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Original Contribution

S-Nitrosylation of the epidermal growth factor receptor: A regulatory mechanism of receptor tyrosine kinase activity

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ABSTRACT

Nitric oxide (NO) donors inhibit the epidermal growth factor (EGF)-dependent auto(*trans*)phosphorylation of the EGF receptor (EGFR) in several cell types in which NO exerts antiproliferative effects. We demonstrate in this report that NO inhibits, whereas NO synthase inhibition potentiates, the EGFR tyrosine kinase activity in NO-producing cells, indicating that physiological concentrations of NO were able to regulate the receptor activity. Depletion of intracellular glutathione enhanced the inhibitory effect of the NO donor 1,1-diethyl-2-hydroxy-2-nitrosohydrazine (DEA/NO) on EGFR tyrosine kinase activity, supporting the notion that such inhibition was a consequence of an S-nitrosylation reaction. Addition of DEA/NO to cell lysates resulted in the S-nitrosylation of a large number of proteins including the EGFR, as confirmed by the chemical detection of nitrosothiol groups in the immunoprecipitated receptor. We prepared a set of seven EGFR(C → S) substitution mutants and demonstrated in transfected cells that the tyrosine kinase activity of the EGFR(C166S) mutant was completely resistant to NO, whereas the EGFR(C305S) mutant was partially resistant. In the presence of EGF, DEA/NO significantly inhibited Akt phosphorylation in cells transfected with wild-type EGFR, but not in those transfected with C166S or C305S mutants. We conclude that the EGFR can be posttranslationally regulated by reversible S-nitrosylation of C166 and C305 in living cells.

Nitric oxide (NO) is an intracellular and intercellular messenger that produces a large variety of cellular responses, depending on its concentration in the cell microenvironment and on the cell type and functional status, among other factors. Cell proliferation can be modulated by the presence of NO. Although both proliferative and antiproliferative effects have been described [see for reviews 1,2], most studies have concentrated on the antiproliferative action of NO in many normal and tumor cell types [3–13].

Proliferation is a complex process that can be regulated at different levels. Interactions of NO with several systems involved in cell proliferation such as ornithine decarboxylase [14]; ribonucleotide reductase [8]; the $p21^{Cip1/Waf1}$/pRb pathway [13,15,16]; other proteins relevant for the control of the cell cycle at the G_1/S transition, such as the activity and/or expression of Cdk2 and several cyclins, among others [13,17,18]; or the Ras/MAPK pathway [13,19–21] have been described. We have reported that, at least in part, the antiproliferative effect of NO may be due to its interaction with the EGFR. This has been shown in stably transfected mouse fibroblasts that overexpress the human EGFR [9], as well as in various tumor cells that naturally express this receptor [11–13]. In all these cases, NO donors inhibited EGFR auto(*trans*)phosphorylation and EGF-dependent cell proliferation. Furthermore, we have recently reported that murine neural precursor cells (NPC), which spontaneously produce NO, increase their proliferation rate and the phosphorylation status of the EGFR when treated with a NO synthase (NOS) inhibitor [22].

Some of the physiological actions of NO, such as vasodilatation [23] and neuromodulation [24], among others, are mediated by activation of a soluble guanylyl cyclase, through interaction of NO with the heme group of the enzyme [25]. However, the inhibitory effect of NO on EGF-induced cell proliferation did not require cGMP production [9,11,12]. Stamler and collaborators [26,27] and others [28,29] have demonstrated that S-nitrosylation (also called S-nitrosation by some authors) of specific cysteine residues is a reversible and regulated mechanism that can modify protein activity and that it may occur at

Abbreviations: biotin-HPDP, *N*-(6-(biotinamido)hexyl)-3′-(2′-pyridyldithio)propionamide; BrdU, 5-bromo-2′-deoxyuridine; BSO, L-buthionine sulfoximine; DEA/NO, 1,1-diethyl-2-hydroxy-2-nitrosohydrazine; DMEM, Dulbecco's modified Eagle's medium; EGF, epidermal growth factor; EGFR, EGF receptor; EGFR–GFP, EGFR–green fluorescence protein chimera; FBS, fetal bovine serum; GSH, reduced glutathione; GSNO, S-nitrosylated glutathione; IgG, immunoglobulin G; L-NAME, N^{ω}-nitro-L-arginine methyl ester; MAPK, mitogen-activated protein kinase; NOS, nitric oxide synthase; NPC, neural precursor cells; PAE, porcine aorta endothelium; PI3K, phosphatidylinositol 3-kinase; PMSF, phenylmethylsulfonyl fluoride; PVDF, polyvinylidene difluoride; SNAP, *S*-nitroso-*N*-acetylpenicillamine.

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physiological concentrations of NO. During the past few years, a large number of proteins have been reported to be susceptible to S-nitrosylation in vitro [27], and in some cases, endogenous S-nitrosylation has been demonstrated in tissues [29,30] or cultured cells [31] able to synthesize NO.

Our previous studies using transfected fibroblasts suggested that the EGFR was S-nitrosylated in the presence of NO donors, because NO-induced receptor inhibition could be reversed in the presence of dithiothreitol [9]. Moreover, we also demonstrated that cell-permeative reducing agents were able to reactivate the NO-inhibited EGFR in living NB69 cells [13]. In the present report, we demonstrate that the EGFR is S-nitrosylated in the presence of NO, so inhibiting its tyrosine kinase activity, and we have identified two cysteine residues as the NO targets in the receptor molecule.

Experimental procedures

Reagents

The following reagents were used: 1,1-diethyl-2-hydroxy-2-nitrosohydrazine (DEA/NO), 2,2′-(hydroxynitrosohydrazino)bis-ethanamine (DETA/NO), *S*-nitroso-*N*-acetylpenicillamine (SNAP), N^{ω}-nitro-L-arginine methyl ester (L-NAME), L-buthionine sulfoximine (BSO), and 5-bromo-2′-deoxyuridine (BrdU) from Sigma–Aldrich (St. Louis, MO, USA); [*methyl*-^{3}H]thymidine and EcoLite(+) liquid scintillation cocktail from ICN Pharmaceuticals (Costa Mesa, CA, USA); EGF (human recombinant) from Upstate Biotechnology (Lake Placid, NY, USA); PVDF membranes (Immobilon-P) from Millipore Corp. (Bedford, MA, USA) or BioTrace from Pall Laboratory (Ann Arbor, MI, USA); GIBCO culture media, saline solutions, glutamine, FBS, trypsin/EDTA solution, gentamicin, bovine serum albumin, and Lipofectamine 2000 from Invitrogen (Carlsbad, CA, USA); trichloroacetic acid, glycerol, inorganic salts, and concentrated acids, bases, and alcohols from Merck (Darmstadt, Germany); SDS, acrylamide:bisacrylamide solutions, and protein assay kits from Bio-Rad Laboratories (Hercules, CA, USA); and Supersignal enhanced chemiluminescence substrate (ECL) kit and EZ-Link biotin-HPDP from Pierce Biotechnology (Rockford, IL, USA). All other chemicals used were of analytical grade. The following antibodies were used: polyclonal antibodies against phospho-EGFR (Tyr992), phospho-EGFR (Tyr1045), phospho-Akt (Ser473), and total Akt from Cell Signaling Technology (Boston, MA, USA); polyclonal antibodies against total EGFR from Santa Cruz Biotechnology (Santa Cruz, CA, USA) or Cell Signaling Technology; monoclonal peroxidase-conjugated anti-phosphotyrosine antibody (RC20 H) from Transduction Laboratories (Lexington, KY, USA); anti-BrdU monoclonal antibody from Dako Denmark A/S (Glostrup, Denmark); monoclonal anti-actin, monoclonal anti-α-tubulin, peroxidase-conjugated monoclonal anti-biotin, and secondary peroxidase-conjugated anti-mouse or anti-rabbit IgG antibodies from Sigma–Aldrich or Pierce Biotechnology; and secondary anti-mouse IgG labeled with fluorescein from Jackson ImmunoResearch Laboratories (West Grove, PA, USA).

Cell cultures

Human neuroblastoma NB69 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 15% (v/v) FBS, 2 mM L-glutamine, and 40 mg/ml gentamicin. Once a week, cells were detached with trypsin/EDTA and seeded at a density of 9000 cells/cm^2, and the medium was changed 3 days afterward. Porcine aorta endothelial cells stably transfected with a chimeric human EGFR with the green fluorescence protein at its C-terminus (PAE/EGFR–GFP cells) were obtained from Dr. Carlos Enrich (Universitat de Barcelona) and were grown in Ham's medium supplemented with 10% (v/v) FBS, 2 mM L-glutamine, and 40 μg/ml gentamicin. NPC were obtained from the subventricular zone of 7-day postnatal CD1 mice and maintained in culture as previously described [22]. HEK293FT cells were commercially obtained from Invitrogen and cultured in DMEM supplemented with 10% (v/v) FBS, 2 mM L-glutamine, 40 μg/ml gentamicin, and 500 μg/ml geneticin. EGFR-T17 cells were obtained and cultured as previously described [9]. All cells were maintained in a humidified atmosphere of 5% CO_2 in air at 37°C. Experiments were performed in cultures that had been maintained in serum-free medium for the preceding 24 h.

Cell proliferation assays

Incorporation of [*methyl*-^{3}H]thymidine into DNA was performed in EGFR-T17 and NB69 cells in the absence and presence of 20 ng/ml EGF as previously reported [9]. Incorporation of BrdU into DNA was detected in fixed and immunostained NPC using a specific anti-BrdU antibody as previously described [22].

Glutathione depletion experiments

NB69 cells were seeded in six-well culture clusters at a density of 4×10^5 cells/well and grown to confluence for 3 days. Then, cells were changed to a serum-free medium in the absence or presence of 20 μM BSO, an inhibitor of γ-glutamyl cysteine ligase, the rate-limiting enzyme of glutathione synthesis. After 24 h, the cells were incubated with or without 1 mM DEA/NO for 15 min, followed by a 2-min pulse addition of 20 ng/ml EGF. Controls in the absence of EGF were also included. The incubations were stopped with ice-cold 10% (w/v) trichloroacetic acid; afterward, cellular precipitates were scraped and collected by centrifugation (7500 g, 15 min) and the pellets were processed for Western blotting, to detect the phosphorylation state of the EGFR, using a peroxidase-conjugated anti-phosphotyrosine antibody (1/2000 dilution).

Site-directed mutagenesis

The cvsvHERc expression vector encoding the wild-type human EGFR was a kind gift from Professor Axel Ullrich (Max-Planck-Institut für Biochemie, Martinsried, Germany). Polymerase chain reaction-aided site-directed mutagenesis was directly performed on the cvsvHERc vector using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA) and appropriate sets of complementary oligos designed to substitute a serine for any of the following cysteine residues: C166, C305, C751, C757, C794, C926, and C1025. We chose this particular substitution because these two amino acids are identical except for the sulfhydryl group (–SH) present in cysteine, susceptible to NO-mediated S-nitrosylation, which corresponds to a hydroxyl group (–OH) in serine that is insensitive to NO. The correctness of each site-directed mutation was corroborated by DNA sequencing of the vector using appropriate primers. The *Escherichia coli* strain DH5α was transformed with the different constructs to replicate the plasmidic DNA, which was thereafter isolated using the Genopure Plasmid Midi kit from Roche (Basel, Switzerland).

Cell transfection

HEK293FT cells were seeded at a density of 100,000 cells/cm^2 in the absence of antibiotics and the next day were transfected using Lipofectamine 2000 to introduce the different plasmidic constructs into the cells, according to the manufacturer's instructions. Two days later, the cells were changed to serum-free medium supplemented with superoxide dismutase (15 units/ml) and treated with or without 5 mM DEA/NO for 30 min followed by a 2-min pulse addition of EGF (20 ng/ml). Then, the medium was aspirated and 10% (w/v) ice-cold trichloroacetic acid was added to the cells to precipitate cellular proteins. Finally, the samples were processed for SDS–PAGE and Western blotting as described below.

Western blot analysis

Cell proteins were dissolved in a loading buffer containing 60 mM Tris–HCl (pH 6.8), 10% (w/v) SDS, 0.02% (w/v) bromophenol blue, 5% (v/v) 2-mercaptoethanol, and 20% (w/v) glycerol. After boiling for 5 min, the samples were loaded into SDS–polyacrylamide linear-gradient (5–20%) gels and electrophoresed at 12 mA overnight. The proteins were electrotransferred to PVDF membranes, fixed with 0.2% (v/v) glutaraldehyde, and temporarily stained with the Fast Green FCF dye. The stained membranes were scanned for later measurements of the protein content in each track. Thereafter, the membrane was rinsed, blocked either with Tris-buffered saline (TBS) plus 5% (w/v) bovine serum albumin for 2 h (when the anti-phosphotyrosine antibody was used) or with TBS containing 0.1% (v/v) Tween 20 and 3% (w/v) fat-free dry milk for 30 min (when any other antibody was used). The membranes were then incubated with the various primary antibodies diluted in the same solution as for blocking. When primary antibodies were not directly conjugated to horseradish peroxidase, the membranes were washed and incubated as appropriate with secondary peroxidase-conjugated anti-mouse or anti-rabbit IgG antibodies (1/4000 dilution). The positive bands were developed, after appropriate time exposure, using the ECL method following the instructions of the manufacturer. When required, specific bands were quantified by photodensitometry, using a computer-assisted scanner and the ImageJ program from the NIH. Corrections were made for the total amount of protein present in the electrophoretic tracks as detected by Fast Green FCF staining or by constitutive proteins like α-tubulin or β-actin, although only minor differences in protein content were detected.

Identification of S-nitrosylated proteins

To detect proteins that were S-nitrosylated in cysteine residues by NO, we used the method described by Jaffrey and collaborators [29], a three-step reaction that allows the specific biotinylation of *S*-nitrosothiol groups that can afterward be identified.

Cell lysates were obtained and divided into aliquots with the same amount of total protein (typically, 0.3 mg of protein in 0.5 ml per sample). Proteins were then precipitated by adding 2 volumes of cold acetone (−20°C) to each sample and kept for 10 min at −20°C. After centrifugation (2300*g*, 5 min), the pellets were resuspended in 200 ml of a buffer containing 250 mM Hepes/NaOH (pH 7.7), 1 mM EDTA, and 0.1 mM neocuproine (HEN buffer). Next, samples were treated with or without 1 mM DEA/NO for 15 min at room temperature and the excess DEA/NO was eliminated by acetone precipitation of proteins. These were resuspended in blocking buffer (HEN buffer plus 2.5% (w/v) SDS and 20 mM *S*-methylmethane thiosulfonate) and incubated at 50°C for 20 min, to block free thiol groups. Proteins were again precipitated with acetone, resuspended in biotinylation buffer (HEN buffer plus 1 mM ascorbate and 0.2 mM EZ-Link biotin-HPDP), and incubated for 1 h at room temperature. In this step, nitrosothiol groups in proteins are reduced by ascorbate to give free thiol groups, which can then be specifically biotinylated. Finally, proteins were collected by acetone precipitation and processed in three ways: (i) The proteins were resuspended in loading buffer (reducing agents and boiling must be avoided) and electrophoresed, to visualize by Western blotting the overall protein biotinylation pattern using a peroxidase-conjugated anti-biotin antibody (1/4000 dilution). (ii) The proteins were diluted with a lysis buffer containing 50 mM Tris–HCl (pH 7.4), 1% (v/v) Triton X-100, 0.5% (w/v) sodium deoxycholate, 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml pepstatin A, 10 μg/ml aprotinin, and 2 mM sodium orthovanadate and treated overnight with an anti-EGFR antibody (4 mg per gram of total cell protein), followed by a 3-h incubation with protein A–agarose to immunoprecipitate the EGFR. The biotinylated EGFR was visualized after SDS–PAGE (avoiding reducing agents) by Western blotting, using the anti-biotin antibody described above. (iii) The proteins were resuspended in 300 μl of HEN buffer and then treated for 1 h at room temperature under gentle rocking with 5 μl of streptavidin–agarose, to precipitate all the biotinylated proteins bound to streptavidin. After centrifugation, the precipitate was resuspended in loading buffer and subjected to SDS–PAGE and Western blotting, to detect the presence of the EGFR using a polyclonal anti-EGFR antibody.

Results

NO inhibits proliferation in a wide variety of EGF-responsive cell types

In previous work, we have demonstrated that NO inhibits the proliferation of various cell types and that this inhibitory effect is more prominent when cell division is induced by EGF [9,11]. Fig. 1 shows the concentration-dependent cytostatic effect of several NO donors in three types of EGF-responsive cell lines, namely: EGFR-T17 cells (Fig. 1A), a mouse fibroblast cell line engineered to overexpress the human EGFR; NB69 cells (Fig. 1B), a human neuroblastoma tumor cell line that naturally expresses the EGFR; and primary cultures of NPC (Fig. 1C) obtained from the subventricular zone of postnatal mice and grown as neurospheres, which express the EGFR and are EGF-dependent for their growth in vivo and in vitro. In all these cell types, NO not only decreased the proliferation rate, but also inhibited the EGF-induced activation of EGFR (Fig. 1D), in agreement with results previously reported in these and other cell lines [9,11–13,22].

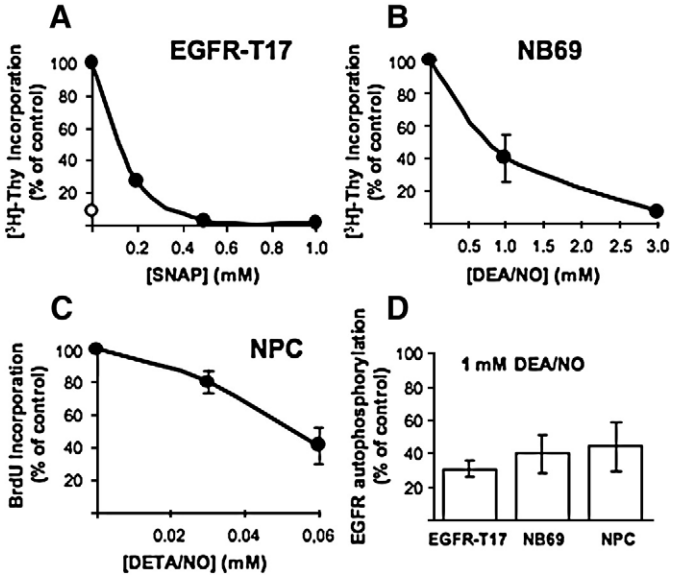

Fig. 1. NO inhibits cell proliferation and EGFR phosphorylation in various EGF-responding cell types. (A) Incorporation of [*methyl*-^{3}H]thymidine into DNA by proliferating EGFR-T17 cells grown for 24 h in the absence of added growth factors (white dot) or in a medium containing EGF (black dots) plus the indicated concentrations of the NO donor SNAP. (B) Incorporation of [*methyl*-^{3}H]thymidine into DNA by NB69 cells grown for 48 h in the presence of EGF plus the indicated concentrations of the NO donor DEA/NO. (C) Number of nuclei that incorporated BrdU during an 8-h period in neural precursor cells (NPC) treated with EGF plus the indicated concentrations of the NO donor DETA/NO for 48 h. (D) The indicated cell types were treated with or without 1 mM DEA/NO for 15 min and then with EGF for 2 min. Thereafter, cell proteins were immediately precipitated with 10% (w/v) trichloroacetic acid, processed for SDS–PAGE, transferred to a PVDF membrane, and probed with an anti-phosphotyrosine antibody to visualize the activation state of the EGFR, as described under Experimental procedures. Data shown are the means±SEM of at least three experiments. Error bars are shown when larger than the symbols.

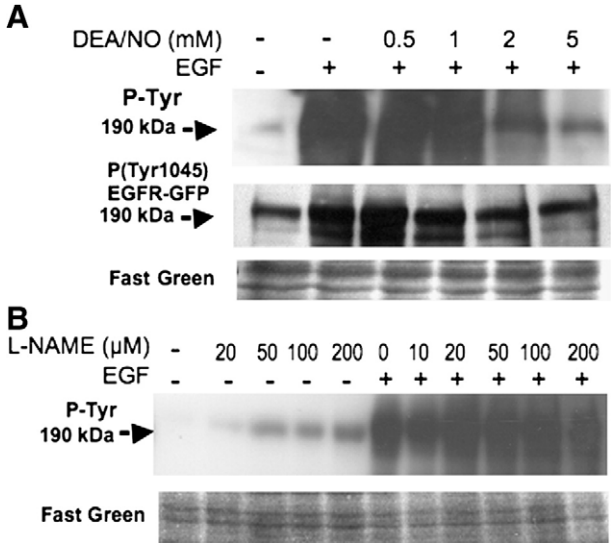

Fig. 2. EGFR–GFP auto(*trans*)phosphorylation was sensitive to exogenously added and endogenously produced NO. NO-producing PAE/EGFR–GFP cells were grown to confluence and serum starved before incubation in the absence (−) and presence (+) of increasing concentrations of (A) the NO donor DEA/NO or (B) the NOS inhibitor L-NAME for 15 min. Thereafter, 20 ng/ml EGF was added and the incubation was prolonged for 2 min. Controls in the absence of EGF are also presented. Samples were processed for Western blotting as described under Experimental procedures. Arrows point to the phosphorylated 190-kDa EGFR–GFP band. A segment of the membrane stained with Fast Green to visualize the total protein loaded in each track is also shown. One of three independent experiments with similar results is shown in each case.

The EGFR is inhibited by physiological concentrations of endogenous NO

Because it is technically difficult to ascertain that physiological concentrations of NO are obtained by the addition of NO donors to cultured cells, we sought to determine whether endogenous NO production was able to inhibit the EGFR phosphorylation. For this purpose, we used a cell culture model consisting of PAE cells stably transfected with a human EGFR–GFP chimera. The advantages of using these cells are that they express the endothelial nitric oxide synthase (eNOS) and produce endogenous NO at a steady rate and the effects of these physiological concentrations of NO on the transfected receptor can be directly tested. As shown in Fig. 2A, we first ascertained that DEA/NO inhibited the EGF-induced EGFR–GFP auto(*trans*)phosphorylation, in accordance with the results obtained in other cell types [9,11–13,22]. In these cells, blocking endogenous NO production by treatment with the NOS inhibitor L-NAME produced EGFR–GFP phosphorylation in the absence of EGF (Fig. 2B).

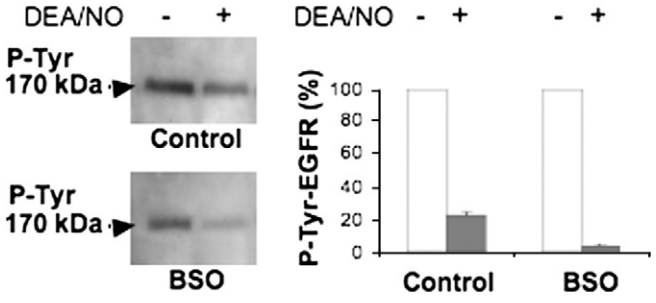

Fig. 3. Intracellular depletion of glutathione potentiates the effect of DEA/NO on EGFR auto(*trans*)phosphorylation. NB69 cells were maintained in serum-free medium for 24 h, in the absence or presence of the glutathione synthesis inhibitor BSO (20 μM). Afterward, cells were washed and incubated with or without 1 mM DEA/NO for 15 min and then with 20 ng/ml EGF for 2 min. Samples were processed as described for Fig. 1D. A representative experiment is shown on the left. The graph on the right represents EGFR phosphorylation as a percentage of the optical density measured in the EGFR band in the absence of DEA/NO. Data are the means±SEM of the values obtained in four independent experiments.

The inhibitory effect of NO on ligand-induced EGFR activation was enhanced by glutathione depletion

Fig. 3 shows that DEA/NO inhibited the auto(*trans*)phosphorylation of EGFR in NB69 cells, as expected. Moreover, when cells were pretreated for 24 h with BSO, an inhibitor of glutathione synthesis, the inhibitory action of the NO donor on EGFR auto(*trans*)phosphorylation was significantly enhanced.

The EGFR was S-nitrosylated in the presence of NO

Exposure of NB69 cell lysates to the NO donor DEA/NO resulted in the S-nitrosylation of a large number of proteins (Fig. 4A). As shown in the magnified inset, a longer exposure time of the film revealed an S-nitrosylated 170-kDa band corresponding to the apparent molecular mass of the EGFR, in samples exposed to DEA/NO. To confirm that this S-nitrosylated band was in fact the EGFR, the receptor was

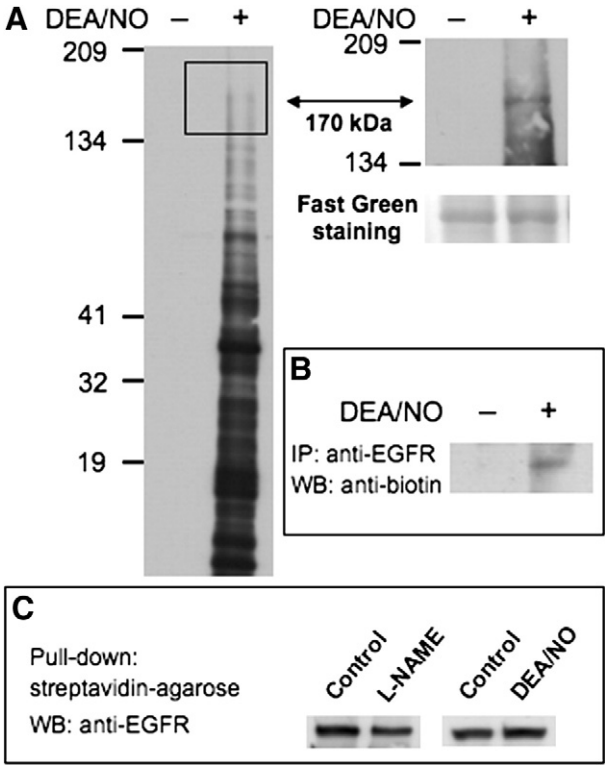

Fig. 4. Nitric oxide S-nitrosylates the EGFR. (A) NB69 cells were depleted of serum for 24 h and lysed. Then, cell lysates were treated with or without 1 mM DEA/NO for 15 min and processed for the *S*-nitrosothiol biotinylation reaction described under Experimental procedures. Whole-cell lysates were directly electrophoresed for Western blotting, using an anti-biotin antibody to visualize the pattern of protein S-nitrosylation. A magnification of the high-molecular-mass bands in the inset, subjected to a longer exposure time of the film, is shown on the right. The double-headed arrow points to the EGFR 170 kDa molecular mass band. Fast Green staining of the membrane is shown as a loading control. (B) Whole-cell lysates (treated as in A) were incubated with an anti-EGFR antibody coupled to protein A–agarose to immunoprecipitate the EGFR, which was then subjected to SDS–PAGE/Western blotting as described under Experimental procedures. An anti-biotin antibody was used to detect the biotinylation status of the EGFR. The appearance of a biotinylated EGFR band when cell lysates were treated with the NO donor indicates that the receptor was S-nitrosylated. One of five independent experiments is shown in each case. (C) NPC were depleted of growth factors and incubated for 1 h in the absence or presence of the NOS inhibitor L-NAME (0.1 mM). Then, cells were lysed and treated as in A. Biotinylated proteins were precipitated with streptavidin–agarose and processed for SDS–PAGE/Western blotting as described under Experimental procedures, using an anti-EGFR antibody to detect the biotinylated/S-nitrosylated EGFR.

immunoprecipitated from NB69 cell lysates with a specific anti-EGFR antibody, and its S-biotinylation (revealing the former presence of nitrosothiol groups) was detected by Western blotting. Fig. 4B shows that the immunoprecipitated EGFR was not S-nitrosylated under control conditions, but a clear band appeared after incubation with DEA/NO. A different result was obtained in NPC, as these cells spontaneously produce NO. Hence, in NPC the EGFR presented a basal S-nitrosylation status, which was slightly reduced after 1 h exposure to the NOS inhibitor L-NAME, as well as slightly enhanced by DEA/NO treatment (Fig. 4C).

Identification of the cysteine residues targeted by NO in the EGFR

Human EGFR possesses 59 cysteine residues, most of which are located in two extracellular cysteine-rich domains forming disulfide bonds. Because it was not affordable for us to make single point mutations of such a high number of residues, and taking into account that for most S-nitrosylated proteins known to date only one or a few cysteine residues in these proteins are targeted by NO, we selected a group of the most likely candidate cysteine residues to be mutated in the first place. Two extracellular cysteine residues (C166 and C305) that matched the S-nitrosylation consensus sequence (K, R, H, D, E)–C–(D, E) described previously [26] were included in the group, even though they formed disulfide bonds with other cysteine partners; also most of the intracellular cysteine residues were mutated, albeit only C926 was surrounded by acidic–basic residues, as deduced from the three-dimensional structure available from crystallographic studies of part of the EGFR intracellular region [32]. The wild-type and a total of seven cysteine-to-serine mutant versions of the human EGFR were expressed in HEK293FT cells and tested for their NO sensitivity. As shown in Fig. 5A, no phospho-EGFR signal was obtained in untreated cells, in cells exposed to DEA/NO alone, or under any condition in mock-transfected cells. EGF induced the auto(*trans*)phosphorylation of the wild type and all EGFR mutants tested (Figs. 5A and B). Pretreatment with DEA/NO inhibited EGFR phosphorylation in the wild type, as well as in the C751S, C757S, C794S, C926S, and C1025S mutants (Figs. 5A and B). However, the EGFR(C166S) mutant was completely resistant to NO (Figs. 5A and B), indicating that this cysteine is targeted by NO, so inducing its inhibitory action. The EGFR (C305S) mutant was still able to respond to NO, although its NO-induced inhibition was significantly reduced compared to the wild-type (WT) EGFR as determined in paired experiments (Figs. 5A and B). Interestingly, the EGFR(C166S) and EGFR(C305S) mutants were activated by EGF to a lesser extent than the WT EGFR (Fig. 5A and C).

NO decreases Akt phosphorylation in EGF-treated cells transfected with the WT EGFR but not with the C166S or C305S mutants

To elucidate whether downstream signaling was affected by the EGFR phosphorylation status in wild-type and NO-resistant EGFR

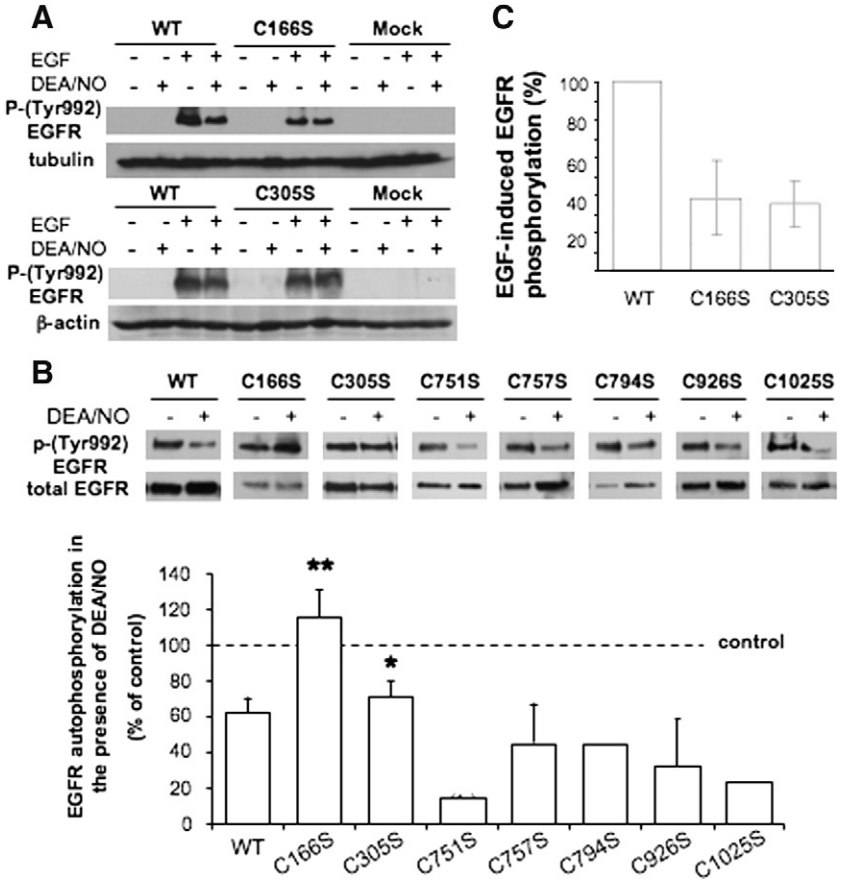

Fig. 5. Identification of the NO-sensitive cysteine residues in the EGFR. (A) HEK293FT cells were transfected with plasmids coding for either the WT EGFR or the EGFR mutants C166S or C305S; mock-transfected cells were used in parallel. Forty-eight hours later, when maximal expression of the exogenous EGFR was obtained, cells were pretreated with or without 5 mM DEA/NO for 30 min and with or without EGF (20 ng/ml) for the last 2 min. Then, the samples were used for SDS–PAGE and Western blotting to detect the phospho-EGFR (Tyr992) band as well as a loading-control protein (as indicated). Typical examples from four independent experiments are shown. (B) HEK293FT cells were transfected and processed following the same procedure as in (A), using different constructs with selected point mutations in the EGFR, all consisting of cysteine-to-serine substitutions. The top shows a typical example of each mutant. The bottom shows the phospho-EGFR/total EGFR band densitometric quantification in the presence of DEA/NO expressed as a percentage of the value obtained in its absence, for the WT EGFR and each mutant. Controls in the absence of EGF were performed for all mutants and were always blank (not shown). (C) The graph shows the densitometric quantification of the phospho-EGFR band, relative to total EGFR; both the C166S and the C305S mutants were activated by EGF to a lesser extent than the WT EGFR. Results were normalized as a percentage of the data obtained for WT EGFR in each independent experiment ($n=4$).

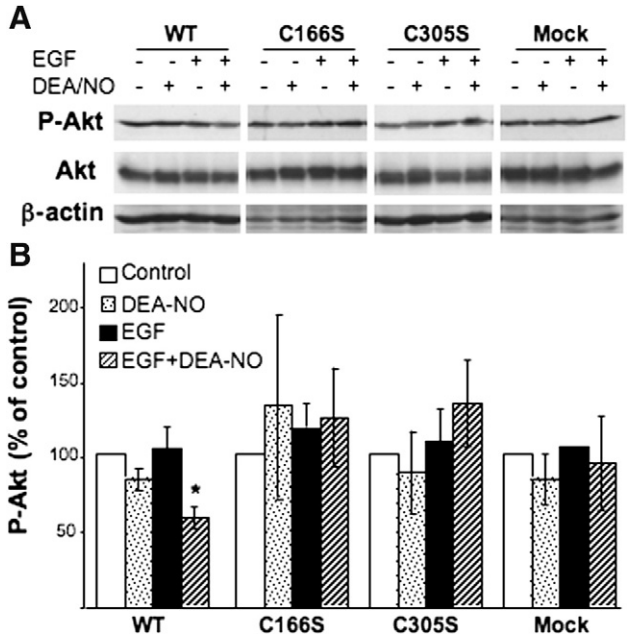

Fig. 6. Nitric oxide inhibits Akt activation in WT-EGFR-expressing cells but not in cells expressing the NO-resistant mutants C166S and C305S. (A) HEK293FT cells were transfected with plasmids coding for the WT EGFR or the C166S or C305S EGFR mutant or were mock transfected. Forty-eight hours later, cells were serum-starved for 5–6 h and pretreated with or without 5 mM DEA/NO for 30 min and with or without EGF (20 ng/ml) for the last 15 min. Then, samples were used for SDS–PAGE and Western blotting to detect phospho-Akt, total Akt, and a loading-control protein (as indicated). Typical examples from three or four independent experiments are shown. (B) The graph represents the densitometric quantification of phospho-Akt in all the conditions presented in (A), normalized for each experiment and cell transfection. Data are the means ± SEM of the values obtained in three or four independent experiments.

mutants, HEK293FT cells transfected with wild-type, C166S, or C305S EGFR, as well as mock-transfected cells, were incubated with DEA/NO for 30 min, EGF for 15 min, or both, and Akt phosphorylation was analyzed. In all cases, including mock-transfected cells, there was a strong basal phospho-Akt signal, which was not modified by addition of DEA/NO or EGF alone. However, DEA/NO in the presence of EGF produced a significant reduction of phospho-Akt in cells transfected with the WT EGFR, but not in those transfected with the two mutant receptors, or in mock-transfected cells (Figs. 6A and B).

Discussion

This study shows in a direct manner and for the first time that the EGFR can be S-nitrosylated in cysteine residues 166 and 305, a process that may explain the inhibition of the receptor tyrosine kinase activity in the presence of NO and, at least in part, the antiproliferative action of NO in different EGF-responding cell types.

Both exogenously added and endogenously produced NO inhibits cell proliferation and EGFR auto(trans)phosphorylation in different cell types

NO released from various NO donors produces a cGMP-independent inhibition of cell proliferation in a variety of cell types, as we have previously reported [9,11–13]. Interestingly, cells grown in defined medium supplemented with EGF are more sensitive to NO than those grown in the presence of serum [9,11], suggesting that NO modulates the activity of proteins involved in the signaling pathways initiated by activation of the EGFR. In the present work, we have emphasized that in both NB69 cells and NPC, as well as in permeabilized EGFR-T17 fibroblasts, NO reduced EGFR auto(*trans*)phosphorylation, in agreement with previously reported results [9,11–13,22], indicating a decrease in the receptor tyrosine kinase activation, which is the first and crucial step in transducing EGFR-mediated signals.

Increased EGFR phosphorylation upon treatment with NO donors, in the absence or presence of added EGF, have been reported recently in A431 and A459 cells [33,34], although no evidence indicates that such activations were due to a direct interaction between NO and the receptor. Indirect ways by which NO could activate EGFR include phosphatase PTP1B inactivation [33] and metalloprotease activation [35], which cause the shedding of endogenous EGFR ligands [36,37]. These mechanisms may prevail in particular cell types and experimental conditions under which EGFR dephosphorylation and/or transactivation play a significant role. Interestingly, several lung tumor cells overexpress EGF/TGFα [38,39], and, specifically, the lung tumor cell line A459 contains the machinery necessary for metalloprotease-mediated ectodomain shedding of EGFR ligands [40].

Although throughout this work high concentrations of DEA/NO (millimolar range) have been used to make sure that the mutant EGFR proteins C166S and C305S were resistant to NO, cells that naturally express the EGFR, such as NPC or NB69 cells, require lower concentrations of NO donors to significantly reduce EGFR phosphorylation and proliferation rate [11,22]. In any event, the physiological significance of the EGFR regulation by NO is supported by the consequences of NOS inhibition in NO-producing cells. The nNOS- and eNOS-expressing NPC increased EGFR auto(*trans*)phosphorylation, Akt phosphorylation, and EGF-dependent proliferation upon NOS inhibition [22], a condition that, as we show now, reduces EGFR S-nitrosylation. A confirmation of the capacity of endogenous NO to inhibit the EGFR tyrosine kinase was obtained using PAE/EGFR-GFP cells, which express eNOS. Interestingly, in these cells, inhibition of NOS activity produced tyrosine phosphorylation of the EGFR–GFP chimera in the absence of EGF, thus suggesting that the receptor could be partially active under basal conditions and that the tonic release of NO prevents this activation.

Both exogenously added and endogenously produced NO S-nitrosylates the EGFR

Most NO reactions are highly sensitive to reduced glutathione (GSH), a major determinant of the intracellular redox state, which can prevent, potentiate, or completely modify NO functions [41–43]. Experimental evidence indicates that NO effects mediated by guanylyl cyclase activation are enhanced in the presence of GSH [44], probably owing to NO protection from oxidative agents or to the formation of stable NO adducts (GSNO), which may thereafter release free NO through a Cu^{2+}-catalyzed mechanism [41]. On the other hand, GSH impairs NO reactions with protein sulfhydryl groups, thereby inhibiting functions derived from protein S-nitrosylation [14,45]. Here we show that inhibition of glutathione synthesis by BSO, a condition under which protein S-nitrosylation is enhanced sixfold upon treatment with NO donors [46], significantly increased the inhibitory effect of NO on the EGFR tyrosine kinase activity. This is consistent with previous work in which we demonstrate that addition of glutathione ethyl ester (a membrane-permeative form of GSH) to the cultures reverts NO-induced inhibition of the EGFR [13]. Altogether, these data suggested to us that the mechanism by which NO affects EGFR function implied S-nitrosylation reactions.

Direct evidence of the molecular interaction between NO and the EGFR was obtained by biotin labeling and subsequent detection of nitrosothiol groups in the receptor molecule, using NB69 cell proteins exposed to DEA/NO. The presence of nitrosothiol groups in the immunoprecipitated EGFR indicated that the receptor contains cysteine residues that can be S-nitrosylated and that the interaction between NO and EGFR is indeed due to direct S-nitrosylation.

Notwithstanding the large number of cysteine residues present in the EGFR, the level of S-nitrosylation observed in the receptor from NB69 cells was remarkably low, which suggested that only one or a low number of cysteine residues are able to react with NO. This is not surprising, as it is now well accepted that only particular nucleophiles within a protein are targeted by NO, whereas others are left unmodified [14,47–49]. Just to mention a few examples, S-nitrosylation of a single cysteine has been shown to be necessary and sufficient to modify the activity of the *N*-methyl-D-aspartate receptor-channel complex [50] or caspase 3 [51].

NO inhibition of EGFR auto(trans)phosphorylation requires C166 and C305 residues

The structural factors that govern the modification of specific cysteine residues in a protein by NO are now well understood. An acid–base-catalyzed SNO/SH exchange reaction, in which the targeted cysteine is surrounded by basic and acidic amino acid residues, has been demonstrated to be the mechanism facilitating this modification [26,52]. Based on these findings, the consensus motif (K, R, H, D, E)-**C**-(D, E), which occurs in either the primary or the tertiary structure of proteins, has been proven useful in identifying S-nitrosylation sites. The extracellular domain of the EGFR contains two cysteine residues surrounded by this motif in its primary sequence: C166 and C305. In the intracellular portion of the EGFR, close to the tyrosine kinase domain, C926 is surrounded as well by a three-dimensional S-nitrosylation motif. Site-directed mutagenesis of these three cysteine residues shows that the C166S and C305S substitutions avoided and reduced, respectively, the NO-mediated inhibition of the EGFR tyrosine kinase activity, whereas the C926S substitution, as well as the serine substitution of four additional cysteine residues located in the intracellular domain and lacking the above-mentioned consensus motif, did not prevent such inhibition. These results indicate that C166 and C305 are required for NO to fully exert its inhibitory action on the EGFR tyrosine kinase activity. Thus, our results strongly suggest that S-nitrosylation of C166 and C305 is the mechanism responsible for the NO-induced inhibition of the EGFR. Of interest, C166 and C305 S-nitrosylation consensus sequences are conserved in the human, mouse, and rat EGFR [53–55], which may underscore their potential physiological significance.

Results obtained using X-ray diffraction analysis of the EGFR extracellular region, as well as mass spectrometry of protease-digested EGFR, have indicated that C166 and C305 residues may form disulfide bonds with C175 and C309, respectively [56]. Energetically it would be extremely difficult for NO to S-nitrosylate a cysteine residue involved in the formation of a disulfide bridge. However, it has been recently established that there is a subclass of disulfide bonds in the extracellular domains of some cell surface receptors that can be cleaved in mature proteins, and when this happens it has significant consequences for protein function [reviewed in 57,58]. The first characterized example was a disulfide bond in the extracellular region of the CD4 receptor, which is reversibly cleaved on the cell surface by thioredoxin, a thiol–disulfide oxidoreductase secreted by $CD4^+$ T cells, and which plays a role in the conformational changes required for fusion of HIV with the cell membrane [57]. Therefore, we propose that the disulfide bridges at C166–C175 and C305–C309 may be reversible and may participate in a dithiolate–disulfide switch, involved in the regulation of EGFR. Only when in the dithiolate conformation would NO be able to S-nitrosylate the corresponding cysteine residue.

In the extracellular domain of the EGFR there is a flexible mobile loop flanked by two cysteine-rich regions [56]. Cysteine residues within these regions form disulfide bonds with other cysteines in the same region. The flexible loop participates in the ligand-induced formation of the EGFR.EGFR dimer and therefore in receptor activation [56,59]. The cysteine residues sensitive to NO, C166 and C305, are located at both sides of this loop. It would be possible for C166–C175 and C305–C309 disulfides to participate in stabilizing the structure of the flexible loop and for S-nitrosylation of these two residues to induce a conformational change in this region, disrupting its capability to form the EGFR dimer and its subsequent activation. According to this hypothesis our results show a lower activation of the EGFR(C166S) and EGFR(C305S) mutants by EGF in the absence of NO, suggesting that the inability of the mutants to form the reversible disulfide bridges may somewhat affect the receptor activity.

Downstream consequences of EGFR inhibition by NO

We have previously reported that in NPC, which express EGFR and are EGF-dependent for survival and proliferation, DEA/NO inhibition of EGFR auto(*trans*)phosphorylation is accompanied by a parallel inhibition of EGF-induced Akt phosphorylation [22]. We tried here to elucidate whether this downstream functional consequence of EGFR inhibition would persist in cells transfected with WT EGFR and be spared in cells transfected with NO-resistant EGFR mutants. A drawback in this experiment was the basal Akt phosphorylation found in the HEK293FT cells used for transfections and the lack of further activation of this intracellular pathway by addition of EGF. However, we consistently found that NO inhibited Akt phosphorylation in WT-EGFR-transfected cells in the presence of EGF, but not in its absence nor in mock-transfected cells. A possible explanation is that, in the absence of the pair EGF/EGFR, the PI3K/Akt pathway is activated by a mechanism independent of EGFR and, therefore, it is not affected by NO. However, in the presence of EGF, the pathway initiated by EGFR activation takes over in controlling PI3K activity in those cells expressing the wild-type receptor, which then become sensitive to the NO inhibitory action. The lack of inhibition of Akt phosphorylation by NO in cells transfected with C166S or C305S EGFR mutants and treated with EGF demonstrated the cause–effect relationship between EGFR and Akt phosphorylations and the functional significance of the control of the receptor activity by NO.

We propose that S-nitrosylation of the EGFR may regulate the signal transmission initiated by EGFR ligands in cells that either express some NOS isoforms or are located in the proximity of other NO-producing cells. These findings may be relevant for a possible pharmacological control of tumor cell growth in those oncogenic processes that are related to dysfunction in EGFR activity or to the expression of constitutively active mutated forms of EGFR, for example, if a vector-mediated NOS transfection could be specifically targeted to tumor cells. S-Nitrosylation of EGFR may also be the molecular mechanism that explains why, in the adult mouse brain subventricular zone, NO exerts a negative regulatory effect on neurogenesis [60], a process that is clearly dependent on EGFR activation [61].

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