Peroxynitrite Induces Covalent Dimerization of Epidermal Growth Factor Receptors in A431 Epidermoid Carcinoma Cells*

(Received for publication, March 6, 1998, and in revised form, September 2, 1998)

Albert van der Vliet, Milena Hristova, Carroll E. Cross, Jason P. Eiserich, and Tzipora Goldkorn†

From the Center for Comparative Respiratory Biology and Medicine, Department of Internal Medicine, University of California, Davis, California 95616

Irreversible tyrosine modifications by inflammatory oxidants such as peroxynitrite (ONOO−) can affect signal transduction pathways involving tyrosine phosphorylation. The epidermal growth factor receptor (EGFR), a member of the c-ErbB receptor tyrosine kinase family, is involved in regulation of epithelial cell growth and differentiation, and possible modulation of EGFR-dependent signaling by ONOO− was studied. Exposure of epidermoid carcinoma A431 cells to 0.1–1.0 mM ONOO− resulted in tyrosine nitration on EGFR and other proteins but did not significantly affect EGFR tyrosine autophosphorylation. A high molecular mass tyrosine-phosphorylated protein (~340 kDa) was detected in A431 cell lysates after exposure to ONOO−, most likely representing a covalently dimerized form of EGFR, based on immunoprecipitation and/or immunoblotting with a-EGFR antibodies and co-migration with ligand-induced EGFR dimers cross-linked with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide. Covalent EGFR dimerization by ONOO− probably involved intermolecular dityrosine cross-linking and was enhanced after receptor activation with epidermal growth factor. Furthermore, irreversibly cross-linked EGFR was more extensively tyrosine-phosphorylated compared with the monomeric form, indicating that ONOO− preferentially cross-links activated EGFR. Exposure of A431 cells to ONOO− markedly reduced the kinetics of tyrosine phosphorylation of a downstream EGFR substrate, phospholipase C-γ1, which may be related to covalent alterations in EGFR. Alteration of EGFR signaling by covalent EGFR dimerization by inflammatory oxidants such as ONOO− may affect conditions of increased EGFR activation such as epithelial repair or tumorigenesis.

Inflammatory conditions are associated with increased production of reactive oxygen metabolites, such as superoxide anion (O2·−) and hydrogen peroxide (H2O2), as well as induction of nitric oxide (NO) synthesis, which is thought to play major roles in host defense but is also presumed to contribute to the development of tissue injury associated with chronic inflammation. The nearly diffusion-limited reaction between nitric oxide (NO) and superoxide (O2·−) produces peroxynitrite (ONOO−), a powerful oxidant and cytotoxic species that has been proposed to importantly contribute to the pathophysiology of a large variety of diseases associated with inflammation (1). Peroxynitrite is reactive toward all classes of biomolecules, including lipids, proteins and nucleic acids, and oxidative reaction products in proteins primarily include modifications of cysteine, methionine, tryptophan, and tyrosine residues (2).

Irreversible covalent modifications of tyrosine residues by ONOO− include the formation of 3-nitrosotyrosine and 3,3′-dityrosine, which represent characteristic “markers” for inflammatory (NO−derived) oxidants (5–8), but these modifications may also have functional consequences and could contribute to the pathophysiological effects of NO−derived reactive nitrogen oxides such as ONOO−. Chemical studies with the nitrating agent tetranitromethane have shown that nitration of critical tyrosine residues inactivates a wide variety of enzymes (9–11) and affects structural proteins (1, 5). Studies with isolated tyrosine kinases have demonstrated that substrate tyrosine residues are not phosphorylated when they are nitrated (12, 13), which implies that tyrosine nitration may interfere with signal transduction pathways involving tyrosine phosphorylation. Alternatively, formation of 3,3′-dityrosine by inflammatory oxidants such as ONOO− can result in inter- or intramolecular covalent cross-linking in e.g. membrane proteins (7, 8) and thereby affect signaling pathways. Despite various recent investigations (13–16), the potential pathophysiological significance of such protein modifications has, however, not been demonstrated conclusively in intact cellular systems.

One group of proteins that may be significantly affected by inflammatory oxidants are membrane receptors for various growth factors or cytokines that are induced and/or activated during inflammation to promote wound healing processes. Among these is the epidermal growth factor receptor (EGFR),1 which is overexpressed and activated in response to epithelial injury to promote epithelial repair processes (17, 18). The EGFR is a member of the receptor tyrosine kinase superfamily and is involved in the regulation of proliferation and differentiation of primarily epithelial cell types (19, 20). The EGFR is a 170-kDa glycoprotein that spans the membrane via one α-hel-

---

1 The abbreviations used are: EGFR, epidermal growth factor receptor; EDAC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; EGF, epidermal growth factor; HPLC, high performance liquid chromatography; PAS, protein A-Sepharose; PBS, phosphate-buffered saline; PLC-γ1, phospholipase C-γ1; PBS, phosphate-buffered saline; HRP, horseradish peroxidase; PAGE, polyacrylamide gel electrophoresis.

* This work was performed under the tenure of a Parker B. Francis Fellowship (to A. v. d. V.) and a Research Fellowship from the California Affiliate of the American Lung Association (to J. P. E.) and was supported by National Institutes of Health Grant HL57452 (to C. E. C.) and grants from the American Lung Association (to A. v. d. V. and T. G.), and the Cystic Fibrosis Foundation (A. v. d. V.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1754 solely to indicate this fact.

† Present address: Dept. of Anesthesiology, University of Alabama, Birmingham, AL 35233.

‡ To whom correspondence may be addressed: Signal Transduction Laboratory, TB149, University of California, Davis, CA 95616. Tel.: 530-752-2988; Fax: 530-752-2949; E-mail: avandervliet@ucdavis.edu.

§ Present address: Dept. of Anesthesiology, University of California, Davis, CA 95616. Tel.: 530-752-6305; Fax: 530-752-4374; E-mail: ttgoldkorn@ucdavis.edu.

¶ To whom correspondence may be addressed: Pulmonary Research Laboratory, 1121 Surge I, University of California, Davis, CA 95616. Tel.: 530-752-6305; Fax: 530-752-4374; E-mail: avandervliet@ucdavis.edu.
Covalent EGF Receptor Dimerization by Peroxynitrite

C. M. O'Halloran, W. T. Kozarich, and C. J. Goss

Journal of Biological Chemistry
Volume 270, Issue 36, September 18, 1995
Page 21051

H2O2. EGFR activation was performed by the addition of EGF (20 nM; 1 μl was added to the incubation medium shortly before the addition of 300 μM H2O2) involving horseradish peroxidase (HRP, type I; Sigma), this enzyme was mixed with the incubation buffer by rapid swirling, in order to assure optimal exposure of the cells to ONOO−

After the various cell treatments, culture dishes were placed on ice, and the cells were washed twice with cold PBS and lysed in 250 μl of solubilization buffer (50 mM HEPES, 250 mM NaCl, 1.5 mM MgCl2, 2 mM Na3VO4, 1% Triton, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 10 μg/ml leupeptin, pH 7.5). Dishes were kept on ice for 20 min on a rocker platform to allow complete solubilization of membrane proteins, and cell lysates were collected and centrifuged (10 min at 10,000 × g) to remove insoluble cellular debris.

Analysis of EGF Tyrosine Phosphorylation and Protein Nitration— Aliquots of cell lysates (100 μl) were mixed with 20 μl of 6% sample reducing buffer (125 mM Tris-HCl, pH 6.8, 20% glycerol, 4% SDS, 10% β-mercaptoethanol) and boiled for 5 min to denature proteins. Proteins were separated on 4% polyacrylamide-SDS gels, and electroblotted on polyvinylidene difluoride (Immobilon-P) Sigma membranes for Western analysis. Membranes were blocked with 1% bovine serum albumin and then incubated with a monoclonal antibody against phosphotyrosine (PY-20; Upstate Biotechnology), a polyclonal antibody against 3-nitrotyrosine (Upstate Biotechnology) or polyclonal antibodies against the intracellular domain of EGFR (kindly donated by Drs. D. Cadena, G. N. Gill, and Dr. J. Dietschy) followed by incubation with secondary antibodies conjugated with horseradish peroxidase. Antibodies were detected by diaminobenzidine staining (Vector Laboratories, Burlingame, CA) or by enhanced chemiluminescence (Amersham Pharmacia Biotech, Buckinghamshire, UK).

Experiments with A431 Plasma Membranes and Purified EGF— A431 plasma membranes were prepared from approximately 106 cells, which were washed with PBS and homogenized in 20 ml of HEPES buffer (20 mM HEPES, 1.5 mM MgCl2, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 100 μM leupeptin, pH 7.4) at 4 °C by brief sonication. Cell homogenates were centrifuged at 1,500 × g for 10 min to remove nuclei and unlysed cells, the supernatant was further centrifuged at 25,000 × g for 30 min, and the pellet containing plasma membranes was washed with PBS and finally resuspended in PBS at approximately 10 mg/ml protein (determined according to Bradford (30) with bovine serum albumin as a standard) and stored at −80 °C until use. Plasma membranes were diluted to 1 mg/ml protein in 50 mM sodium phosphate buffer (pH 7.4), containing 5 mM MgCl2, 1 mM MnCl2 and 100 μM ATP, reacted with ONOO− or other oxidants, and solubilized for SDS-PAGE and Western analysis. Similar experiments were performed with A431 plasma membranes by affinity chromatography (Sigma), which was suspended to 20 μg/ml in 50 mM sodium phosphate buffer (pH 7.4), containing 5 mM MgCl2, 1 mM MnCl2, and 100 μM ATP. Aliquots of 50 μl were reacted with ONOO− (500 μM) before or after receptor stimulation with 20 nM EGF. Incubations were terminated by the addition of 10 μl of 6% sample reducing buffer, and samples were subjected to SDS-PAGE and Western blotting.

Catalytic EGF Receptor Phosphorylation— A431 cells were used in these experiments, which were washed with PBS and monolayered in 20 ml of HEPES buffer (20 mM HEPES, 150 mM NaCl, 0.1% Triton X-100, 10% glycerol, pH 7.5), and aliquots of the PAMAb complex containing 4 μg of mAb 528 were incubated overnight with 200 μl of A431 cell lysate (up to 1 mg of protein) at 4 °C (31). The PAMAb receptor complex was washed three times with HNTG buffer and mixed with 3% sample reducing buffer for analysis by SDS-PAGE.

Analysis of Tyrosine Phosphorylation of Phospholipase C-γ1 (PLC-γ1)— After treatment of A431 cells with EGF and/or ONOO−, cells were solubilized, and aliquots containing 2 mg of protein were immunoprecipitated with overnight incubation at 4 °C with 2 μg of a monoclonal antibody against PLC-γ1 (clone D-7-3; Upstate Biotechnology) followed by incubation for 2 h with 5 μg of protein A-Sepharose (Sigma). The PASmAb complex was washed three times with HNTG buffer, boiled for 5 min in sample reducing buffer, and subsequently subjected to SDS-PAGE and Western blot analysis with α-phosphotyrosine (PY-20) or with a polyclonal antibody against PLC-γ1 (Upstate Biotechnology).

Analysis of Tyrosine Modification Products by HPLC— Cellular proteins were precipitated from cell lysates with 5% trichloroacetic acid, and washed twice with ethanol/ethyl acetate 50/50 (v/v) to remove contaminating lipids. Protein pellets were dissolved in 6 M HCl and hydrolyzed under vacuum for 24 h at 110 °C, after which hydrolysates were dried under N2 and reconstituted in 50 mM phosphate buffer (pH 7.3).
with 0 (□), 0.25 (■), 0.5 (▲), or 1.0 mM (▲) ONOO− or with 5 mM EGF (▲). Cells were subsequently kept in Dulbecco’s modified Eagle’s medium containing 1% newborn bovine serum for the next 4 days (EGF was kept in the medium throughout the experiment). Cell proliferation was followed by cell counting on four consecutive days after these treatments.

3.0% methanol (93/7) (v/v). Tyrosine and its oxidation products were analyzed by HPLC with tandem UV (274 nm) and fluorescence detection (excitation, 284 nm; emission, 410 nm) (3) and quantitated by comparison with external standards.

Results and Discussion

Effects of ONOO− on Cell Viability and Proliferation—A431 cells were seeded at 5 × 10^4 cells/well in six-well plates in Dulbecco’s modified Eagle’s medium with 1% newborn bovine serum and incubated for 24 h before the addition of ONOO− (after replacing the medium with modified PBS as described above; final volume 1 ml) or 20 nM EGF. Cell proliferation was assessed on four consecutive days after these treatments by collection of cells by trypsinization and cell counting using an hemocytometer. In separate experiments, cell viability was assessed 1 h after the addition of ONOO− using Alamar Blue® (Alamar Bioscience, Sacramento, CA), which is metabolized by respiring cells, resulting in increased absorbance at 600 nm. Cell viability was assessed by comparing A570 to untreated cells (100% viable) with those of ONOO−-treated cells.

Nitration of Cellular Proteins and Tyrosine Phosphorylation—The addition of 0.25–1.0 mM ONOO− to subconfluent A431 cells in six-well plates (50,000 cells/well) caused only marginal loss of cell viability as determined using Alamar Blue®. Cell viability was decreased by 20–30%, measured 24 h after the addition of 1 mM ONOO−. As illustrated in Fig. 1, ONOO− causes inhibition of cell proliferation only when added at concentrations higher than 0.5 mM. Continuous stimulation of A431 cells with 5 nM EGF also resulted in inhibition of cell growth, as shown previously (32). Thus, overstimulation of EGFR and the addition of ONOO− both cause a stress response in A431 cells that results in growth arrest and/or apoptosis (33–36).

Nitration of Protein Cellular Proteins and Tyrosine Phosphorylation—The addition of 0.1–1.5 mM ONOO− to confluent A431 cells in 100-mm culture dishes caused a dose-dependent increase in tyrosine nitration of cellular proteins as detected by both HPLC and immunoblotting with an α-nitrotyrosine antibody. The 3-nitrotyrosine content in cellular proteins increased from ~0.02 to 0.23 ± 0.06, 0.32 ± 0.06, and 0.65 ± 0.19 mmol/mmol of tyrosine residues (n = 4) after the addition of 0.5, 1.0, and 1.5 mM ONOO−, respectively. SDS-PAGE followed by immunoblotting with an α-nitrotyrosine antibody also demonstrated dose-dependent nitration of various proteins including EGFR. Nitration of EGFR was confirmed by immunoprecipitation of EGFR (using mAb 528) followed by SDS-PAGE and immunoblotting with α-nitrotyrosine (Fig. 2A).

The EGFR in nonstimulated A431 cells is partly phosphorylated on tyrosine residues, as detected with an α-phosphotyrosine antibody, and the extent of this basal autophosphorylation was only marginally but not significantly affected after exposure to ONOO− for 30 min (Fig. 2B). Stimulation of A431 cells with 20 nM EGF for 10–30 min caused a dramatic increase in EGFR autophosphorylation, but EGF-induced autophosphorylation was not significantly affected after treatment of A431 cells with up to 1.5 mM ONOO− (not shown). Together, these results suggest that the extent of protein tyrosine nitration by nontoxic concentrations of ONOO− is not sufficient to cause significant alteration in EGFR autophosphorylation. Moreover, EGFR tyrosine residues other than the intracellular phosphorylation substrates may have been nitrated. Hence, unless certain tyrosine residues are preferentially nitrated, tyrosine nitration to an extent that is feasible in vivo (37, 38) appears unlikely to cause significant inhibition of tyrosine phosphorylation. In similar recent studies, exposure of various cell types to ONOO− was found to cause variable changes in tyrosine phosphorylation as well as nitration of tyrosine residues, although these events could not be directly related (13–16).

Peroxynitrite-Induced Formation of Dimeric EGFR—Analysis of cell lysates of ONOO− exposed A431 cells by SDS-PAGE revealed the presence of a large phosphorylated protein (approximately 340 kDa) that was not detected in untreated cells.
This protein was also detected by various polyclonal antibodies against EGFR (Fig. 3), indicating that this protein is a covalent aggregate of EGFR with one or more other proteins and most likely represents a homodimer of EGFR. The presumed EGFR dimer was also detected after immunoprecipitation of A431 lysates with mAb 528, followed by SDS-PAGE and immunoblotting with PY-20 or α-EGFR antibodies. Control experiments showed that formation of this putative EGFR dimer was not due to pH changes after the addition of alkaline ONOO⁻ solutions or to contaminating components in the ONOO⁻ stock solution, since similar additions of either 1.2 M NaOH, previously decomposed ONOO⁻ or H₂O₂ were ineffective (Fig. 3).

The putative EGFR homodimer was detectable within 1 min after the addition of ONOO⁻ to A431 cells, and the extent of dimerization did not change significantly for at least 60 min after the addition of ONOO⁻ (not shown), suggesting that EGFR dimerization occurs by direct chemical modification of EGFR, rather than via secondary intracellular pathways induced by ONOO⁻. This notion was confirmed by similar experiments with (partially) purified EGFR; the addition of ONOO⁻ to either A431 plasma membranes or to purified EGFR also resulted in the formation of dimeric EGFR, as assessed by SDS-PAGE (not shown). Furthermore, EGFR dimerization was also detected after the addition of ONOO⁻ to A431 cells at 4 °C, conditions that would have minimized metabolic pathways.

Possible Involvement of Dityrosine Cross-linking—The extracellular domain of EGFR contains cysteine-rich motifs (19, 39), and thiol-reactive reagents have been demonstrated to affect EGFR activation by modification of one or more cysteine residues in the intracellular domain (25–27). Since cysteine residues represent important targets for ONOO⁻ (29), formation of intermolecular disulfide bonds might have been involved in EGFR cross-linking by ONOO⁻. However, SDS-PAGE analyses were performed under reducing conditions that would have cleaved intermolecular disulfide bonds. Furthermore, neither H₂O₂ (Fig. 3) nor the inflammatory oxidant HOCl (not shown), oxidants capable of oxidizing cysteine residues to the disulfide, were able to induce similar EGFR cross-linking, indicating that ONOO⁻ acts by a unique mechanism distinct from these other oxidants. Characteristic products from reaction of ONOO⁻ with cysteine residues may include S-nitrosothiols (40–42), which may somehow have been involved in ONOO⁻-induced EGFR cross-linking. However, the addition of 1 mM S-nitroso-glutathione to subconfluent A431 cells, which would be ex-
were subsequently exposed to 1 mM ONOO\textsuperscript{−} to inhibit GSH synthesis (43) did not significantly affect the extent of EGFR cross-linking by ONOO\textsuperscript{−}. This latter finding further substantiates that ONOO\textsuperscript{−} causes EGFR cross-linking by direct receptor modification at the cell surface rather than by modifications of intracellular targets, which would be prevented by intracellular GSH.

The EGF receptor contains 15 tyrosine residues in its extracellular region, some of which are exposed and involved in ligand binding (44, 45) and hence may represent susceptible targets for oxidation by ONOO\textsuperscript{−}. A major product of reaction of ONOO\textsuperscript{−} with tyrosine residues is 3,3'-dityrosine, formed via combination of two tyrosyl radicals (3, 4); hence, EGFR could theoretically be covalently dimerized via intermolecular dityrosine cross-links. Proteins recovered from ONOO\textsuperscript{−}-treated A431 cells were found to have increased levels of 3,3'-dityrosine, and protein 3,3'-dityrosine content and the extent of EGFR cross-linking were both found to increase proportionately with increasing concentrations of ONOO\textsuperscript{−} (Fig. 4, A and B), consistent with the notion that EGFR cross-linking is due to intermolecular dityrosine formation. Moreover, irreversible EGFR cross-linking was also observed after incubation of A431 cells with 100 μM H\textsubscript{2}O\textsubscript{2} in the presence of HRP (Fig. 4C), or other heme peroxidases such as myeloperoxidase (not shown).

In the presence of these heme peroxidases, H\textsubscript{2}O\textsubscript{2} is known to be capable of oxidizing tyrosine via a one-electron mechanism to form 3,3'-dityrosine (46). In contrast, no detectable covalent EGFR cross-linking was observed in response to H\textsubscript{2}O\textsubscript{2} or HOCl, oxidants that are unable to cause significant oxidation of tyrosine to 3,3'-dityrosine. Collectively, these findings strongly suggest involvement of intermolecular 3,3'-dityrosine cross-links in covalent EGFR dimerization by ONOO\textsuperscript{−} and HRP/H\textsubscript{2}O\textsubscript{2}.

Formation of protein tyrosyl radicals was recently demonstrated in human blood plasma after reaction with ONOO\textsuperscript{−} using electron paramagnetic resonance spectroscopy (47). As protein tyrosyl radicals can be relatively long lived with half-lives of up to several minutes, protein cross-linking by combination of two protein tyrosine radicals is highly feasible.

Oxidant-induced EGFR Cross-linking Depends on Receptor Stimulation—Ligand-induced activation of EGFR is known to be mediated via receptor dimerization, which enhances ligand affinity (48–50). A431 cells release transforming growth factor-α as an endogenous ligand for EGFR (51), which stimulates autocrine receptor activation and cell proliferation. The formation of dimerized EGFR during receptor activation has been visualized with the use of chemical cross-linking agents such as disuccinimidyl suberate, EDAC, or glutaraldehyde (21, 52, 53). The addition of 10 mM EDAC to unstimulated A431 cells was found to result in cross-linking of a small fraction of the EGFR population, reflecting basally activated EGFR by autocrine receptor stimulation. Furthermore, SDS-PAGE analysis demonstrated that EDAC-cross-linked EGFR comigrates with the ONOO\textsuperscript{−}-induced EGFR dimer, and EGFR-cross-linking by ONOO\textsuperscript{−} and EDAC were found to be additive (Fig. 5A). These findings indicate that both agents act by covalently cross-linking ligand-induced EGFR dimers. Similar results were also obtained with A431 plasma membranes treated with ONOO\textsuperscript{−}, HRP/H\textsubscript{2}O\textsubscript{2}, or EDAC (not shown).

Increased EGFR dimerization by cell stimulation with EGF has been demonstrated with the use of various cross-linking agents (21, 52, 53) and was confirmed in the present study. The extent of ONOO\textsuperscript{−}-induced EGFR cross-linking was similarly found to be enhanced after pretreatment of A431 cells with 20 nM EGF (Fig. 5B). Additionally, significantly less EGFR cross-linking by ONOO\textsuperscript{−} was detected after prolonged serum starvation of A431 cells, which diminishes EGFR activation and autophosphorylation. Finally, experiments in which receptor-bound ligands were removed by acid wash treatment revealed that, whereas EGF-dependent receptor autophosphorylation and dimerization was diminished after acid wash treatment, ONOO\textsuperscript{−}-induced EGFR cross-linking could not be reversed (not shown).

Potential Effects on EGFR Activation—The apparent relationship between ligand-induced EGFR activation/dimerization and irreversible cross-linking by ONOO\textsuperscript{−} or HRP/H\textsubscript{2}O\textsubscript{2} suggests that these oxidant systems preferentially cross-link activated EGFR. Consistent with this notion, determination of the extent of EGFR tyrosine phosphorylation, by quantitation of immunostaining of monomeric and dimeric EGFR with either PY-20 or α-EGFR antibodies, demonstrated that covalently cross-linked EGFR, by either ONOO\textsuperscript{−} or HRP/H\textsubscript{2}O\textsubscript{2}, was more extensively tyrosine-phosphorylated than the monomeric form (Fig. 6). Similarly, covalently cross-linked EGFR using EDAC has also been found to have a higher degree of autophosphorylation and possesses higher tyrosine kinase activity than the monomeric form (21, 22). It is recognized, however, that this argument is based on assumed similar binding affinities of these antibodies to both the monomeric and dimeric form of EGFR.

To study potential changes in EGFR signaling by exposure of A431 cells to ONOO\textsuperscript{−}, we investigated the kinetics of EGFR phosphorylation as well as phosphorylation of a downstream substrate for EGFR, PLC-γ1. As demonstrated in Fig. 7, EGF-induced EGFR tyrosine phosphorylation displayed similar ki-
FIG. 6. Oxidant-induced covalent EGFR dimers are more extensively tyrosine-phosphorylated than monomeric EGFR. A431 cells were treated with 1 mM ONOO\(^{-}\) or with 100 \(\mu M\) H\(_2\)O\(_2\) and 1 \(\mu M\) HRP for 30 min. Cell lysates were subsequently analyzed by SDS-PAGE and Western blotting using either \(\alpha\)-phosphotyrosine (PY-20) or \(\alpha\)-EGFR (RK-2). Antibody binding to either monomeric or dimeric EGFR was quantified by densitometry, and the extent of EGFR phosphorylation was calculated as the ratio of PY-20 over RK-2 binding. Mean values and S.D. from three separate experiments are shown.

FIG. 7. ONOO\(^{-}\) alters kinetics of tyrosine phosphorylation of phospholipase C-\(\gamma\)-1. A, subconfluent A431 cells in 100-mm culture dishes were treated with 1 mM ONOO\(^{-}\) for 10 min (lanes 2, 4, and 6) and subsequently stimulated with 20 nM EGF for the indicated time periods. PLC-\(\gamma\)-1 was immunoprecipitated from A431 cell lysates and immunoblotted with either \(\alpha\)-phosphotyrosine (PY-20) or \(\alpha\)-PLC-\(\gamma\)-1. B and C, A431 cells were treated with ONOO\(^{-}\) and subsequently stimulated with 20 nM EGF for the indicated time period. The extent of tyrosine phosphorylation was assayed by densitometry, which was performed on Western immunoblots with PY-20 of PLC-\(\gamma\) (immunoprecipitated from A431 cell lysates; \(B\)) or EGFR (\(C\)), and normalized for immunostaining of either \(\alpha\)-PLC-\(\gamma\)-1 or \(\alpha\)-EGFR (RK-2). Results represent the average of two or three separate experiments.

Acknowledgments—We thank Drs. D. Cadena, G. N. Gill, J. Schlessinger, and J. Mendelsohn for providing the various \(\alpha\)-EGFR antibodies, and Carmen Leticia Lopez for technical assistance.

REFERENCES

1. Beckman, J. S., and Koppenol, W. H. (1996) Am. J. Physiol. 271, C1424–C1437
2. Pryor, W. A., and Squadrito, G. L. (1995) Am. J. Physiol. 268, L699–L722
3. van der Vliet, A., Eiserich, J. P., O’Neill, C. A., Halliwell, B., and Cross, C. E. (1995) Arch. Biochem. Biophys. 319, 341–349
4. Li, X., De Sarno, P., Song, L., Beckman, J. S., and Jope, R. S. (1998) Biochem. J. 331, 1055–1063
5. Beckman, J. S. (1996) Chem. Res. Toxicol. 9, 836–844
6. van der Vliet, A., Eiserich, J. P., Kaur, H., Cross, C. E., and Halliwell, B. (1996) Methods Enzymol. 269, 175–184
7. Giulivi, C., and Davies, K. J. A. (1994) Methods Enzymol. 233, 363–371
8. Leeuwenburgh, C., Rasmussen, J. E., Hsu, F. F., Mueller, D. M., Pennathur, S., and Heinecke, J. W. (1997) J. Biol. Chem. 272, 35220–35228
9. Riedl, J. F., and Vallee, B. L. (1992) Methods Enzymol. 225, 145–192
10. Miersza, S., and Chan, S. K. (1987) Biochem. J. 246, 37–42
11. Haddad, I. Y., Zhu, S., Ischiropoulos, H., and Matalon, S. (1996) Am. J. Physiol. 270, L281–L288
12. Kong, S.-K., Yim, M. B., Stadtman, E. R., Chock, P. B. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 3377–3382
13. Gow, A. J., Duran, D., Malcolm, S., and Ischiropoulos, H. (1996) FEBS Lett. 385, 63–66
14. Menders, T. H., Shafer, B. C., and Vostal, J. G. (1997) Faseb J. 11, 1281–1290
15. Li, X., De Sarno, P., Song, L., Beckman, J. S., and Jope, R. S. (1998) Biochem. J. 331, 599–606
16. Rappolee, D. A., Mark, D., Banda, M. J., and Werb, Z. (1988) Science 241,
