

Activation of Epidermal Growth Factor Receptor and Its Downstream Signaling Pathway by Nitric Oxide in Response to Ionizing Radiation

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Abstract

Epidermal growth factor receptor (EGFR) is activated by ionizing radiation (IR), but the molecular mechanism for this effect is unknown. We have found that intracellular generation of nitric oxide (NO) by NO synthase (NOS) is required for the rapid activation of EGFR phosphorylation by IR. Treatment of A549 lung cancer cells with IR increased NOS activity within minutes, accompanied by an increase of NO. 2-Phenyl-4,4,5,5-tetramethylimidazole-1-oxyl-3-oxide, an NO scavenger, and N^G-monomethyl-L-arginine, an NOS inhibitor, abolished the increase in intracellular NO and activation of EGFR by IR. In addition, an NO donor alone induced EGFR phosphorylation. Transient transfection with small interfering RNA for endothelial NOS reduced IR-induced NO production and suppressed IR-induced EGFR activation. Overexpression of endothelial NOS increased IR-induced NO generation and EGFR activation. These results indicate a novel molecular mechanism for EGFR activation by IR-induced NO production via NOS. (Mol Cancer Res 2008;6(6):996–1002)

Introduction

Nitric oxide (NO) acts as an intercellular and intracellular messenger in a vast array of physiologic phenomena. NO is produced during the conversion of L-arginine to citrulline by NO synthase (NOS; ref. 1). Many contradictory reports have

shown both beneficial and harmful effects of endogenous NO: promoting or inhibiting apoptosis (2), killing tumors or increasing the potential for metastasis or vascularization (3), increasing or protecting against damage after stroke (4), and triggering ischemic preconditioning in the heart and mediating the late effects of preconditioning (5). Direct interactions between NO and target proteins, such as guanylate cyclase, account for its physiologic properties; however, its secondary reactions with reactive oxygen species, which generate reactive nitrogen species, are likely to account for indirect pathologic effects (3). Interestingly, both increasing and decreasing NO have been used to treat cancer (6).

Increased expression of epidermal growth factor (EGF) has been observed in a wide variety of tumors, including non-small cell lung cancer and squamous cell carcinoma of the head and neck (7, 8). When EGF or transforming growth factor- α binds to EGF receptor (EGFR), the receptor is autophosphorylated and associates with additional molecules of EGFR or other family members to form dimers or oligomers (9). These activated receptor tyrosine kinases phosphorylate several cellular signaling proteins and form receptor complexes with Src homology and collagen (Shc), growth factor receptor binding protein 2, and Sos (10). Activation of the EGFR signal transduction pathway has been shown to enhance cellular processes involved in tumor growth and progression, angiogenesis, invasion, and metastasis (11). A positive correlation has also been found between EGFR expression and tumor resistance to radiation therapy (12).

It has been shown that 1 to 5 Gy ionizing radiation (IR) activates EGFR to the same extent and with similar kinetics as growth factors, as measured by receptor tyrosine phosphorylation (13, 14). IR-induced EGFR activation promotes cell proliferation after single and/or repeated exposures, a response that is blocked by selective inhibition of EGFR tyrosine phosphorylation (15). This response is likely the mechanism underlying accelerated repopulation in tumors (16), implicating IR-induced EGFR activation as the initiating event. This conclusion is supported by studies that provide strong preclinical evidence that EGFR inhibition enhances the antitumor activity of IR (17, 18). The mechanism by which IR activates EGFR phosphorylation has not been determined and has, therefore, been the subject of speculation. In this report, we show that IR activates EGFR and its downstream pathway via production of NO generated by NOS.

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Results and Discussion

The phosphorylation of receptor tyrosine kinases in the absence of ligand binding may be mediated by oxidative molecules and likely contributes to the initiation of specific signaling cascades and stress-related responses within cells (19). It has been suggested that reactive oxygen species function as intracellular second messengers that participate in the activation of intracellular signaling pathways (20) and, therefore, may mediate ligand-independent phosphorylation of cell surface receptors.

To determine whether NO is involved in IR-induced EGFR phosphorylation, we measured the effect of IR on NO production in A549 cells using a NO-sensitive fluorescent probe, 3-amino, 4-aminomethyl-2',7'-difluorofluorescein diacetate, and Griess reagent. As shown in Fig. 1A, at doses up to 2 Gy, IR dose-dependently induced NO production. Furthermore, at 5 Gy of IR, NO production increased with time (Fig. 1B). Because NO is formed during the NOS-catalyzed conversion of arginine to citrulline, we measured NOS activity with an arginine-citrulline conversion assay after irradiation of cells with IR. We found that IR caused a significant dose-dependent increase in NOS (Fig. 1C). At 5 Gy of IR, NOS activity peaked after 15 minutes and returned to baseline after 60 min (Fig. 1D). We then examined the effect of IR on the

EGFR Y¹¹⁷³ autophosphorylation site by immunoblotting. A549 cells express the wild-type EGFRs. As shown in Fig. 1E, IR caused a dose-dependent increase in the level of EGFR Y¹¹⁷³ phosphorylation that paralleled NO formation and NOS activity (Fig. 1A and C). At 5 Gy of IR, EGFR phosphorylation peaked after 15 minutes and returned to baseline after 60 minutes, which was consistent with NOS activity (Fig. 1D) but not with NO production (Fig. 1B).

Our observations are consistent with data reported by Leach et al. (21) stating that activation of NOS by 2 Gy of IR peaked in 5 minutes, slowly decreased, and returned to basal levels in 30 minutes, whereas NO production increased constantly. Although we cannot exclude the possibility of other intracellular compounds being targeted by IR and directly generating NO, it is likely that IR initiates intracellular NO generation and EGFR phosphorylation. These processes may then activate downstream signaling pathways, resulting in steady accumulation of intracellular NO, whereas NOS activity and EGFR phosphorylation fall back to their basal levels.

We determined the phosphorylation of EGFR by EGF versus IR (Supplementary Fig. S1). Phosphorylation by EGF was slightly higher (1.2-fold increase) than that by IR, implying that use of NO scavengers and IR in combination with a series of EGFR inhibitors, such as cetuximab, gefitinib, and erlotinib,

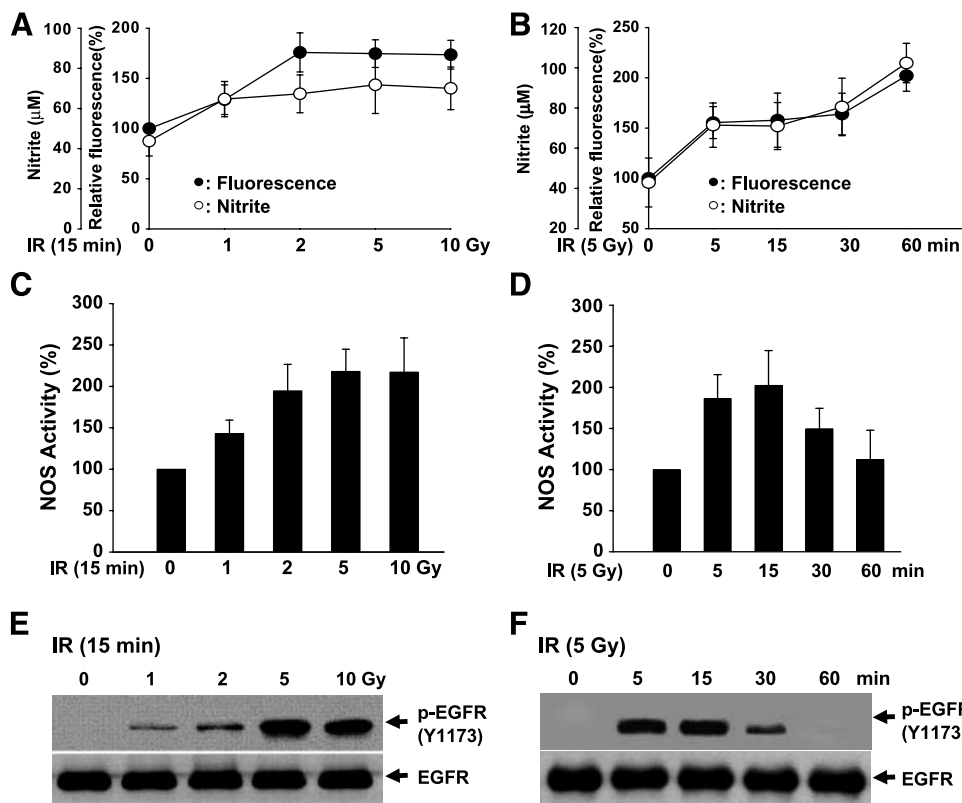


FIGURE 1. IR-induced NO generation and EGFR phosphorylation in A549 lung adenocarcinoma cells. **A.** Cells were exposed to 1 to 10 Gy IR and cellular nitrite concentration was assayed after 1 h. **B.** After exposure to 5 Gy IR, cells were collected at the indicated time (5–60 min) and nitrite (○) and relative NO (●) were detected. **C.** Cellular NOS activity was assayed after 15 min of exposure to the indicated dose of IR (1–10 Gy). **D.** After exposure to 5 Gy of IR, cells were collected at the indicated time (5–60 min) and NOS activity assay was done. Expression levels and status of phosphorylation of EGFR (on both Tyr¹¹⁷³ and total tyrosine) were assayed by immunoblot using anti-pEGFR (Y¹¹⁷³) and EGFR antibodies after collection of cells at 15 min after exposure to the indicated dose (5–10 Gy) of IR (**E**) or at the indicated time (5–60 min) after exposure to 5 Gy IR (**F**). All results were repeated six times. All measurements (**A–D**) for each condition were done in triplicate and quantitative data were expressed as mean ± SD.

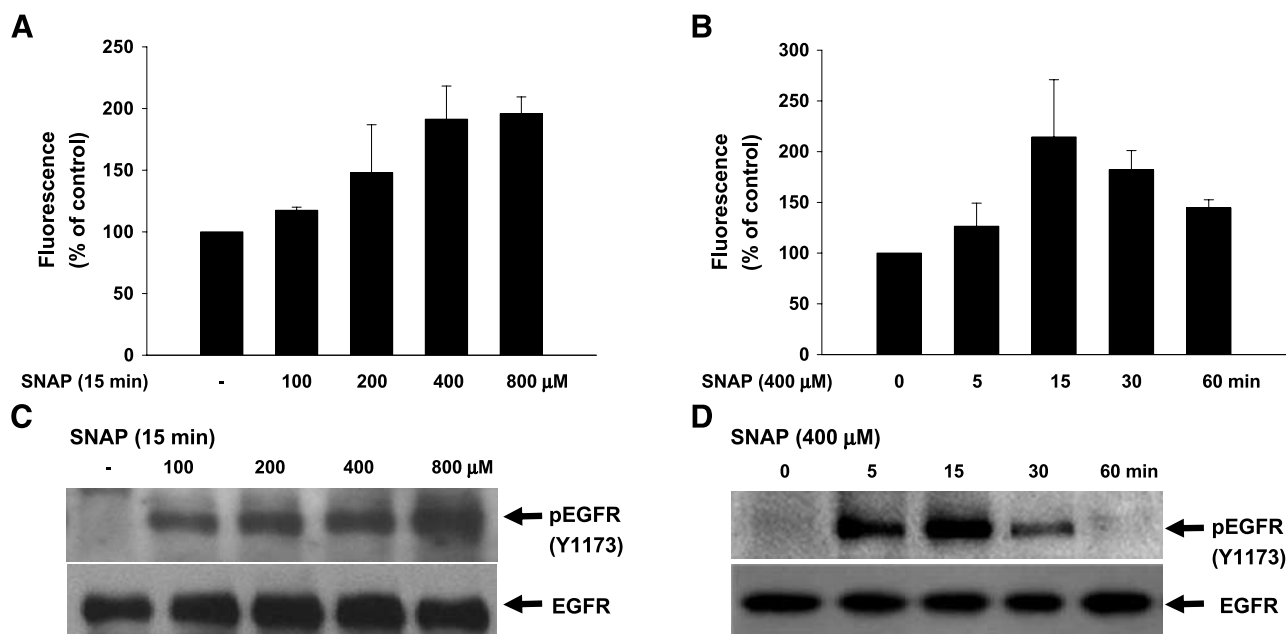


FIGURE 2. SNAP induced NO production and EGFR phosphorylation. Cells were exposed to SNAP (100–800 $\mu\text{mol/L}$) for 15 min (**A**) or to SNAP (400 $\mu\text{mol/L}$) for 5 to 60 min (**B**) and relative NO amount was determined. All measurements for each condition were done in triplicate and quantitative data were expressed as mean \pm SD. Immunoblot analysis was done with anti-pEGFR (Y¹¹⁷³) or EGFR antibodies after exposure to SNAP (100–800 $\mu\text{mol/L}$) for 15 min (**C**) or to SNAP (400 $\mu\text{mol/L}$) for 5 to 60 min (**D**). All results were repeated six times.

might achieve better treatment of EGFR-overexpressing cancer patients. To determine whether the observed phosphorylation of EGFR on Y¹¹⁷³ was due to receptor autophosphorylation or to phosphorylation by Src (22, 23), we examined the effect of PP1, a cell permeable and selective inhibitor of Src, on IR-induced EGFR phosphorylation (Supplementary Fig. S2) and clearly found that Src is not involved.

Because IR induced NO production and EGFR phosphorylation, we examined the direct effect of NO on EGFR using the NO donor, *S*-nitroso-*N*-acetylpenicillamine (SNAP). In A549 cells, SNAP dose-dependently generated NO, which peaked after 15 min (Fig. 2). We also found that SNAP dose-dependently induced tyrosine phosphorylation of EGFR on Y¹¹⁷³. At 400 $\mu\text{mol/L}$ SNAP, EGFR tyrosine phosphorylation was stimulated after 5 minutes and peaked after 15 minutes, which was consistent with NO generation (Fig. 2A and B). These results suggest that NO can directly induce EGFR activation. Although it has been reported that NO inhibits EGF-dependent EGFR activation in different cell types (24, 25), our data in Supplementary Fig. S3 showed that NO generated by SNAP can enhance the EGF-induced EGFR phosphorylation in A549 cells.

To investigate whether IR-induced NO generation participates in EGFR phosphorylation, we examined the effect of 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (PTIO), a specific scavenger of NO, on IR-induced NO production and EGFR tyrosine phosphorylation. At 50 $\mu\text{mol/L}$, PTIO almost completely suppressed IR-induced NO production (Fig. 3A) and dose-dependently inhibited IR-induced EGFR phosphorylation (Fig. 3B). Furthermore, *N*^G-monomethyl-L-arginine (L-NMMA), a specific inhibitor of NOS, abrogated IR-induced NO production and EGFR

phosphorylation, whereas *N*^G-monomethyl-D-arginine, a negative control for L-NMMA, affected neither (Fig. 3C and D). These results suggest that NO generation is involved in NOS activation. When phosphorylated, Y¹¹⁷³ on EGFR provides a direct binding site for Shc (10). To determine whether IR-induced phosphorylation of EGFR on Y¹¹⁷³ mediates the signaling downstream of EGFR, we immunoprecipitated proteins with EGFR antibody and analyzed Shc coprecipitation by immunoblotting. As shown in Fig. 3E, IR stimulated the association of Shc with tyrosine phosphorylated EGFR. In addition, PTIO and L-NMMA reduced the amount of Shc that coprecipitated with EGFR after IR treatment, consistent with the ability of these agents to inhibit IR-induced phosphorylation of EGFR on Y¹¹⁷³. These compounds also inhibited the phosphorylation of the downstream signaling molecules Akt and Erk1/2. Together, these results suggest that IR-generated NO mediates the activation of EGFR and its downstream signaling pathway.

To further examine the role of NOS in IR-induced EGFR phosphorylation, we transiently transfected A549 cells with a Myc-tagged endothelial NOS (eNOS) vector (Fig. 4A and B). eNOS-transfected cells treated with 5 Gy IR showed an increase in NO production, EGFR phosphorylation on Y¹¹⁷¹, and Akt and Erk1/2 activation compared with control cells (Fig. 4A). The amount of Shc immunoprecipitated with EGFR was also significantly increased in the eNOS-transfected cells (Fig. 4B). These results suggest that IR activation of eNOS plays an important role in the activation of EGFR phosphorylation and its downstream signaling pathways. We also examined the effect of transiently transfecting A549 cells with an eNOS-specific small interfering RNA (siRNA) to inhibit eNOS expression. Figure 4C shows that transfection with an siRNA

that corresponds to the eNOS mRNA sequence inhibited eNOS expression, IR-induced NO production, and NOS activity by ~70% (Fig. 4C and D) and IR-induced EGFR phosphorylation was also suppressed (Fig. 4E). In addition, there was a significant decrease in the amount of Shc immunoprecipitated with EGFR in eNOS siRNA-transfected cells compared with control cells. These results further support the idea that endogenous NOS is required for IR-induced NO production and stimulation of EGFR tyrosine phosphorylation.

It has been shown that unstimulated A549 cells express low levels of neuronal NOS and very low levels of inducible NOS (iNOS) and that iNOS, but not neuronal NOS, is up-regulated and produces NO in response to IFN- γ , interleukin 1 β , and

tumor necrosis factor- α (26, 27). Lo et al. (28) recently reported that EGF treatment induces early activation of iNOS transcription at 1 h and the level of iNOS mRNA increases for 6 hours in MDA-MB-468 human breast carcinoma cells. Therefore, to determine whether other isoforms are also involved in activation of EGFR and production of NO in A549 cells, we examined the expression levels of *iNOS* gene in response to IR by semiquantitative reverse transcription-PCR. As shown in Supplementary Fig. S4, we failed to detect *iNOS* transcript in unstimulated A549 cells. Cells exposed to 5 Gy of IR did not show increased levels of *iNOS* mRNA even after 120 min; the same cells treated with tumor necrosis factor- α showed a dramatic increase of *iNOS*. In contrast to *iNOS*, A549 cells

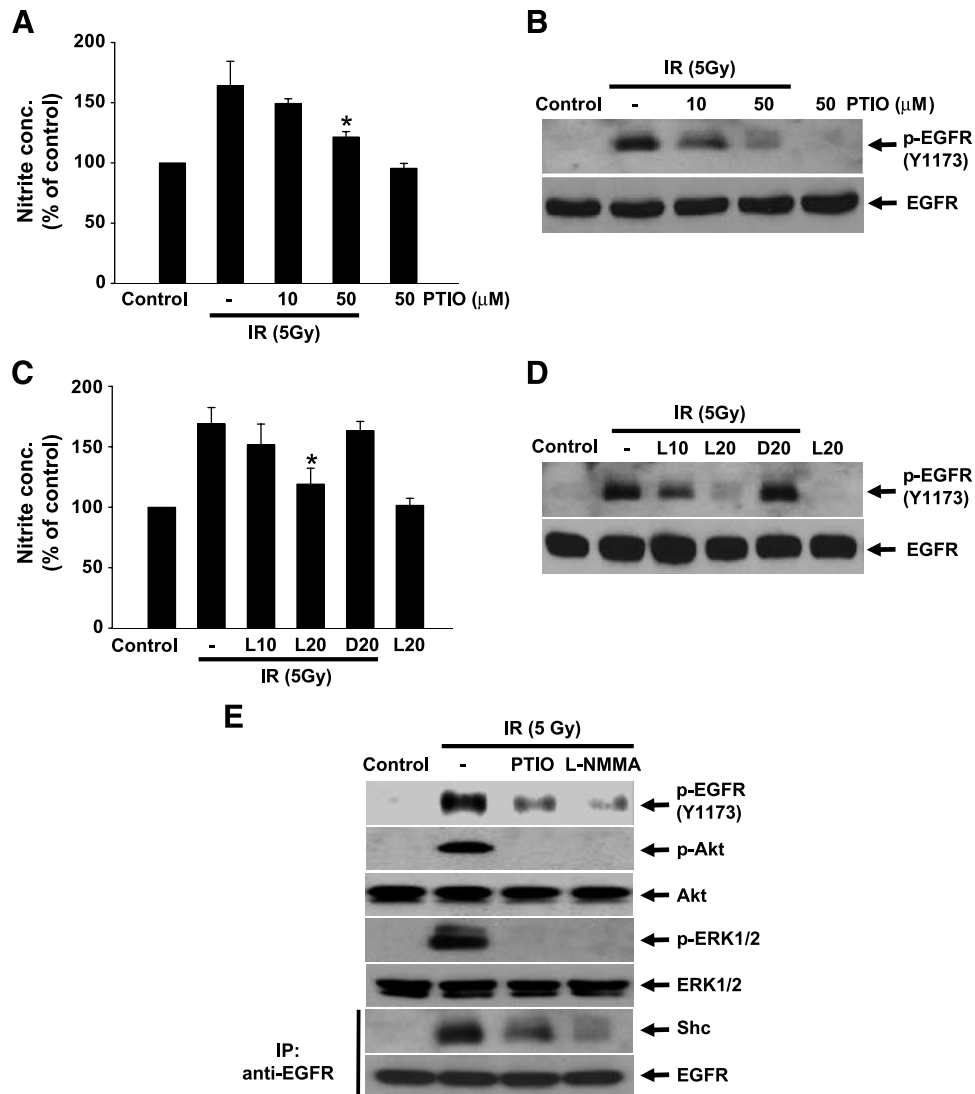


FIGURE 3. Suppression of NO decreased IR-induced NO generation and EGFR phosphorylation. **A.** Cells were treated with PTIO (10 or 50 $\mu\text{mol/L}$) for 30 min and exposed to 5 Gy IR for 15 min, and the relative amount of NO was measured. **B.** Immunoblot analysis using anti-pEGFR (Y¹¹⁷³) and EGFR antibodies. **C.** Cells were pretreated with 10 or 20 $\mu\text{mol/L}$ of L-NMMA (L10 or L20) or 20 $\mu\text{mol/L}$ of a D-isomer of NMMA (D20) as a negative control and then exposed to 5 Gy IR for 15 min. NO generation was analyzed. **D.** Same as for **C** except EGFR phosphorylation was analyzed. **E.** Cells were pretreated with 50 $\mu\text{mol/L}$ PTIO and 20 $\mu\text{mol/L}$ L-NMMA for 30 min and then exposed to 5 Gy IR for 15 min. Immunoblot analysis was done using anti-pEGFR (Y¹¹⁷³), phosphorylated Akt (p-Akt; Ser⁴⁷³), Akt, phosphorylated Erk1/2 (p-Erk1/2), and Erk1/2 antibodies. Immunoprecipitation with anti-EGFR antibody and immunoblot with anti-Shc and EGFR antibodies. All measurements (**A** and **C**) for each condition were done in triplicate and quantitative data were expressed as mean \pm SD. All results were repeated five times. Statistical significance of differences was assessed using a two-tailed homoscedastic Student's *t* test. Asterisk (*, $P < 0.05$) was considered to be statistically significant.

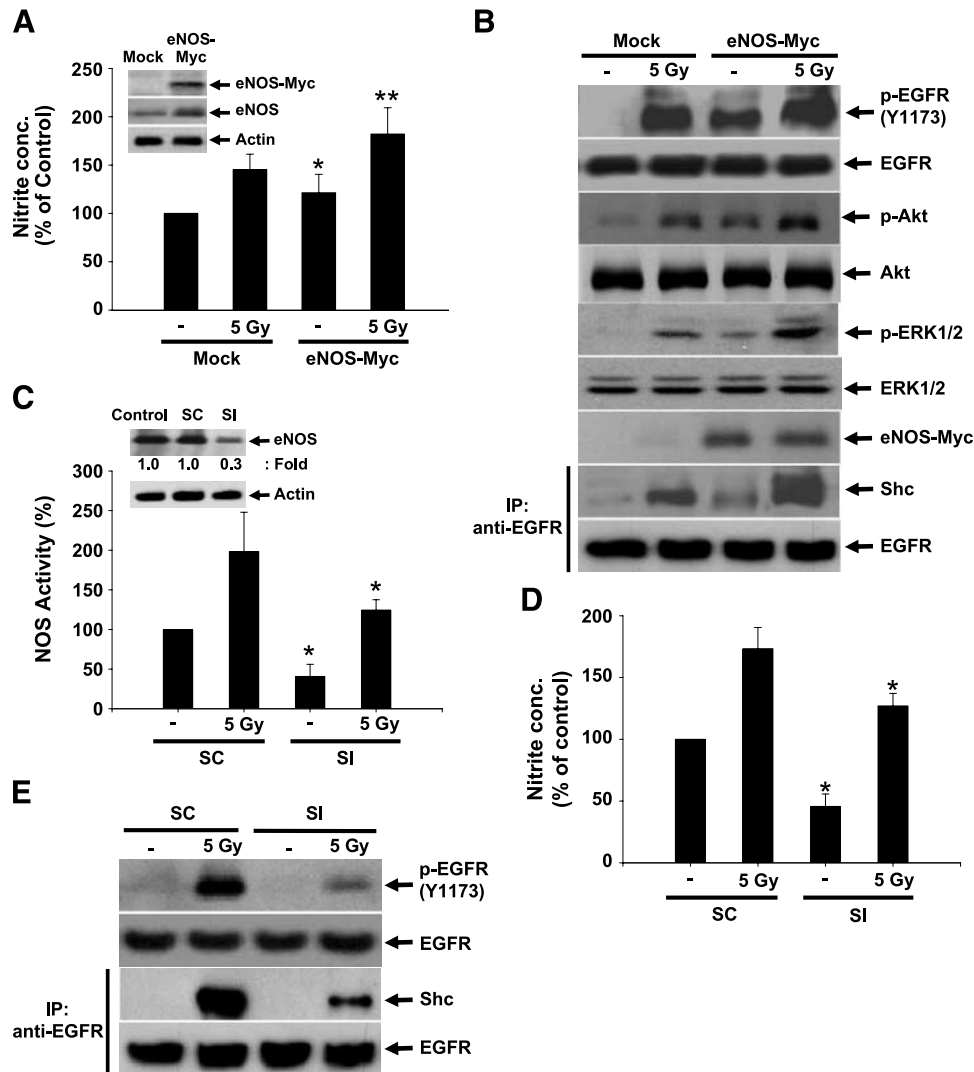


FIGURE 4. eNOS regulated IR-induced NO generation and EGFR signal activation. **A.** After transfection with pcDNA3-eNOS-Myc (*eNOS-Myc*) or empty vector (*Mock*), cells were exposed to 5 Gy IR for 15 min and nitrite concentration was determined. Immunoblots with anti-Myc and eNOS antibodies, using anti-actin as a loading control. **B.** After transfection, immunoblot analysis was done using anti-pEGFR (Y1173), EGFR, phosphorylated Akt (*p-Akt*; Ser⁴⁷³), Akt, phosphorylated Erk1/2 (*p-Erk1/2*), and Erk1/2 antibodies. Exogenously induced eNOS was measured with anti-Myc antibodies. Immunoprecipitation with anti-EGFR and immunoblot with anti-Shc and EGFR antibody. **C.** After transfection with scrambled (SC) and eNOS (SI) siRNAs, cells were exposed to 5 Gy IR for 15 min and NOS activity was assayed. Immunoblot analysis was done using anti-eNOS and actin antibodies. **D.** The nitrite concentration was determined. **E.** Cells transfected with scrambled (SC) and eNOS (SI) siRNAs were harvested after 15 min of exposure to 5 Gy IR. Immunoblot analysis with anti-pEGFR (Y1173) and anti-EGFR antibodies. Immunoprecipitation with anti-EGFR antibody and immunoblot with anti-Shc and EGFR antibodies. Data (C-E) are representative of two different siRNAs of eNOS experiments done with similar results. All measurements (A, C, and D) for each condition were done in triplicate, and quantitative data were expressed as mean \pm SD. All results were repeated five times.

expressed modest basal levels of eNOS protein (Fig. 4A and C) that stayed constant during the same time course and tumor necrosis factor- α treatment (Supplementary Fig. S4). Taken together, these observations imply that eNOS has a role in activating EGFR and producing intracellular NO.

EGFR has been identified as an immediate early response gene product that is activated by radiation. Similar to the effect of physiologic ligands, IR mediates activation of downstream cytoplasmic protein kinase cascades (13, 14). Because these responses to IR contribute to tumor cell radioresistance, EGFR blockade has been proposed to enhance the cytotoxicity of IR (17). EGFR autophosphorylation is thought to involve

dimerization or oligomerization, followed by tyrosine phosphorylation of an adjacent receptor molecule in the complex. Thus, radiation could act by releasing EGFR from intracellular inhibitors or structural elements attached to the plasma membrane that prevent dimerization under normal physiologic conditions. Our results provide evidence that intracellular generation of NO by NOS is required for rapid activation of EGFR and its downstream pathways by IR. Based on these results, we propose the following sequence of events in response to IR: (a) NOS is activated; (b) NOS generates NO; (c) EGFR autophosphorylates; and (d) downstream signaling pathways of EGFR are activated.

The present study provides several lines of evidence that intracellular accumulation of NO is required for rapid activation of EGFR phosphorylation by IR. Also, EGFR was in cytoplasmic fraction after exposure to IR (Supplementary Fig. S7). Furthermore, it is suggested that changes of phosphorylated Akt, phosphorylated ERK, and Shc be, at least in part, due to EGFR phosphorylation induced by IR (Supplementary Fig. S8). Although the existence of target molecules to generate NO directly by IR cannot be excluded, we suggest that IR-activated eNOS plays an important role in producing NO. Our data also indicate that EGFR tyrosine phosphorylation is induced in the absence of ligand binding by NO and is significantly increased when NO and IR is cotreated (Supplementary Fig. S11). Although the mechanism by which NO mediates tyrosine phosphorylation of EGFR remains to be determined, our studies suggest that nitrosylation of EGFR is critical because the IR and NO donor SNAP increased the level of nitrotyrosine of EGFR (Supplementary Fig. S12). In summary, our data show that, in A549 lung carcinoma cells, transient production of NO in response to IR is mediated by the activation of NOS. Although the precise mechanism of how IR activates NOS remains to be determined, our present findings strongly suggest that NO plays a key role in the activation of EGFR and its downstream pathways. Also, inhibition of NOS/EGFR in combination with IR significantly decreased cell proliferation (Supplementary Fig. S9). Therefore, these novel findings provide a potential strategy for enhancing the effectiveness of radiotherapy by targeting EGFR.

Materials and Methods

Cell Culture, Transfection, and Reagents

Human lung adenocarcinoma cell line A549 (American Type Culture Collection) was cultured in RPMI 1640 supplemented with 10% fetal bovine serum (Invitrogen). Cells were transiently transfected with pcDNA3-eNOS-Myc (gift from Dr. Dimmeler, University of Frankfurt), empty plasmids (Invitrogen), or siRNAs (Santa Cruz Biotech and Ambion), using LipofectAMINE 2000 transfection reagent (Invitrogen). SNAP, PTIO, L-NMMA, and *N*^G-monomethyl-D-arginine were purchased from Calbiochem, and EGF was from R&D Systems. IR source was ¹³⁷Cs γ -ray (Atomic Energy of Canada, Ltd.) at a dose rate of 3 Gy/min. 3-Amino, 4-aminomethyl-2',7'-difluorofluorescein diacetate was from Invitrogen. Anti-phosphorylated EGFR (pEGFR; Tyr¹¹⁷³), phosphorylated tyrosine, EGFR, Shc, and Myc antibodies were from Santa Cruz Biotech; anti-phosphorylated Akt (Ser⁴⁷³), Akt, phosphorylated Erk1/2, Erk1/2, and eNOS antibodies were from Cell Signaling; and anti-actin antibody was from Sigma-Aldrich.

Detection of NO and NOS

Exponentially growing cells (10⁵) were seeded in 60-mm dishes, serum-starved for 12 h, and treated as indicated. The medium was collected, and the nitrite concentration was measured by the Griess reagent system (Promega) and calculated from sodium nitrite (NaNO₂) standards. For fluorescent detection of NO, the cells were pretreated with 3-amino,4-aminomethyl-2',7'-difluorofluorescein diacetate

(Invitrogen) and measured by fluorescence-activated cell sorting analysis (Becton Dickinson). Determination of cellular NOS catalytic activity was assayed with the nitric oxide synthase assay kit (Calbiochem). Relative NOS activity was calculated to determine the conversion of L-[³H]arginine to L-[³H]citrulline (Perkin-Elmer).

Western Blot and Immunoprecipitation Analysis

Total cell lysates (20–40 μ g of protein) were immunoprecipitated with specific antibodies, separated by SDS-PAGE, and transferred to nitrocellulose membranes. The membranes were incubated with primary antibodies and horseradish peroxidase-conjugated secondary antibodies, and immunoreactive bands were visualized with enhanced chemiluminescence reagents (Pierce). The density of immunoreactive bands was quantified by Densitometry System (Bio-Rad).

Statistical Analysis

Quantitative data were presented as mean \pm SD. Statistical significance of differences was assessed using a two-tailed homoscedastic Student's *t* test. Asterisk (**P* < 0.05, ***P* < 0.01) was considered to be statistically significant.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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