Epidermal Growth Factor (EGF)-induced Generation of Hydrogen Peroxide

ROLE IN EGF RECEPTOR-MEDIATED TYROSINE PHOSPHORYLATION*

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Yun Soo Bae‡, Sang Won Kang‡, Min Seok Seo‡, Ivan C. Baines‡, Ephrem Tekle†, P. Boon Chock†, and Sue Goo Ree‡**

From the ‡Laboratory of Cell Signaling, the §Laboratory of Cell Biology, and the ¶Laboratory of Biochemistry, NHLBI, National Institutes of Health, Bethesda, Maryland 20892

Recent evidence indicates that reactive oxygen species (ROS) may function as intracellular messengers in receptor signaling pathways. The possible role of ROS in epidermal growth factor (EGF) signaling was therefore investigated. Stimulation of A431 human epidermoid carcinoma cells with EGF resulted in a transient increase in the intracellular concentration of ROS, measured with the oxidation-sensitive fluorescent probe 2′,7′-dichlorofluorescein diacetate and laser-scanning confocal microscopy. The predominant ROS produced appeared to be H₂O₂, because the EGF-induced increase in fluorescence was completely abolished by incorporation of catalase into the cells by electroporation. The elimination of H₂O₂ by catalase also inhibited the EGF-induced tyrosine phosphorylation of various cellular proteins including the EGF receptor and phospholipase C-γ1. The dependence of H₂O₂ production on the intrinsic tyrosine kinase activity of the EGF receptor and the autophosphorylation sites located in its COOH-terminal tail was investigated. EGF failed to induce H₂O₂ generation in cells expressing a kinase-inactive EGF receptor. However, normal H₂O₂ generation was observed in cells expressing a mutant receptor from which the 126 COOH-terminal amino acids had been deleted to remove four (out of the total of five) autophosphorylation sites. These results suggest that EGF-induced H₂O₂ formation requires the kinase activity but probably not the autophosphorylation sites of the EGF receptor and that inhibition of protein tyrosine phosphatase activity by H₂O₂ may be required for EGF-induced protein tyrosine phosphorylation to be manifested. For example, hydrogen peroxide (H₂O₂) mimics the stimulatory effects of insulin on glucose transport and lipid synthesis in adipocytes (2, 3). Recently, the production of ROS has been detected in a variety of cells stimulated with cytokines such as transforming growth factor-β1 (4, 5), interleukin-1 (6), and tumor necrosis factor-α (6, 7), with peptide growth factors such as platelet-derived growth factor (PDGF) (8, 9) and basic fibroblast growth factor (7, 9), with agonists of receptors with seven transmembrane spans such as angiotensin II (10) and lysophosphatidic acid (11) or with phorbol ester (12).

The term ROS encompasses many species including singlet oxygen, the superoxide anion radical (O₂⁻), H₂O₂, lipid peroxides, nitric oxide, peroxynitrite (ONOO⁻), the thiyl peroxy radical (R₅O₂⁻), the ferryl radical (FeO₂⁺) and the hydroxyl radical (OH⁻) (13–16). However, the chemical nature of ROS generated in response to the activation of various receptors has not been well characterized. H₂O₂ was shown to be a major component of ROS in cells activated by transforming growth factor-β1 or PDGF (4, 8). The generation of ROS in response to various external stimuli has been related to the activation of transcription factors such as NF-κB (17) and AP-1 (7, 18), mitogen-activated protein (MAP) kinases (8, 11), and phospholipase A₂ (19) to the triggering of apoptosis (20), and to the inhibition of protein tyrosine phosphatases (PTPases) (21, 22). H₂O₂ is a small, diffusible, and ubiquitous molecule that can be synthesized, as well as destroyed, rapidly in response to external stimuli. As such it fulfills the important prerequisites for an intracellular messenger. We have now investigated the role of ROS in epidermal growth factor (EGF) signal transduction by the EGF receptor (EGFR) protein.

EXPERIMENTAL PROCEDURES

Materials—Bovine catalase was obtained from Boehringer Mannheim; Dulbecco’s modified Eagle’s medium (DMEM), modified Eagle’s medium without phenol red, fetal bovine serum (FBS), penicillin, and streptomycin were from Life Technologies, Inc.; enhanced chemiluminescence (ECL) reagents were from Amersham Corp.; antibodies to phosphotyrosine and the EGFR were from Upstate Biotechnology; antibodies to catalase and α-tubulin were from Calbiochem and Oncogene Science, respectively; protein A-Sepharose beads were from Pharmacia Biotech Inc.; and 2′,7′-dichlorofluorescein diacetate (DCFH-DA) was from Molecular Probes. A mixture of monoclonal antibodies that was used for immunoprecipitation of phospholipase C-γ1 (PLC-γ1) was prepared as described (23).

Cell Culture, Electroporation, and Stimulation with EGF—Human A431 epidermoid carcinoma cells were maintained at 37 °C under an atmosphere of 5% CO₂ in 150-mm dishes containing DMEM supplemented with 10% FBS. At 80–90% confluency, cells were deprived of serum for 24 h and then harvested by trypsin treatment. Harvested cells were suspended in serum-free DMEM at a density of ~1 × 10⁵ cells/ml, and 100-μl portions of the cell suspension were placed in an electroporation chamber in the absence or the presence of catalase (8 mg/ml). Electroporation was performed by subjecting cells to six pulses,
at intervals of 1 or 2 s, at a field strength of 850 ± 50 V/cm and a single-pulse width of 250 μs. Cell viability, assessed by trypan blue exclusion, was typically 70–80% after electroporation under these conditions. The construction, operation, and efficiency of the electroporation apparatus have been described previously (24). The electroporated cells were transferred to DMEM supplemented with 1% FBS and the same concentration of catalase as was present in the electroporation chamber and were placed in an incubator for 18 h.

For analysis of EGF-induced tyrosine phosphorylation, cells were stimulated with EGF (500 ng/ml) for 10 min and then exposed to lysis buffer (20 mM Hepes-NaOH (pH 7.2), 1% Triton X-100, 10% glycerol, 50 mM NaF, 1 mM Na3VO4, leupeptin (5 μg/ml), aprotonin (5 μg/ml), and 1 mM phenylmethylsulfonfyl fluoride). The lysates were incubated on ice for 5 min and then centrifuged for 5 min at 10,000 × g. Protein concentration of the supernatant was measured with the Bio-Rad detergent-compatible assay kit and bovine serum albumin as standard.

**Immunoprecipitation and Immunoblot Analysis**—The lyse supernatants were incubated with monoclonal antibodies to PLC-γ1 or to phosphotyrosine for 8 h, after which protein A-Sepharose beads were added, and the incubation was continued for an additional hour. The beads were pelleted at 10,000 × g for 5 min, washed three times with ice-cold lysis buffer, and subjected to SDS-polyacrylamide gel electrophoresis on an 8% gel. The separated proteins were transferred to a nitrocellulose membrane and probed with antibodies to PLC-γ1, to phosphotyrosine, or to EGFR. Immune complexes were detected with appropriate secondary antibodies and ECL reagents.

**Assay of Intracellular ROS**—Intracellular ROS production was measured by the method of Bass et al. (25) as modified for confocal microscopy by Ohba et al. (4). Briefly, dishes of confluent cells at various times after stimulation with EGF were washed with modified Eagle’s medium without phenol red and incubated in the dark for 5 min in Krebs-Ringer solution containing 5 mM DCFH-DA. DCFH-DA is a non-polar compound that readily diffuses into cells, where it is hydrolyzed to the nonfluorescent polar derivative DCFH and thereby trapped within the cells (25). In the presence of a proper oxidant, DCFH is oxidized to the highly fluorescent 2′,7′-dichlorofluorescein (DCF). Culture dishes were transferred to a Zeiss Axiovert 135 inverted microscope, equipped with a 20× Neofluor objective and Zeiss LSM 410 confocal attachment, and ROS generation was detected as a result of the oxidation of DCFH (excitation, 488 nm; emission, 515–540 nm). The effects of DCFH photo-oxidation was minimized by collecting the fluorescent image with a single rapid scan (line average, 4; total scan time, 4.33 s) and identical parameters, such as contrast and brightness, for all samples. The cells were then imaged by differential interference contrast microscopy. Five groups of 20–50 cells each were randomly selected from the image in the digital interference contrast (DIC) channel for each sample, the fluorescence intensity was then measured for each group from the fluorescence image, and the relative fluorescence intensity was taken as the average of the five values. Therefore, the relative fluorescence intensity (given in arbitrary units) reflects measurements performed on a minimum of 100 cells for each sample. All experiments were repeated at least three times.

**RESULTS**

Intracellular generation of ROS in A431 cells was measured with DCFH-DA and laser-scanning confocal microscopy. Exposure of quiescent A431 cells to EGF (500 ng/ml) resulted in a rapid increase in DCF fluorescence, with the maximal, 2-fold increase apparent 5 min after stimulation (Fig. 1); fluorescence had returned to the baseline value after 20 min. Introduction of catalase, an enzyme that specifically catalyzes the dismutation of H2O2 to O2 and H2O into A431 cells by electroporation, prevented EGF-induced DCFH oxidation (Fig. 2), suggesting that the latter is mainly mediated by H2O2. The amount of catalase incorporated into cells was about five times that of the endogenous enzyme (see below). The introduction of catalase also reduced DCFH oxidation in cells not exposed to EGF.

We next assessed the effect of incorporated catalase on EGF-induced tyrosine phosphorylation. EGF induced a rapid increase in tyrosine phosphorylation of several proteins in control cells as revealed by immunoblot analysis of cell lysates with antibodies to phosphotyrosine (Fig. 3A). Increased tyrosine phosphorylation of a broad band with an apparent molecular mass of ~160 kDa that was recognized by antibody to the EGFR was particularly prominent. However, in cells containing exogenous catalase, EGF had no apparent effect on tyrosine phosphorylation of the EGFR or other proteins. Phosphorylation of PLC-γ1, a well characterized target of the EGFR kinase, was studied further. Immunoblot analysis, with antibodies to PLC-γ1, of PLC-γ1 immunoprecipitated with antibodies to PLC-γ1 to immunoblot analysis with antibody to phosphotyrosine (Fig. 3C). Tyrosine phosphorylation of PLC-γ1 was apparent from control EGF-treated cells but not with that from EGF-treated cells containing exogenous catalase. As demonstrated previously (26, 27), coprecipitation of autophosphorylated EGFR with tyrosine-phosphorylated PLC-γ1 was observed. The requirement for H2O2 of the tyrosine phosphorylation of PLC-γ1 was also demonstrated by precipi-
EGFR using 125I-EGF. The binding of 125I-EGF was saturable with electroporation, we measured the number of receptors affected by electroporation or by the addition of catalase containing exogenous catalase. Whether this background DCFH oxidation is caused by nonphagocytic cells also generate ROS (4–12). To identify the ROS responsible for the intracellular oxidation of DCFH, Ohba et al. (4) added catalase to the culture medium of osteoblastic cells. The addition of catalase completely inhibited the transforming growth factor-β1-induced increase in DCF fluorescence, suggesting that H2O2 was important in DCFH oxidation in response to transforming growth factor-β1. Because the cells are not permeable to catalase, the researchers proposed that H2O2 was released into the medium from the plasma membrane, the site of its production, and then diffused into the cells. Whether this diffusion was promoted by the trapped DCFH is not clear. Catalase was also used to characterize the ROS generated in PDGF-treated rat vascular smooth muscle cells (8). Unlike most other cells, these muscle cells incorporated, by an unknown mechanism, catalase that was added to the culture medium. Catalase incorporation completely blocked the PDGF-stimulated increase in H2O2 production, suggesting that H2O2 is also the predominant ROS induced by PDGF in these cells.

We have now shown that EGF induces ROS production in A431 cells and that the increase in DCF fluorescence was primarily attributable to H2O2 on the basis of its sensitivity to catalase introduced into the cells by electroporation. With incorporation of catalase by electroporation, no ambiguity arises as to whether H2O2 is released first into the medium and is then taken up by the cells. Unlike rat vascular smooth muscle cells, the simple addition of catalase to the culture medium did not result in uptake of the enzyme and a consequent decrease in DCF fluorescence (data not shown). The incorporation of catalase also reduced DCFH oxidation in A431 cells not exposed to EGF, indicating that substantial amounts of H2O2 are generated in the absence of EGF, probably as a result of respiratory activity and the presence of various growth factors in 1% FBS. Overnight incubation of electroporated cells in medium containing 1% FBS was necessary for cells to recover from the electroporation procedure and to exhibit a tyrosine phosphorylation response to EGF. A high background of DCF fluorescence was apparent even in cells containing exogenous catalase. Whether this background DCFH oxidation is caused by...
Induced hydrolysis of phosphatidylinositol 4,5-bisphosphate. Treatment of smooth muscle cells with H$_2$O$_2$ was previously shown to release Ca$^{2+}$ from intracellular stores that are sensitive to inositol 1,4,5-trisphosphate (33). We also observed a rapid increase in intracellular Ca$^{2+}$ following addition of 1 mM H$_2$O$_2$ to A431 cells in a Ca$^{2+}$-free medium (figure not shown). These observations are consistent with the notion that H$_2$O$_2$ inhibits PTPases and thereby causes activation (tyrosine phosphorylation) of PLC-γ1 (see below).

Our study with EGF, together with previous studies with basic fibroblast growth factor (7, 9) and PDGF (8, 9), suggests that the generation of H$_2$O$_2$ is a common signaling event for peptide growth factors. However, the role of H$_2$O$_2$ in growth factor signaling is not clear. Exogenously added H$_2$O$_2$ was previously shown to elicit tyrosine phosphorylation in several cell types (34, 35), whereas inhibition of the PDGF-induced increase in H$_2$O$_2$ blocked various steps in signaling by this growth factor, including tyrosine phosphorylation of MAP kinase (8). Furthermore, H$_2$O$_2$ directly inhibits PTase activity in vitro, and this inhibition is completely reversed by incubation with dithiothreitol (21). All PTases contain one essential sulphydryl group at their active site that is susceptible to oxidation because of its unusually low $pK_a$ (<5) (36). These observations suggest that PTases may be targets of intracellularly generated H$_2$O$_2$. Inactivation of PTases would result in increased tyrosine phosphorylation. Furthermore, the specific activities of PTases in vitro are 10–1000 times those of protein tyrosine kinases (37). Therefore, in most cells, the activation of a receptor tyrosine kinase by the binding of a growth factor may not be sufficient to increase the steady-state level of protein tyrosine phosphorylation; concurrent inhibition of PTases might be necessary, and this inhibition may be achieved through H$_2$O$_2$.

Binding of various peptide growth factors to their cognate receptors activates multiple signaling pathways, including those mediated by PLC-γ1, phosphatidylinositol 3-kinase, signal transducer and activator of transcription protein (STAT), and MAP kinase (38, 39). The ligand-bound receptors dimerize and transphosphorylate each other at several tyrosine residues, thereby creating binding sites for cellular proteins that contain Src homology 2 (SH2) domains, including PLC-γ1, GTPase-activating protein of RAS, the 85-kDa subunit of phosphatidylinositol 3-kinase (p85), and SH2-containing collagen protein. Studies with autophosphorylation site mutants of the receptors for PDGF (40), colony-stimulating factor (41), fibroblast growth factor (42), and nerve growth factor (43) have shown that elimination of specific individual sites selectively abrogates the association of one or two SH2-containing proteins with the receptors, suggesting that individual autophosphorylation sites mediate the binding of specific SH2-containing proteins. However, the association of PLC-γ1, GTPase-activating protein, p85, or SH2-containing collagen protein with the EGFR, which contains five autophosphorylation sites (residues 992, 1068, 1086, 1148, and 1173) in the COOH-terminal region, does not appear to stringently require individual autophosphorylation sites, but decreases gradually as the sites are removed one by one by COOH-terminal truncation (44).

The intrinsic tyrosine kinase activity and autophosphorylation sites of the EGFR are not required for all signaling pathways activated by EGF. The activation of MAP kinase can occur independently of EGFR kinase activity (45, 46), and the activation of signal transducer and activator of transcription proteins requires none of the autophosphorylation sites (47). Our data with the kinase-inactive mutant indicate that EGFR-dependent H$_2$O$_2$ generation requires the intrinsic kinase activ-
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ity of the receptor. The CD-126 mutant receptor, which lacks four of the five autophosphorylation sites, was as effective as the wild-type receptor in inducing H$_2$O$_2$ generation, suggesting that the tyrosine phosphorylation sites of the EGFR are not required for this effect. However, the possibility that the remaining autophosphorylation site (Tyr$^{992}$) in CD-126 specifically serves as the binding site for a signaling molecule that mediates H$_2$O$_2$ generation cannot be excluded.

The mechanism by which H$_2$O$_2$ is generated has been studied extensively in neutrophils and macrophages (48). In these phagocytic cells, O$_2^-$ is first produced from the reduction of O$_2$ by a multicomponent NADPH oxidase-like system, which consists of a membrane-bound cytochrome b, a 22-kDa subunit (p22-phox), and a 90–110-kDa glycoprotein subunit (gp91-phox). In addition to these membrane-bound components, NADPH oxidase activity requires various cytosolic factors including SH3-containing proteins (p47-phox and p67-phox) and the small GTP-binding protein Rac.

The mechanism of O$_2^-$ and H$_2$O$_2$ generation in nonphagocytic cells is unknown. Neither the xanthine oxidase system nor the mitochondrial respiratory chain appears to mediate receptor-triggered ROS generation (6). Evidence suggests involvement of an NADPH oxidase-like system, which might be linked to the wild-type receptor in inducing H$_2$O$_2$ generation, suggesting that the tyrosine phosphorylation sites of the EGFR are not containing proteins (p47-phox and p67-phox) and the small GTP-binding protein Rac.

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