incubated with the indicated concentrations of DEA-NO for 30 min before treatment with 10 nM EGF for 5 min (A and B, and C only where indicated). Phosphorylation of EGFR (A) and NOIPP-58 (B and C) were determined using an antibody to phosphotyrosine as described in Experimental procedures. The arrows indicate the position of migration of EGFR (A) and NOIPP-58 (B and C). Typical experiments of a total of five performed under similar conditions are presented.

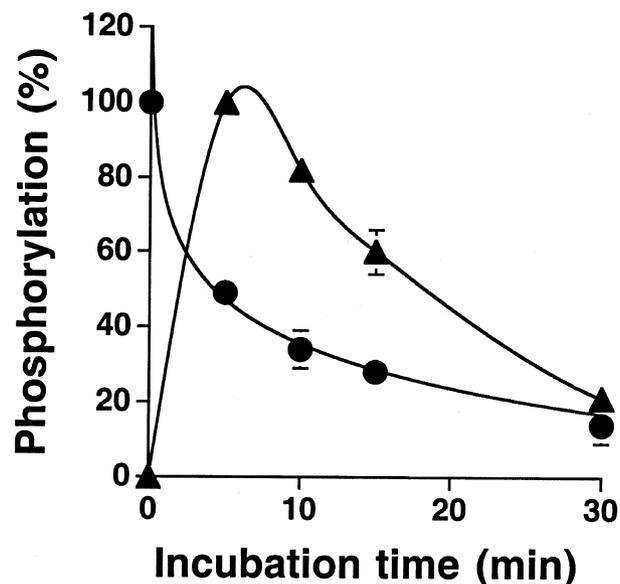


Fig. 3. NO[•] inhibits phosphorylation of EGFR and induces phosphorylation of NOIPP-58 with different kinetics. Cells were treated with 5 mM DEA-NO for the indicated times. Thereafter, 10 nM EGF was added, and 1 min later phosphorylation of EGFR (●) and NOIPP-58 (▲) were determined as described in Experimental procedures. Results are from two separate experiments, and the error bars represent the range of values obtained.

immunoprecipitated Src in its phosphorylated form does not match that of NOIPP-58. Moreover, the addition of PP1, a highly potent inhibitor of the Src tyrosine kinase family, including Lck, Lyn, Hck, and Src itself [40], did not significantly affect the phosphorylation of NOIPP-58 (results not shown).

NO[•] appears to also have a small effect on the apparent activation constant of EGF for its receptor. Thus, we determined from experiments performed using different concentrations of EGF and measuring the phosphorylation of the receptor, that in A431 tumour cells $K'_{a[EGF]}$ varies from ≈ 0.2 nM to ≈ 1 nM in the absence and presence of DEA-NO, respectively. Similarly, in EGFR-T17 fibroblasts, we found $K'_{a[EGF]}$ values of ≈ 0.05 nM and ≈ 1.5 nM in the absence and presence of DEA-NO, respectively, under similar experimental conditions.

The NO[•]-promoted inhibition of the phosphorylation of both EGFR and NOIPP-58 are cGMP-independent processes

To study whether the actions of NO[•] on the phosphorylation of EGFR and NOIPP-58 require an increase in intracellular cGMP, we performed experiments using different concentrations of the guanylate cyclase inhibitor ODQ [37]. Figure 4 shows that EGFR phosphorylation in the absence of DEA-NO was partially inhibited ($\approx 40\%$) by ODQ (open circles). However, the residual phosphorylation of the receptor observed in the presence of DEA-NO ($\approx 30\%$ of the control) did not increase in the presence of ODQ (filled circles). Moreover, the EGF-dependent NO[•]-induced phosphorylation of NOIPP-58 was not affected by

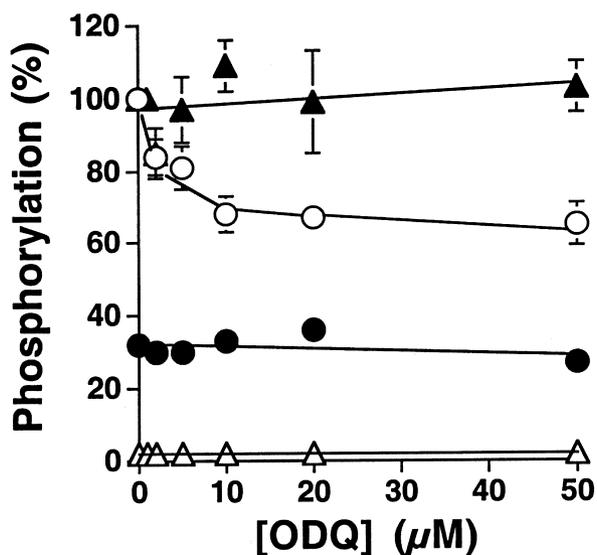


Fig. 4. NO[•] inhibits phosphorylation of EGFR and induces phosphorylation of NOIPP-58 by cGMP-independent mechanisms. Cells were treated for 15 min with the indicated concentrations of ODQ. Thereafter, the cells were incubated in the absence (open symbols) and presence (filled symbols) of 5 mM DEA-NO for another 15 min. Then 10 nM EGF was added and 5 min later phosphorylation of EGFR (circles) and NOIPP-58 (triangles) were determined as described in Experimental procedures. Results are from four (EGFR) and six (NOIPP-58) separate experiments, and the error bars represent SD.

ODQ (filled triangles), nor was this guanylate cyclase inhibitor able to promote any phosphorylation of NOIPP-58 in the absence of DEA-NO and presence of EGF (open triangles). These experiments show that both the NO[•]-elicited inhibition of EGFR phosphorylation and the EGF-dependent NO[•]-induced phosphorylation of NOIPP-58 are cGMP-independent processes.

Activation of the p38MAPK pathway by NO[•]

As different MAPKs are central to signalling by EGFR, we tested whether NO[•] regulates the different MAPK pathways. Figure 5 shows that addition of DEA-NO to A431 tumour cells does not significantly affect the phosphorylation level of ERK1/2. The clone of A431 tumour cells used in this study has an already activated ERK1/2 pathway in the absence of EGF. This is consistent with the high proliferation rate of this cell line in the absence of added growth factors (results not shown). Therefore, the addition of EGF does not increase the level of ERK1/2 phosphorylation. In contrast, DEA-NO somewhat decreases the active form of JNK1/2 in the absence or presence of EGF, whereas this NO[•] donor strongly activates p38MAPK both in the absence and presence of EGF, as determined by measuring the phosphorylation levels of these MAPKs. Additional phosphorylated bands of lower molecular mass are recognized by the antibody to P-JNK1/2 in the presence of DEA-NO. This may represent proteolytic products of these kinases and/or the cross-detection of the phosphorylated form of p38MAPK. Control experiments showed that the level of total p38MAPK was somewhat decreased after DEA-NO treatment but was not significantly affected by EGF. Figure 6 shows the time courses of phosphorylation of EGFR (Fig. 6A), NOIPP-58 (Fig. 6B), and p38MAPK

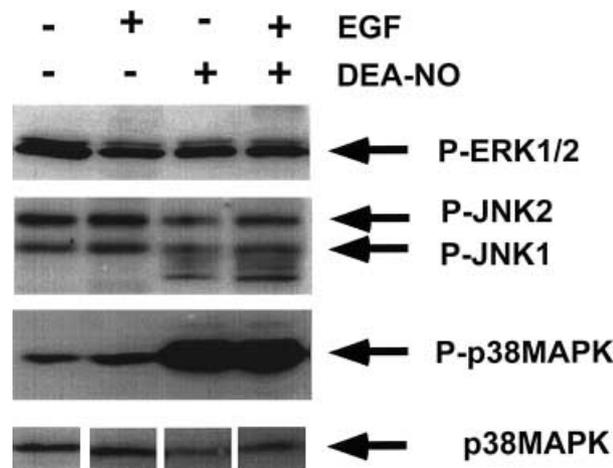


Fig. 5. NO[•] activates p38MAPK but does not activate ERK1/2 or JNK1/2 pathways. Cells were incubated in the absence and presence of 5 mM DEA-NO for 15 min and then stimulated with 10 nM EGF for 5 min as indicated. The active phosphorylated forms of the different MAPKs (P-ERK1/2, P-JNK1/2 and P-p38MAPK) were determined as described in Experimental procedures. The arrows indicate the phosphorylated forms of these kinases. A control showing total p38MAPK is also presented. Typical experiments from a total of 11 performed under similar conditions are presented.

Fig. 6. Time course of EGF-induced phosphorylation of EGFR, NOIPP-58, and p38MAPK in the absence and presence of NO[•]. Cells were incubated in the absence (open symbols) and presence (filled symbols) of 5 mM DEA-NO for 15 min. Thereafter, 10 nM EGF was added at time zero, and phosphorylation of EGFR (A), NOIPP-58 (B), and p38MAPK (C) was determined at the indicated times as described in Experimental procedures. Results are from two separate experiments, and the error bars represent the range of values obtained.

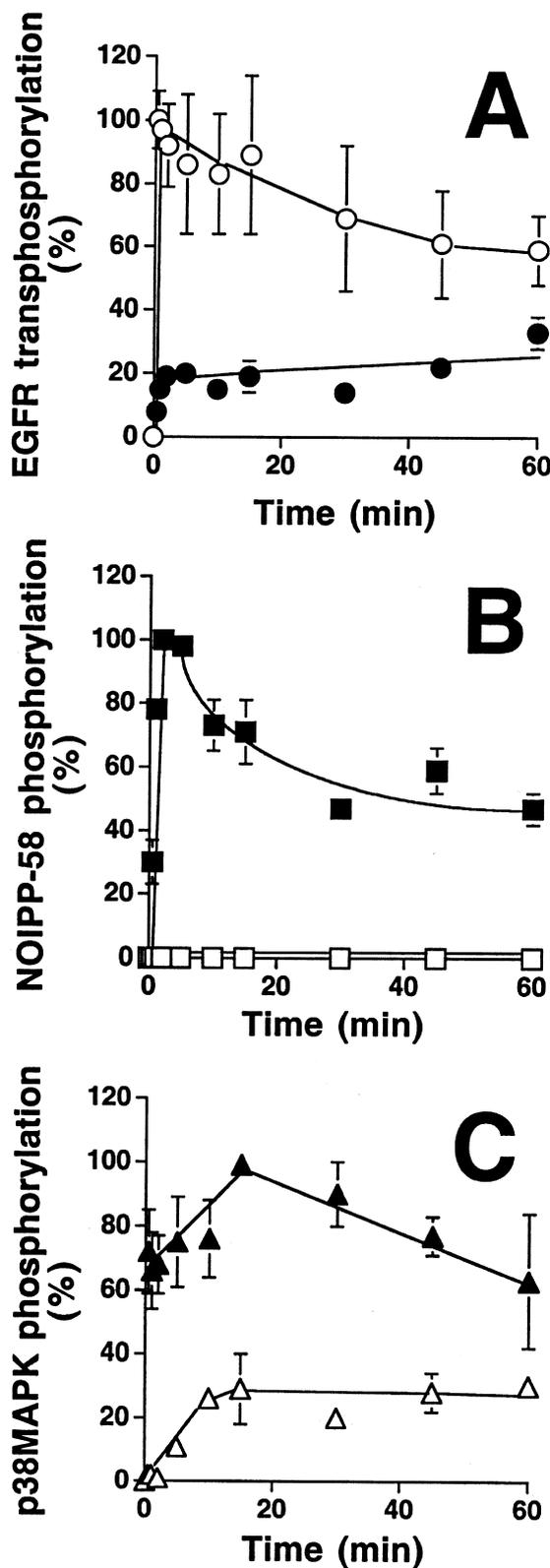
(Fig. 6C) in the absence (open symbols) and presence (filled symbols) of DEA-NO. It is apparent that activation of the p38MAPK pathway, although very prominent in the presence of DEA-NO, also occurs to a lesser extent in its absence, most significantly after 10 min of exposure to EGF, as previously demonstrated [24].

EGFR and NOIPP-58 are both dephosphorylated by a p38MAPK-regulated PYPP

To test whether the p38MAPK pathway regulates the phosphorylation state of both EGFR and NOIPP-58, the tyrosine phosphorylation levels of these proteins were monitored before and after addition of SB202190 to EGF-stimulated cells treated with DEA-NO. Figure 7 shows that addition of SB202190 induces rapid dephosphorylation of EGFR (left panel) and NOIPP-58 (right panel). The dephosphorylation of EGFR induced by SB202190 also occurs in the absence of DEA-NO (results not shown). The effect of SB202190 on the tyrosine phosphorylation levels of these proteins was also assayed in the absence and presence of the PYPP inhibitors vanadate and peroxovanadate [35,41]. As shown in Fig. 7, both inhibitors prevent the dephosphorylation of EGFR and NOIPP-58 induced by the addition of SB202190, although peroxovanadate was far more efficient than vanadate, in accordance with its higher capacity to permeate cell membranes [35]. Overall, these results illustrate that the dephosphorylation of EGFR and NOIPP-58 is under the control of a vanadate/peroxovanadate-sensitive p38MAPK-regulated PYPP.

Discussion

We have previously shown that NO[•] inhibits the proliferation of EGFR-T17 fibroblasts and NB69 neuroblastoma cells by a cGMP-independent pathway [10,12]. The effect of NO[•] was slightly more pronounced when the cells were grown in the presence of EGF than when grown in the presence of fetal bovine serum, suggesting that EGFR may be a target for NO[•] [10,12]. Moreover, using an *in vitro* permeabilized-cell system, we showed that NO[•] targets EGFR inhibiting its tyrosine kinase activity, a process that was reversed by dithiothreitol, suggesting S-nitrosylation of the receptor [10]. We now demonstrate that addition of DEA-NO also inhibits the proliferation of A431 tumour cells by a cGMP-independent mechanism, but in a more efficient fashion than in the other cell lines tested (see Fig. 1 and [10,12]). In contrast, the sensitivity of the EGFR tyrosine kinase to NO[•] in whole A431 tumour cells (this work) and permeabilized EGFR-T17 fibroblasts [10] was within the same order of magnitude ($K'_{i[DEA-NO]} \approx 1-2$ mM).



The concentration of NO[•] donor required to achieve substantial inhibition of EGFR phosphorylation in both cell types appears to be rather high. However, although we did not determine the concentration of free NO[•] in our experimental system, this is expected to be several orders of

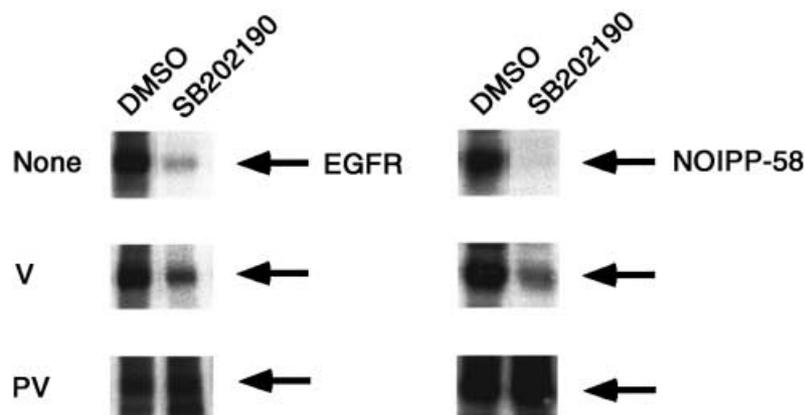


Fig. 7. EGFR and NOIPP-58 are dephosphorylated by a p38MAPK-regulated vanadate/peroxovanadate-sensitive PYPP. Cells incubated for 30 min in the absence (None) and presence of 1 mM vanadate (V) or 1 mM peroxovanadate (PV) were treated with 5 mM DEA-NO for 15 min. The cells were then stimulated with 10 nM EGF for 4 min, and thereafter 100 μ M SB202190 or the solvent dimethyl sulfoxide was added as indicated. Phosphorylation of EGFR (left panel) and NOIPP-58 (right panel) were determined 1 min later as described in Experimental procedures. A typical experiment from a total of three performed in similar conditions is presented.

magnitude lower than the actual concentration of NO \cdot donors used. There are several reasons including: (a) the low solubility of NO \cdot in water [42]; (b) the high reactivity of NO \cdot with different cellular targets that are S-nitrosylated and may act as molecular scavengers [43–49]; and (c) the rapid transformation of NO \cdot into peroxynitrite and other metabolites by different cellular systems [50].

We have observed that the $t_{1/2}$ for the release of NO \cdot from donors of the NONOate family [39] inversely correlates with the magnitude of the observed inhibition of the phosphorylation of EGFR in intact cells. NONOates, in contrast with other NO \cdot donors such as SNAP (*S*-nitroso-*N*-acetylpenicillamine), SIN-1 [3-(morpholin)sydnimine hydrochloride] and sodium nitroprussiate, have a simple mechanism of decomposition in aqueous solution and hence release NO \cdot into the medium without the need of any metabolic transformation by the cell and/or the formation of any NO \cdot -derived byproduct [39]. Thus, our results suggest that NO \cdot itself, and not NO \cdot -derived metabolites, is probably the active species inhibiting the phosphorylation of EGFR in whole A431 cells, in agreement with our observations in permeabilized EGFR-T17 fibroblasts [10] and intact NB69 neuroblastoma cells [12]. We performed Western blots using an antibody to nitrotyrosine in cells treated with DEA-NO and SPER-NO. Despite the high background yield by this antibody, we detected no labelled band at 170 kDa, suggesting that tyrosine residues in EGFR were not nitrated (results not shown).

Interestingly, NO \cdot does not inhibit the binding of [125 I]EGF to its receptor in EGFR-T17 fibroblasts [10]. However, we have demonstrated in both A431 tumour cells and EGFR-T17 fibroblasts that NO \cdot slightly increases the apparent activation constant of EGF for tyrosine phosphorylation of the receptor when monitored in whole cells. This suggests that the binding of EGF to the receptor is not impaired by NO \cdot , but the bound EGF cannot activate the NO \cdot -modified EGFR with the same efficiency as it does the native receptor.

Our experiments also show that the NO \cdot -promoted inhibition of EGFR phosphorylation in A431 tumour cells

and other cell lines is a cGMP-independent process (this work and [10,12]). We have found, however, that phosphorylation of EGFR in the absence of NO \cdot is partially sensitive to the guanylate cyclase inhibitor ODQ (Fig. 4). The inhibition of EGFR phosphorylation by ODQ is a concentration-dependent process up to 10 μ M, conditions under which the guanylate cyclase is fully inhibited [37]. However, higher concentrations of ODQ do not further affect the phosphorylation of the receptor, suggesting that only a part (\approx 40%) of this process is dependent on cGMP. The interplay between cGMP and EGFR appears to be quite complex, as it has been shown that cGMP inhibits the EGF-induced activation of the MAPK pathway via phosphorylation of Raf by a cGMP-dependent protein kinase [51,52] and through the induction of MAPK phosphatase 1 [52]. The effect of ODQ on EGFR phosphorylation described in this work is a new and unexpected observation that may underscore a potent activation of the receptor by a regulatory cGMP-dependent protein kinase or another cGMP-dependent system.

The NO \cdot -dependent phosphorylation of NOIPP-58 is strictly dependent on the presence of EGF, and therefore requires a partially active EGFR. As no phosphorylation of NOIPP-58 was detected in the absence of NO \cdot , either in the absence or presence of EGF, we propose that the partially active EGFR may be directly responsible for the phosphorylation of NOIPP-58. This is supported by the fact that the $K'_{i[\text{DEA-NO}]}$ for EGFR phosphorylation and the $K'_{a[\text{DEA-NO}]}$ for NOIPP-58 phosphorylation have comparable values (1–2 mM). An NO \cdot -modified NOIPP-58 is probably the actual substrate of EGFR.

We have also shown, as previously reported by others [25–28], that NO \cdot induces the activation of the p38MAPK pathway, not only in A431 tumour cells (Figs 5 and 6), but also in several murine fibroblast cell lines such as EGFR-T17 and N7xHERc, which overexpress human EGFR, Swiss 3T3 and NIH 3T3, which, respectively, express a moderate and low number of EGFR molecules, and clone 2.2, which does not express this receptor (results not shown). This demonstrates that the NO \cdot -mediated activation of

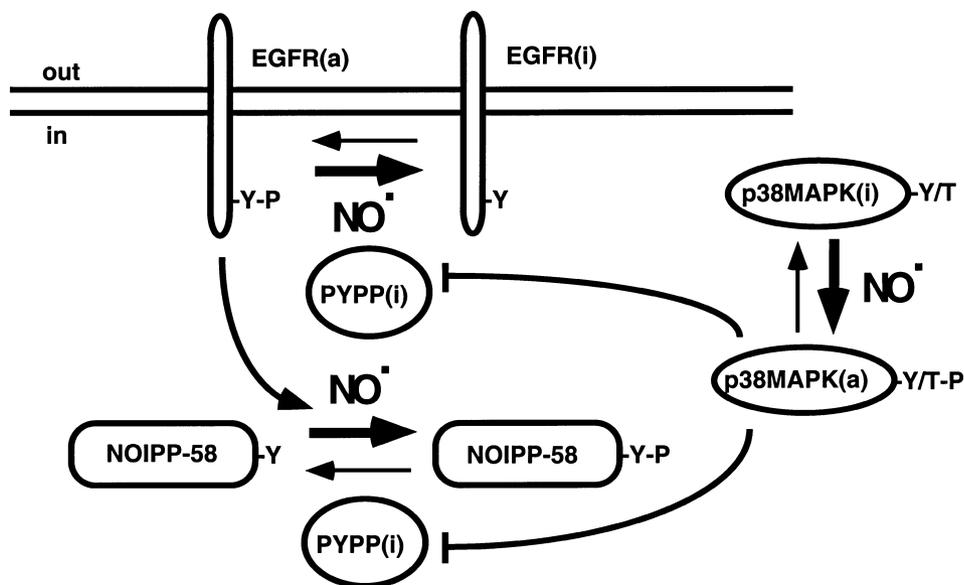


Fig. 8. Signalling events during nitrosative stress in A431 tumour cells. NO[•] induces partial inactivation of EGFR, probably by S-nitrosylation [10], and activation of the p38MAPK pathway. The partially active EGFR in the presence of NO[•] favours the phosphorylation of NOIPP-58, perhaps because of inactivation of a p38MAPK-regulated PYPP that has as substrates both NOIPP-58 and EGFR. The symbols -Y-P and -Y/T-P represent the phosphorylated form of either EGFR/NOIPP-58 or p38MAPK, respectively, at tyrosine or tyrosine/threonine residues. The active (a) and inactive (i) forms of the different proteins are indicated. Nitrosative stress in A431 tumour cells inactivates a p38MAPK-regulated PYPP, thereby favouring phosphorylation of NOIPP-58 by the partially active EGFR and avoiding total dephosphorylation of the receptor.

p38MAPK is an EGFR-independent process. The NO[•]-dependent activation of the p38MAPK pathway may contribute to the arrest of the cell cycle, as it has been shown in a different system on activation of the activin receptor pathway [53]. Although our results do not allow us to establish a direct correlation between the NO[•]-induced inhibition of cell proliferation and the phosphorylation/dephosphorylation events under study, as the two processes are achieved at different concentrations of DEA-NO, we cannot exclude the possibility that low concentrations of DEA-NO during long exposure times, such as those required for the inhibition of [*methyl*-³H]thymidine incorporation into DNA, may affect the phosphorylation state of the relevant proteins during the long period required to complete a full cell cycle. Nevertheless, it is likely that distinct systems involved in cell proliferation are affected by NO[•].

Inhibition of the p38MAPK pathway activates a vanadate/peroxovanadate-sensitive PYPP which dephosphorylates EGFR. In cells exposed to NO[•] and in the presence of EGF, conditions in which NOIPP-58 is phosphorylated, p38MAPK inhibition results in dephosphorylation of both NOIPP-58 and EGFR by the same mechanism (Fig. 7). This suggests that, under normal physiological conditions, when cells are stimulated by EGF, or during nitrosative stress generated by activation of NOS, the activated p38MAPK pathway signals to down-regulate the activity of the PYPP acting on EGFR and NOIPP-58 (see model in Fig. 8). This system may therefore be a mechanism for keeping EGFR and the potential signalling capacity of the phosphorylated form of NOIPP-58 partially operative by preventing their dephosphorylation.

To the best of our knowledge, this is the first demonstration of the existence of a p38MAPK-regulated PYPP modulating the activity of both EGFR and the phosphorylation state of NOIPP-58, a protein substrate of this receptor. Further studies should uncover the physiological function of NOIPP-58, as well as the molecular characteristics of the p38MAPK-regulated phosphatase involved in dephosphorylation of EGFR and NOIPP-58, and whether similar dephosphorylation pathways act on other activated receptors of the ErbB family and/or other unrelated tyrosine kinase receptors.

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