Nitric oxide stimulates tyrosine phosphorylation in murine fibroblasts in the absence and presence of epidermal growth factor

Tereza M. S. PERANOVICH,* Aline M. DA SILVA,† Diana M. FRIES,* Arnold STERN‡ and Hugo P. MONTEIRO*§

*Fundação Hemocentro São Paulo, Brazil, †Departamento de Bioquímica, Instituto de Química da Universidade de São Paulo, São Paulo, Brazil, and ‡New York University Medical Center, Department of Pharmacology, New York, NY, U.S.A.

In the present study, utilizing anti-phosphotyrosine monoclonal antibodies, sodium nitroprusside (SNP) and *S*-nitroso-*N*-acetylpenicillamine (SNAP) as sources of NO and murine fibroblasts expressing the human epidermal growth factor (EGF) receptor (HER14 cells), we showed that tyrosine phosphorylation of a set of proteins (126, 56 and 43 kDa) was stimulated when cells were incubated with either SNP or SNAP and abolished by Methylene Blue and oxyhaemoglobin. Inhibition by Methylene Blue suggested an involvement of cyclic GMP in the process, which was evidenced by the effects of 8-bromo cyclic GMP. This analogue of cyclic GMP stimulated tyrosine phosphorylation of the same set of proteins phosphorylated after incubation with the NO source. Tyrosine phosphorylation of the same set of proteins was stimulated when cells were incubated simultaneously with SNP and EGF, showing that NO also potentiates EGF-evoked tyrosine kinase activity in HER14 cells. However, stimulation of the autophosphorylation of the EGF receptor, above the levels obtained for EGF alone, was not observed under those conditions. Additionally, we investigated the effects of NO on EGF-receptor tyrosine phosphatase activities in HER14 cells. Increasing concentrations of NO correlate with a gradual inhibition of these activities in HER14 cells, either in intact cells or in cell lysates. Taken together, these observations suggest that NO modulates tyrosine phosphorylation in HER14 cells.

INTRODUCTION

The mitogenic response to epidermal growth factor (EGF) is mediated by a 170 kDa transmembrane glycoprotein, the EGF receptor. The EGF receptor is a protein tyrosine kinase (PTK) receptor that catalyses the phosphorylation of exogenous substrates as well as tyrosine residues within its own polypeptide chains. Tyrosine phosphorylation associated with the control of both mitogenic signal pathways and oncogenic transformation [1,2] is a reversible process controlled by the opposing actions of PTK and protein tyrosine phosphatases (PTPs). Together, PTK and PTP activities control the amount of phosphotyrosine in the cell [3,4]. Sequence analysis of PTP isoforms from several sources revealed the presence of a conserved catalytic domain and an essential cysteine residue in this domain [4].

Previously, it was shown that oxidative-stress-inducing systems, such as the thiol-group oxidant diamide [5], the vanadate/H_2O_2 system [6] and autoxidizing ascorbic acid in the presence of sub-micromolar concentrations of iron [7], were able to inhibit PTP activities in a number of cell lines. PTK activities are also subject to redox regulation. Chan et al. [8] have shown that oxygen-derived free radicals generated through redox cycling of naphthoquinones were stimulatory of tyrosine phosphorylation in rat liver plasma membranes. The redox regulation of a transmembrane PTK homologous to the cytoplasmic portion of the insulin receptor and localized in the endoplasmic reticulum of B lymphocytes, the Ltk receptor, has been reported [9]. The thiol-group-oxidizing and -alkylating agent *N*-ethylmaleimide was shown to be a modulator of PTK activity in T lymphocytes [10]. Thus PTP activities and PTK activities could be redox modulated.

NO, a reactive free-radical gas, was found to be generated enzymically from L-arginine and molecular oxygen by NO synthases in activated macrophages, endothelium, adrenal glands, neutrophils, platelets, hepatocytes, Kupffer cells and neuronal cells [11]. NO plays several physiological roles in mammalian systems: (a) mediator of endothelial-derived relaxation of smooth-muscle cells in the artery; (b) synaptic neuronal messenger; (c) cytotoxic agent released by macrophages; and (d) signalling molecule that acts by binding the haem iron at the active site of soluble guanylate cyclase, stimulating the enzyme to generate cyclic GMP [12]. Furthermore, under appropriate conditions, NO reacts with thiol groups to form *S*-nitroso compounds in a reversible manner [13]. These observations led us to investigate whether, like the previously described oxidative systems, NO could also modulate PTP and PTK activities. Using sodium nitroprusside (SNP) and *S*-nitroso-*N*-acetylpenicillamine (SNAP) as NO donors, we have analysed its effects on tyrosine-phosphorylation levels in NIH 3T3 cells expressing the human EGF receptor, HER14 cells [14], in the absence and presence of EGF. The effects of NO on PTP activities evaluated as the EGF-receptor tyrosine phosphatase activity, from whole extracts of these cells, were also investigated.

MATERIALS AND METHODS

Reagents

EGF from mouse submaxillary glands was obtained from Toyobo Co. (New York, NY, U.S.A.). SNP, 8-bromo cyclic GMP (8-Br-cGMP), Protein A–Sepharose CL4B and other

Abbreviations used: EGF, epidermal growth factor; PTK, protein tyrosine kinase; PTP, protein tyrosine phosphatase; SNP, sodium nitroprusside; SNAP, *S*-nitroso-*N*-acetylpenicillamine; MB, Methylene Blue; Hb, oxyhaemoglobin; 8-Br-cGMP, 8-bromo cyclic GMP; anti-P-Tyr, anti-phosphotyrosine; DMEM, Dulbecco's modified Eagle's medium.

§ To whom correspondence should be addressed, at: Fundação Hemocentro São Paulo, Av. Dr. Enéas de Carvalho Aguiar 255, PAMB CEP 05403-900, São Paulo, SP, Brazil.

routine reagents were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Methylene Blue (MB) was from Merck (Darmstadt, Germany). SNAP was kindly provided by Dr. Lakshmi Devi (New York University Medical Center, NY, U.S.A.). Oxyhaemoglobin (Hb) was obtained from haemolysates of freshly drawn human blood donated by healthy volunteers. Briefly, 20 ml of blood was centrifuged to obtain the erythrocytes. Erythrocytes (10 ml) were washed twice with phosphate-buffered saline and lysed with an equal volume of ultrapure deionized cold water. Haemolysates were centrifuged, and the supernatant was collected and eluted through a Sephadex G-50 column equilibrated with 20 mM Hepes buffer, pH 7.5. The Hb concentration of the eluate was determined by reading the A_{577} (ϵ_{577} = 15300 $M^{-1} \cdot cm^{-1}$ [15]). Cell culture reagents and tissue-culture flasks were from Gibco/BRL (Gaithersburg, MD, U.S.A.). Electrophoresis reagents were from Pharmacia Biotechnology (Uppsala, Sweden). Autoradiographic films X-OMAT AR and X-RP were from Eastman Kodak (Rochester, NY, U.S.A.). [γ-^{32}P]ATP (250 μCi/ml), ^{125}I-labelled Protein A (100 μCi/ml) and ^{125}I-labelled EGF (20 μCi/ml) were from Amersham International (Amersham, Bucks., U.K.). A monoclonal antibody specific for the human EGF receptor (mAB 108.1) was kindly provided by Dr. Yuri Ivaschenko of Rorer Rhone–Poulenc Central Research (King of Prussia, PA, U.S.A.). Affinity-purified anti-phosphotyrosine (anti-P-Tyr) antibodies were generously given by Dr. Ben Margolis (New York University Medical Center).

Cell cultures

HER14 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal-bovine serum in 75 cm^2 plastic culture flasks and maintained in a humidified atmosphere of air/CO_2 (19:1) at 37 °C.

Treatment of intact cells

Cells grown in DMEM supplemented with 10% fetal-bovine serum had their medium changed and were then starved for approx. 12 h or for 48 h in DMEM supplemented with 0.5% fetal-bovine serum before they were incubated with or without agents in DMEM supplemented with 20 mM Hepes, pH 7.5, and 1 mg/ml BSA for 30 min at 25 °C.

Immunoprecipitation of the EGF receptor

Cells were solubilized in lysis buffer A (20 mM Hepes, pH 7.5, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM $MgCl_2$, 1 mM EGTA, 1 μg/ml aprotinin, 1 μg/ml leupeptin and 1 mM phenylmethanesulphonyl fluoride) and immunoprecipitated with mAB 108.1 against the human EGF receptor, conjugated with Protein A–Sepharose CL4B [14].

Phosphorylation of the EGF receptor *in vitro*

After washing the immunoprecipitated receptors with kinase buffer (20 mM Hepes, pH 7.5, 5 mM $MnCl_2$ and 2 mM $MgCl_2$), 8 μCi of [γ-^{32}P]ATP was added to immunoprecipitates, and the mixture was incubated for 5 min at 25 °C. The reaction was stopped by addition of HNTG buffer (20 mM Hepes, pH 7.5, 150 mM NaCl, 10% glycerol, 0.1% Triton X-100), followed by three washes in this buffer. The labelled immunoprecipitate was resuspended in the same buffer and kept at 4 °C for further use.

Immunoblotting

For immunoblotting experiments cells were lysed with lysis buffer A containing tyrosine as well as serine/threonine phosphatase inhibitors (2 mM sodium orthovanadate, 50 mM NaF and 10 mM sodium pyrophosphate). The protein content of lysates was determined by the Bradford method [16], and 75 μg of protein was boiled with 3-fold-concentrated SDS/PAGE sample buffer and resolved by SDS/PAGE (7.5% or 10% gels) [17]. The gels were transferred to 0.45 μm-pore nitrocellulose sheets by using the Pharmacia Novablot system. The transfer was carried out for 2 h at 200 mA in 48 mM Tris base/39 mM glycine/0.037% SDS/20% methanol. The nitrocellulose blots were saturated with TBS (10 mM Tris, pH 7.5, 150 mM NaCl) and 5% BSA for 2 h at room temperature and incubated overnight with anti-P-Tyr antibodies at room temperature. After four washes with TBS and TBS/0.05% Triton X-100, the blots were incubated for 1 h with ^{125}I-labelled Protein A (approx. 300000 c.p.m./ml) in TBS containing 5% BSA. The blots were subsequently washed with the TBS solutions described above. After drying, the nitrocellulose sheets were exposed to autoradiographic films for 2–7 days at −80 °C. Band intensities were quantified by densitometric analysis of linear-range autoradiograms by using a LKB Ultroscan enhanced laser densitometer.

Assay of PTP activity

Cells were either untreated or treated with agents as described above and lysed with lysis buffer A. After incubation for 30 min on ice, lysates were centrifuged at 4 °C for 10 min at 12000 ***g***. The supernatants were collected and served as the source of PTP activities. Protein concentration was determined, and the volume of cell lysate corresponding to 200 μg of protein was mixed with 25 μl of a suspension containing [^{32}P]phosphorylated immunoprecipitated EGF receptors, prepared as described above. Reactions were carried out for 30 min at 37 °C with agitation, and terminated by centrifugation (12000 ***g*** for 1 min) of the immunoprecipitates. After centrifugation, the supernatant was immediately aspirated and 50 μl of 2-fold-concentrated SDS/PAGE sample buffer was added to the immunoprecipitates. Samples were boiled for 5 min and resolved by SDS/PAGE (7.5% gels). After drying, gels were exposed to autoradiographic films for 4–12 h at room temperature. The intensity of the band corresponding to the EGF receptor was determined by enhanced laser densitometry as above.

Binding studies

^{125}I-EGF-binding studies in intact HER14 cells were performed essentially as described by Lax et al. [18].

Assay for NO

NO generation by autoxidation of SNP and SNAP was quantified by measuring the accumulation of nitrite in the reaction medium by using the Greiss reagent [19].

RESULTS

Production of NO by autoxidation of SNP and SNAP

SNP and SNAP, which are two NO donors, autoxidize and generate NO [20,21]. Autoxidation of both compounds was studied in 20 mM Hepes, pH 7.5, supplemented with 1 mg/ml BSA. DMEM was not added to the reaction medium because it contains Phenol Red, which interferes with the spectrophoto-

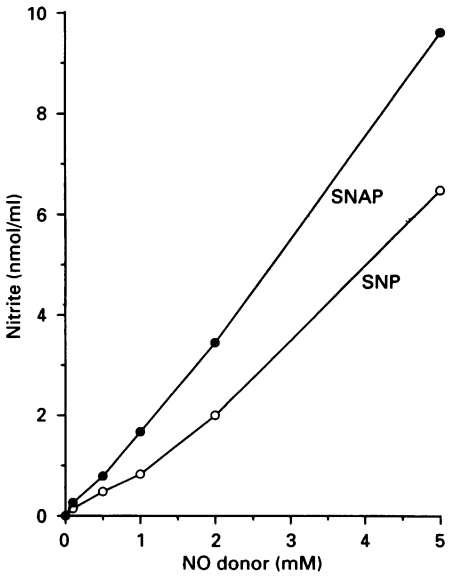

Figure 1 NO generation by autoxidation of SNP (○) and SNAP (●)

Increasing concentrations of both NO donors (0.1–5 mM) were incubated for 30 min at 25 °C in 20 mM Hepes, pH 7.5, supplemented with 1 mg/ml BSA. After this period, the nitrite content of the reaction medium was assayed as described in the Materials and methods section.

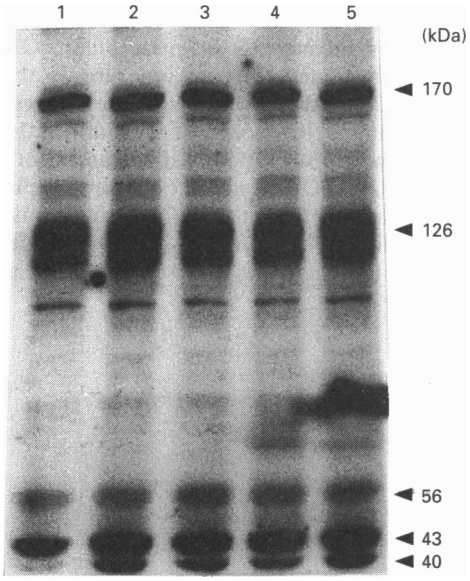

Figure 2 Effects of SNP on tyrosine phosphorylation in HER14 cells starved for 12 h

HER14 cells were starved for 12 h, and after this period were incubated in the presence of increasing concentrations (0–5 mM) of SNP. After 30 min incubation, cells were lysed. The same amount of protein from lysates (75 μg) was resolved by SDS/PAGE (7.5% gel) and transferred to nitrocellulose membranes. Immunoblotting was performed with anti-P-Tyr antibodies as described in the text. The autoradiograph of one out of three independent experiments is shown. Lane 1, control; lane 2, 0.1 mM SNP; lane 3, 0.5 mM SNP; lane 4, 1 mM SNP; lane 5, 5 mM SNP.

metric determination. NO production, determined as accumulation of nitrite in the reaction medium, was linearly dependent on the concentration of the NO donors (Figure 1).

Table 1 Effects of SNP on tyrosine-phosphorylation levels in HER14 cells

HER14 cells were starved for 12 or 48 h and treated with increasing concentrations of SNP, as described in the legend of Figure 2. Tyrosine-phosphorylation levels in each protein band were determined by laser-densitometry analysis of the autoradiographed immunoblots. The data represent the means ± S.E.M. of three independent experiments. Results are expressed as percentage of the area for each protein band from untreated cell lysates (% of control). Values are significantly different from control ($P < 0.05$), unless indicated by * (n.d. = not determined).

	Tyrosine phosphorylation (% of control)					
	Starvation 12 h			Starvation 48 h		
SNP (mM) Protein...	126 kDa	56 kDa	43 kDa	126 kDa	56 kDa	43 kDa
0.1	156 ± 15	192 ± 21	218 ± 20	295 ± 24	186 ± 2	260 ± 14
0.5	100 ± 7*	207 ± 15	196 ± 25	235 ± 31	199 ± 10	206 ± 16
1.0	95 ± 11*	184 ± 25	146 ± 10	222 ± 16	185 ± 20	249 ± 26
5.0	100 ± 8*	181 ± 10	146 ± 11	n.d	n.d	n.d

Effects of SNP and SNAP on levels of phosphotyrosine-containing proteins in HER14 cells

HER14 cells were allowed to grow in DMEM supplemented with 10% fetal-bovine serum for 24 h and then subjected to starvation in DMEM supplemented with 0.5% fetal-bovine serum for approx. 12 h. Cells were subsequently treated with increasing concentrations of SNP (0.1–5 mM) and were analysed for their content on tyrosine-phosphorylated proteins by immunoblotting with anti-P-Tyr antibodies. The specificity of the antibody used in these experiments was demonstrated by competitive displacement from the blots by 1 mM phosphotyrosine, but not by 1 mM phosphoserine, 1 mM phosphothreonine or 1 mM nitrotyrosine (results not shown). Incubation of cells with 0.1–5 mM SNP stimulated tyrosine phosphorylation of 126, 56 and 43 kDa proteins to different extents (Figure 2). At 0.1 mM, SNP stimulated tyrosine phosphorylation of the 126 kDa protein above basal levels, by approx. 1.4-fold; at higher concentrations stimulation was not observed (Table 1). The 56 kDa protein had its tyrosine-phosphorylation levels enhanced to approx. 2.0-fold above basal levels by 0.1–5 mM SNP (Table 1). Stimulation to approx. 1.5–2.0-fold above basal levels of tyrosine phosphorylation was also observed for the 43 kDa protein on treatment with the four different SNP concentrations. SNP-dependent stimulation of tyrosine phosphorylation was also investigated in cells starved for a longer period (48 h). As shown on Table 1, this condition caused a greater stimulation of tyrosine phosphorylation. After incubation with 0.1, 0.5 and 1 mM SNP, the tyrosine phosphorylation of the three proteins was enhanced, on average, 2.0–3.0-fold above basal levels. A protein of 40 kDa also appears to have its tyrosine-phosphorylation level enhanced after treatment with SNP. However, the changes were not as reproducible as those observed for the 126, 56 and 43 kDa bands. In either starvation condition (12 or 48 h), treatment with SNP concentrations lower than 0.1 mM had no detectable effect on the tyrosine-phosphorylation levels of the bands analysed. In order to exclude a direct effect of the nitroprusside anion on tyrosine phosphorylation, we performed the experiments with SNAP (0.1–1 mM), a NO-releasing substance which does not contain cyanide or ferricyanide groups in its structure. SNAP also promoted tyrosine phosphorylation of the same group of proteins phosphorylated after incubation with SNP (Figure 3). Stimulation of tyrosine-phosphorylation levels of the three proteins ranged from 2.0- to 4.0-fold above basal levels (Figure 3).

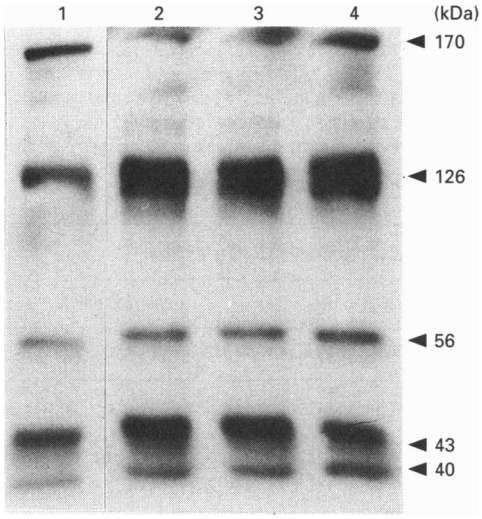

Figure 3 Effects of SNAP on tyrosine phosphorylation in HER14 cells starved for 48 h

HER14 cells were starved for 48 h and then incubated with increasing concentrations (0–1 mM) of SNAP. After 30 min incubation, cells were lysed. The same amount of protein from lysates (75 μg) was resolved by SDS/PAGE (7.5% gel) and transferred to nitrocellulose membranes. Immunoblotting was performed with anti-P-Tyr antibodies as described in the text. The autoradiograph of one out of two independent experiments is shown. Lane 1, control; lane 2, 0.1 mM SNAP; lane 3, 0.5 mM SNAP and lane 4, 1 mM SNAP. Quantification of the SNAP-dependent increase in tyrosine-phosphorylation levels in HER14 cells was performed by laser-densitometry analysis of the autoradiographs. Data represent the means ± S.E.M. of two independent experiments and are significantly different from control (untreated) cells ($P < 0.05$).

SNAP (mM)	Protein...	Tyrosine phosphorylation (% of control) 126 kDa	56 kDa	43 kDa
0.1		176 ± 3	136 ± 42	397 ± 20
0.5		170 ± 14	180 ± 56	388 ± 50
1.0		194 ± 19	154 ± 7	433 ± 23

With either 12 h or 48 h of starvation, or with SNP or SNAP, we did not observe an increase in tyrosine phosphorylation of the 170 kDa protein, which corresponds to the EGF receptor.

Effects of MB and Hb on tyrosine phosphorylation induced by SNP

To verify that the SNP effect was due to NO generated during its autoxidation, we studied the effects of Hb and MB on SNP-stimulated tyrosine phosphorylation in HER14 cells starved during 12 h. Cells were first incubated in the absence or presence of 0.1 mM MB for 1 h. Medium was then replaced, and cells were incubated for 30 min with increasing concentrations of SNP (0–1 mM). Preincubation of cells with MB for 1 h before addition of SNP abolished the SNP-induced tyrosine phosphorylation of the 126, 56 and 43 kDa proteins (Figure 4, lanes 6–8). In the absence of SNP, MB slightly decreased the basal levels of tyrosine phosphorylation of the 43 kDa protein, but had no significant effects on the other proteins (Figure 4, lane 2). Cells were preincubated for 30 min with 20 μM Hb, before they were stimulated with different concentrations of SNP (0, 0.5 and 1 mM). Similarly to that seen with MB, SNP-stimulated tyrosine phosphorylation was abolished by preincubation of cells with Hb

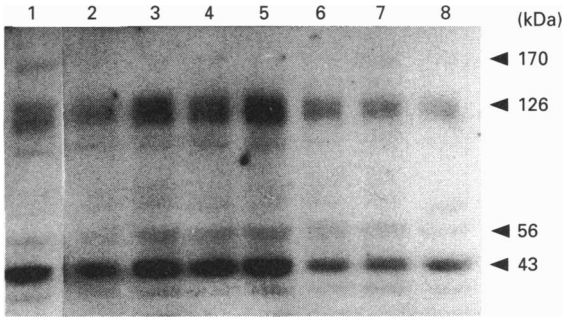

Figure 4 Effects of preincubation with MB on SNP-stimulated tyrosine phosphorylation in HER14 cells

Her14 cells were incubated for 1 h with or without 0.1 mM MB. Then, medium was replaced and cells were incubated with increasing concentrations (0–1 mM) of SNP (lanes 1, 3–5, without MB; lanes 2, 6–8, with MB). Cells were lysed, and the same amount of protein (75 μg) was separated on SDS/PAGE (10% gel). Immunoblotting was performed with anti-P-Tyr antibodies. Lane 1, control; lane 2 control plus MB; lane 3, 0.1 mM SNP; lane 4, 0.5 mM SNP; lane 5, 1 mM SNP; lane 6, as in lane 3, plus MB; lane 7, as in lane 4, plus MB; lane 8 as in lane 5, plus MB. This experiment was reproduced once.

(Figure 5, lanes 5 and 6). In the absence of SNP, Hb had no effect on the basal levels of tyrosine phosphorylation (Figure 5, lane 2).

Effect of 8-Br-cGMP on tyrosine phosphorylation in HER14 cells

NO can activate the soluble form of the enzyme guanylate cyclase, resulting in an increase in intracellular cGMP levels [22,23]. On the other hand, MB inhibits directly the enzyme guanylate cyclase [24], and Hb, which is an effective scavenger of NO [25], can also inhibit NO-dependent activation of the enzyme. Taken together, these observations led to the hypothesis that cGMP could mediate NO-dependent tyrosine phosphorylation in HER14 cells. Experimental support for this idea was obtained in experiments utilizing 8-Br-cGMP, a stable analogue of cyclic GMP that preferentially activates cyclic-GMP-dependent protein

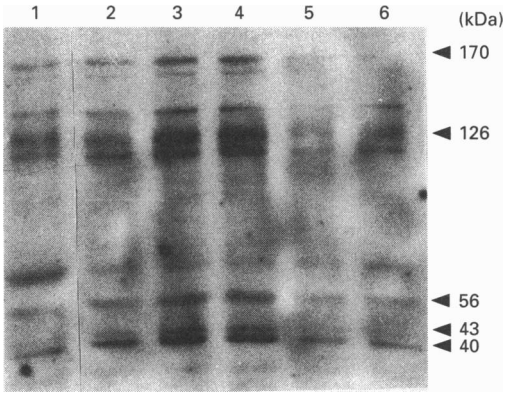

Figure 5 Effects of preincubation with Hb on SNP-stimulated tyrosine phosphorylation in HER14 cells

Cells were preincubated with and without 20 μM Hb for 30 min. After this period, cells were treated or not with SNP (0.5 and 1 mM) for a further 30 min. Subsequently cells were lysed, and lysates (75 μg of protein) were resolved on SDS/PAGE (7.5% gel). Immunoblotting was performed with anti-P-Tyr antibodies. Lane 1, control; lane 2, control plus Hb; lane 3, 0.5 mM SNP; lane 4, 1 mM SNP; lane 5, as in lane 3, plus Hb; lane 6, as in lane 4, plus Hb. This experiment was reproduced twice.

Table 2 Effects of 8-Br-cGMP on tyrosine phosphorylation in HER14 cells

HER14 cells were starved for 48 h and then incubated with the indicated concentrations of 8-Br-cGMP for 30 min. After incubation, cells were lysed and the same amount of protein (75 μg) was resolved on SDS/PAGE (7.5% gel). After transfer to nitrocellulose sheets, immunoblotting was performed with anti-P-Tyr antibodies. Increased tyrosine-phosphorylation levels in each band were determined by laser-densitometry analysis of the autoradiographs. The data represent the means ± S.E.M. of two independent experiments and are significantly different from control (untreated) cells ($P < 0.05$).

8-Br-cGMP (mM)	Tyrosine phosphorylation (% of control)		
Protein...	126 kDa	56 kDa	43 kDa
0.1	207 ± 5	195 ± 18	155 ± 5
1.0	188 ± 12	218 ± 20	158 ± 4

Table 3 Effects of SNP on EGF-induced tyrosine phosphorylation in HER14 cells

After 48 h starvation in DMEM supplemented with 0.5% fetal-bovine serum, cells were stimulated with EGF (83 nM) for 30 min and incubated simultaneously in the absence or presence of increasing concentrations of SNP (0–5.0 mM). At the end of the incubation, cells were lysed and 75 μg of total protein from lysates was resolved by SDS/PAGE (7.5% gel), transferred to nitrocellulose sheets and incubated with anti-P-Tyr antibodies as described in the text. Increased tyrosine-phosphorylation levels in each band were determined by laser-densitometry analysis of the autoradiographs. Data represent the means ± S.E.M. of two independent experiments and are significantly different from control (EGF-stimulated) cells ($P < 0.05$) unless indicated by *.

	Tyrosine phosphorylation (% of control)			
Protein...	170 kDa	126 kDa	56 kDa	43 kDa
EGF	100	100	100	100
EGF + SNP (0.1 mM)	95 ± 7*	130 ± 16	132 ± 17	136 ± 17
EGF + SNP (0.5 mM)	105 ± 3*	168 ± 7	163 ± 11	158 ± 20
EGF + SNP (1.0 mM)	98 ± 11*	153 ± 31	136 ± 10	133 ± 10
EGF + SNP (5.0 mM)	99 ± 13*	156 ± 5	125 ± 1	124 ± 1

kinases [26]. At concentrations of 0.1 and 1 mM this compound stimulated tyrosine phosphorylation of the same group of proteins that were phosphorylated after incubation with the NO donors (Table 2): stimulation to approx. 2.0-fold above basal levels was obtained for the 126 and 56 kDa proteins, but a more modest stimulation (~ 1.5-fold) was found for the 43 kDa protein.

Effects of SNP on EGF-stimulated tyrosine phosphorylation in HER14 cells

Analysis of phosphotyrosine-containing proteins in whole cell lysates of HER14 cells showed a faint band corresponding to a 170 kDa protein, the EGF receptor, in cells which were starved for 48 h and received no treatment (results not shown). Here we selected the longest starvation period, in order to obtain the lowest basal levels of tyrosine phosphorylation. Addition of 83 nM EGF and incubation of cells with EGF for 30 min, as expected, stimulated phosphorylation of the band corresponding to EGF-receptor tyrosine kinase (170 kDa), as well as of other proteins of 126, 56 and 43 kDa (Table 3). Co-incubation of the growth factor with 0.1 mM, 0.5 mM, 1 mM and 5 mM SNP for 30 min enhanced the EGF-stimulated tyrosine phosphorylation of the 126, 56 and 43 kDa proteins above the levels detected with EGF alone (Table 3). By contrast, no enhancement was observed for the 170 kDa protein corresponding to the EGF receptor. SNP-dependent stimulation of tyrosine phosphorylation above the levels evoked by EGF ranged from 1.3- to 1.6-fold in all combinations (Table 3).

Table 4 Inhibition of PTPase activity by SNP-derived NO

HER14 cells were starved for 48 h in DMEM containing 0.5% fetal-bovine serum, before incubation with the indicated concentrations of SNP for 30 min. Reaction was terminated by aspirating the cell media, and cells were lysed with 1 ml of ice-cold lysis buffer A. After 30 min on ice, lysates were centrifuged and supernatants had their protein concentration determined. Protein contents were matched (200 μg) and PTP activity in lysates was measured as described in the Materials and methods section. The corresponding area for the EGF-receptor band was determined by laser-densitometry analysis. Results are expressed as a percentage of this area and represent means ± S.E.M. of three independent experiments.

Treatment	PTP activity (% of control)
Control	100
+ SNP (0.1 mM)	71 ± 17
+ SNP (0.5 mM)	50 ± 10
+ SNP (1.0 mM)	16 ± 7
+ SNP (5.0 mM)	0

Effects of NO on binding of EGF to its receptor

The EGF receptor has a characteristic structural feature of two cysteine-rich repeat sequences in the extracellular domain at the ligand-binding site [1]. *N*-Ethylmaleimide, a thiol-group oxidant and alkylating agent, was shown to be able to alter the binding capacity of EGF to its receptor [27]. Moreover, NO also reacts with thiols to generate *S*-nitrosothiols [13]. Thus, to determine whether SNP-derived NO was potentiating EGF-dependent tyrosine phosphorylation by affecting binding of the growth factor to its receptor, we performed experiments on binding of ^{125}I-labelled EGF to its receptor. Binding was affected only marginally (13% decrease) by incubation with 5 mM SNP, the maximal concentration used in this study. At lower concentrations of SNP, binding was not affected (results not shown).

Effects of NO on PTP activities in HER14 cells

Experiments to verify whether NO, like other cell-permeant oxidants [5–7], could inhibit PTP activities in HER14 cells were performed. Cells were incubated with increasing concentrations of SNP and had their PTP activities assayed as the EGF-receptor tyrosine phosphatase activity. Gradual inhibition was observed when cells were treated with increasing concentrations of SNP (Table 4). Similarly to the effects on intact cells, incubation of HER14-cell lysates with increasing concentrations of SNP led to increasing inhibition of PTP activities on lysates (results not shown).

DISCUSSION

Recent studies have established that NO is an important mediator of intercellular signalling [11,12,22,23]. Acting in a different manner from regular growth factors, this free radical reacts with its targets covalently, depending on their redox potential. Some cellular targets of NO have already been described, among them the soluble form of guanylate cyclase [23]. NO-dependent nitrosation of the guanylate cyclase haem group led to elevated levels of cyclic GMP [22]. Furthermore, protein phosphorylation has been suggested as one possible mechanism through which activation of soluble guanylate cyclase by NO could mediate cell signalling. Indeed, a recent study by Tsou and co-workers [28]

demonstrated that SNP-derived NO increased the threonine-phosphorylation levels of the dopamine- and cyclic AMP-regulated 32 kDa phosphoprotein from brain tissue. The present paper showed for the first time that NO derived from two NO-releasing compounds, SNP and SNAP, stimulated tyrosine phosphorylation levels on a series of endogenous proteins in murine fibroblasts. Essentially, three proteins, 126, 56 and 43 kDa had their levels of phosphotyrosine increased by the action of NO. However, stimulation patterns were quite different, depending on the NO donor used. SNAP strongly stimulated tyrosine phosphorylation of the 43 kDa protein ($\sim$ 4.0-fold), but stimulated phosphorylation of the 126 and 56 kDa proteins to a lesser extent. On the other hand, under the same experimental context, SNP was less efficient as a stimulant of tyrosine phosphorylation. Two basic features could account for the observed differences: (a) the amount of NO released by the NO donor in the reaction medium; (b) the nature of the redox-related forms of NO generated after autoxidation of each compound. Under our experimental conditions, SNAP was a better releaser of NO than was SNP, as showed by the nitrite accumulation in the reaction medium. However, in any case, stimulation of tyrosine phosphorylation was not directly proportional to the concentration of the NO donor used in the experiment. Considering the redox forms of NO released by each compound, SNP donates primarily the nitrosonium cation, NO^+ [18], whereas SNAP delivers NO as a *S*-nitrosothiol, RS-NO [21]. Under neutral physiological conditions the NO^+ species is very reactive, and will participate in addition and substitution reactions with nucleophiles, producing nitrosamines, alkyl and aryl nitrites, nitrogen oxides and *S*-nitrosothiols [18]. On the other hand, *S*-nitrosothiols are a more stable redox form of NO which can participate in *S*-nitrosylation and thiol/disulphide exchange reactions [18], and are the predominant redox form of NO in human plasma [29]. Thus SNAP, which is a *S*-nitrosothiol, could deliver this stable redox form of NO into cells more efficiently than SNP.

Stimulation by NO was obtained in cells which were in either a quiescent or a non-quiescent stage. Others [5,30] have shown that oxidants such as vanadate, H_2O_2 and sodium selenate were capable of stimulating tyrosine phosphorylation in cell lines which respond to insulin or EGF. Nevertheless, differently from NO, this stimulation was only achieved in cells which were in a non-quiescent stage.

SNP-evoked tyrosine phosphorylation was abolished by pre-treatment of the cells either with Hb, a haem protein which is an efficient NO scavenger [25], or with MB, an inhibitor of soluble guanylate cyclase [24]. Abolition by Hb indicates that the SNP effect is due to NO. Abolition by MB would suggest that the observed NO-stimulated tyrosine phosphorylation could be related to guanylate cyclase activation. This hypothesis is supported by the experiments with the cGMP analogue 8-Br-cGMP, showing that the activation of cGMP-dependent protein kinases alone is sufficient to produce a pattern of tyrosine phosphorylation similar to that observed when cells were incubated with the NO donors.

We also observed that NO can stimulate EGF-induced protein tyrosine phosphorylation in HER14 cells. Co-incubation of the NO source together with the growth factor led to an enhancement of phosphotyrosine cellular content above the levels observed for incubation with the growth factor alone. NO neither stimulated nor inhibited EGF-dependent receptor autophosphorylation, but enhanced EGF-dependent protein tyrosine phosphorylation towards endogenous substrates. These observations suggest that NO could regulate distal components of the EGF-initiated signal-transduction pathway. The mechanism of action of NO in this situation is not understood, but may be related to its reaction with essential thiol groups in regulatory proteins. Reactions of NO with thiol groups have been described in the literature [13,18]. However, the absence of significant effects on EGF binding, which is dependent on cysteine-rich domains, suggests that NO would target preferentially intracellular critical thiol groups. PTP activities, by promoting tyrosine dephosphorylation, are regulatory of growth-factor PTK activity [3,4]. Furthermore, PTPs have a conserved cysteine residue in their catalytic domains, which is essential for their activities [3,4]. As mentioned above, the NO^+ species is released during SNP autoxidation and can be promptly reduced by thiols, generating *S*-nitrosothiols [13,18]. Thus NO produced from SNP autoxidation possibly inhibits PTP activities through *S*-nitrosylation reactions with their critical cysteine residue. However, by contrast with the NO-evoked inhibition of PTP activities, the stimulation of tyrosine phosphorylation was not linearly dependent on the concentration of SNP. These observations imply that NO could exert either inhibitory effects towards PTP activities or its stimulatory effects on tyrosine phosphorylation, independently.

The effect of NO on tyrosine phosphorylation and PTP activities reported here may have some implications on cell proliferation. NO has been reported to inhibit proliferation of keratinocytes and smooth-muscle cells, and to be involved in neuronal cell death [31–33]. Here we report that NO is able to induce tyrosine phosphorylation in a number of endogenous proteins in murine fibroblasts, but not at the EGF-receptor level. These results suggest that NO could exert its anti-proliferative effects by subverting the regular growth-factor-associated signal-transduction pathway. EGF otherwise counteracts the NO-derived growth-inhibitory effects [31,33]. Accordingly, we show that NO in the presence of EGF had no effects on the growth-factor-mediated tyrosine autophosphorylation levels of its receptor, and stimulated tyrosine phosphorylation of other proteins. Thus, in the vasculature, the simultaneous presence of NO synthesized by endothelial cells and of EGF may have consequences on the modulation of smooth-muscle-cell proliferation.

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