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EGF receptor–ligand interaction generates extracellular hydrogen peroxide that inhibits EGFR-associated protein tyrosine phosphatases

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Abstract

Hydrogen peroxide (H_2O_2) has been shown to be an important modulator of intracellular phosphatase activity involved in cell signaling pathways, including signaling by members of the receptor tyrosine kinase family of receptors such as the epidermal growth factor receptor (EGFR). Intracellular H_2O_2 can be generated by mitochondria-dependent pathways, whereas we recently showed that H_2O_2 could be generated extracellularly by receptor–ligand interaction. Here, we show that H_2O_2 produced by EGF–EGFR interaction can modulate the activity of intracellular protein tyrosine phosphatases (PTPs). Using purified proteins, we found that EGFR–ligand interaction generates H_2O_2 that is capable of inhibiting the activity of PTP1B in vitro. Furthermore, the addition of catalase rescued phosphatase inhibition consequent to EGF–EGFR interaction. Using cells that overexpress EGFR, we found that the addition of extracellular catalase prevented EGF-induced inhibition of EGFR-associated phosphatase activity. Our findings suggest that extracellular H_2O_2 generated by EGFR–ligand interaction permeates the plasma membrane and inhibits EGFR-associated tyrosine phosphatase activity, thereby modulating downstream signal transduction pathways.

Keywords: Receptor tyrosine kinase signaling; ROS; Phosphatase; Hydrogen peroxide; Redox; Epidermal growth factor receptor

The epidermal growth factor receptor (EGFR) is a member of the receptor tyrosine kinase (RTK) superfamily of receptors that possess intrinsic protein tyrosine kinase activity [1,2]. The EGFR was the first RTK discovered and it is an important regulator of cellular homeostasis, including the regulation of cellular growth, proliferation, and differentiation in normal and pathological states [1,3–6]. RTK signaling dysfunction by receptor overexpression or activating mutations can result in a multitude of serious diseases, including cancers such as breast carcinomas, squamous cell carcinomas, and glioblastomas [1,7].

Growth factor-dependent RTK signaling occurs through the activation of intrinsic kinase activity following ligand binding [1,6,8]. This signaling can be modulated by protein tyrosine phosphatases (PTPs) that counterbalance the actions of activated receptor kinases [9,10]. PTPs are receptor-like and non-receptor enzymes possessing highly conserved catalytic domains specific for phosphotyrosine hydrolysis, which make them attractive molecules in the regulation of RTK-mediated signal transduction [10,11]. PTPs contain essential cysteine residues in a signature active site motif [HC*XX*G*XX*R(S/T)] that must be in a reduced state for proper catalytic activity [9,12]. A key PTP in the regulation of EGFR-dependent signaling is PTP1B [10]. PTP1B is a ubiquitously expressed non-transmembrane

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tyrosine phosphatase that can bind to activated EGFR in vitro and has been shown to co-immunoprecipitate with EGFR in cells [13,14].

The EGFR has been shown to be modulated by numerous redox mechanisms in cells, including hydrogen peroxide (H_2O_2) [15]. PTP1B possesses a low pK_a active site cysteine 215 [16], rendering it sensitive to cellular reactive oxygen species (ROS) including H_2O_2 [17,18]. Specifically, it has been shown that H_2O_2 targets and can reversibly inactivate PTPs in cells that have been stimulated by peptide growth factors [9,17,19–21]. The mechanism of PTP1B inactivation by H_2O_2 involves the oxidation of the catalytic site cysteine 215 of PTP1B [22]. Cysteine 215 may therefore serve as a regulatory switch, whereby in the presence of ROS, the tyrosine phosphatase activity is reversibly inhibited, resulting in a modulation of RTK signal transduction.

PTP1B has been shown to be reversibly inactivated in A431 human epidermoid carcinoma cells stimulated with EGF [20]. It was also found in cells that H_2O_2 added extracellularly caused the reversible inactivation of PTPs [20]. We recently reported that receptor–ligand interaction, including EGF–EGFR, can generate extracellular H_2O_2 [23]. To extend our findings, we sought to elucidate a mechanism by which extracellular H_2O_2 generated by EGF–EGFR interaction may influence cell signaling pathways. Here, we show that receptor–ligand-generated extracellular H_2O_2 modulates the activity of PTPs that associate with the EGFR.

Materials and methods

Proteins. Recombinant human soluble EGFR (sEGFR; Research Diagnostics) was approximately 80% pure. Recombinant human EGF (R&D Systems) was >97% pure. Recombinant human granulocyte–macrophage-colony stimulating factor (GM-CSF) was a gift from Amgen, Inc. Bovine liver catalase was obtained from Sigma. Active recombinant human full-length PTP1B was obtained from Upstate Biotechnology.

H_2O_2 quantitation. H_2O_2 generated by receptor–ligand interaction was quantitated using a PeroxiLuminol Kit (World Precision Instruments) and Lumat LB9501 luminometer (Berthold). H_2O_2 was obtained from World Precision Instruments.

Cell culture and transfections. A431 (human epidermoid carcinoma) and 293T (human kidney epithelial) cells were grown in Dulbecco's modified Eagle's medium in high glucose supplemented with 10% heat-inactivated fetal bovine serum, 1% L-glutamine, and 1% penicillin/streptomycin. Transient transfections of 293T cells at ~30% confluence were carried out in 150-mm cell culture dishes using GeneJammer transfection reagent (Stratagene) and 8 μg EGFR cDNA. Human full-length EGFR was in the pUSE expression vector (Upstate Biotechnology).

Antibodies and immunoblotting. The following primary antibodies were used for immunoblotting and immunoprecipitation: anti-EGFR (rabbit polyclonal; Santa Cruz Biotechnology), anti-phospho-EGFR (Y-1173; rabbit polyclonal; Santa Cruz Biotechnology), and anti-PTP1B (mouse monoclonal, clone AE4-2J; EMD Biosciences/Calbiochem). For immunoblotting, proteins were resolved by SDS–PAGE using 7.5% ReadyCast gels (Bio-Rad), transferred onto nitrocellulose membranes (Bio-Rad), and incubated with appropriate primary and secondary antibodies. Proteins were visualized using Enhanced Chemiluminescence (ECL) Plus Detection Kit (Amersham Biosciences).

Protein tyrosine phosphatase assays. Activity of purified PTP1B (Upstate) was assayed with a RediPlate 96 EnzChek Tyrosine Phosphatase Assay Kit (Molecular Probes) and quantitated by fluorescence spectroscopy with a Fluoroskan Ascent FL fluorometer (Thermo-Labsystems). Excitation wavelength was 320 nm and emission wavelength was 460 nm. Tyrosine phosphatase activity associated with the EGFR was detected for A431 cells and 293T cells transiently transfected with EGFR. Cells were serum-starved overnight, washed with PBS, and pre-incubated for 10 min with or without catalase (3×10^3 U/mL) at 37 °C and then treated with or without EGF (1 μg/mL) for 5 min at 37 °C. Cells were harvested and washed in PBS, resuspended in tyrosine assay buffer (25 mM Hepes, pH 7.2, 50 mM NaCl, 5 mM DTT, and 2.5 mM EDTA; Upstate Biotechnology), and then sonicated. Cleared lysates were incubated overnight at 4 °C with 1 μg of anti-EGFR antibody. The next day, protein G beads, washed in PBS and resuspended in RediPlate tyrosine phosphatase reaction buffer (25 mM Mops, pH 7.0, 50 mM NaCl, 1 mM DTT, and 0.05% Tween 20), were added to each lysate and rocked for 2 h at room temperature. Beads were spun down, washed three times in PBS, and resuspended in RediPlate reaction buffer. Equal amounts of beads were then added to wells of a RediPlate and tyrosine phosphatase activity quantitated by fluorescence spectroscopy. Experiments with 293T cells used 358 nm excitation and 452 nm emission wavelengths on a Gemini fluorometer (Molecular Devices). Experiments with A431 cells used 320 nm excitation and 460 nm emission wavelengths on a Fluoroskan fluorometer. For A431 experiments, total phosphatase activity was normalized for protein content by adding beads to RediPlate wells containing 20 mM DTT.

Results and discussion

On the basis of our previous finding that EGFR–ligand interaction could generate extracellular hydrogen peroxide (H_2O_2) [23], we sought to examine the role of H_2O_2 in modulating the activity of phosphatases involved with EGFR signaling. First, we utilized purified EGF ligand and the sEGFR that possesses the extracellular binding domain of the receptor. We quantitated H_2O_2 production by EGF–sEGFR interaction in vitro using a luminometry-based assay. The PeroxiLuminol assay system determines H_2O_2 levels based on peroxidase-catalyzed luminol chemiluminescence. We found that at receptor and ligand concentrations of 500 nM, approximately 1.1 μM H_2O_2 was generated by cognate receptor–ligand interaction (Fig. 1A). Conversely, using GM-CSF as a non-binding ligand to the sEGFR, we observed no increase in H_2O_2 production as compared to receptor alone (Fig. 1A). Likewise, cognate EGF–sEGFR interaction resulted in approximately a 60% increase in H_2O_2 over that produced by the sum of EGF and sEGFR individually, whereas the incubation with GM-CSF and sEGFR generated no increase (Fig. 1B). On the basis of these findings, we examined whether H_2O_2 produced by receptor–ligand interaction could directly inhibit the activity of PTP1B in vitro. PTP1B is a phosphatase known to dephosphorylate the EGFR and serve as a modulator of EGFR-dependent signal

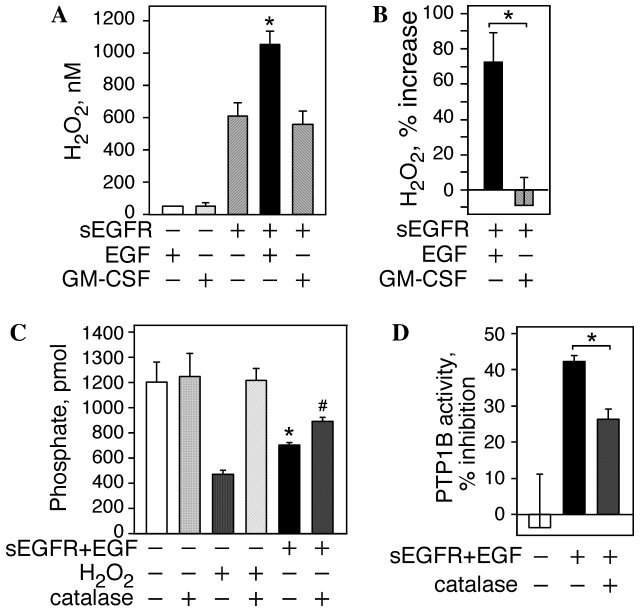

Fig. 1. EGF–sEGFR interaction generates H_2O_2 that inhibits phosphatase activity of PTP1B in vitro. (A) 500 nM of EGF, GM-CSF, and/or sEGFR were incubated for 30 min at room temperature and H_2O_2 quantitated by luminometry. (*) Statistically significant increase in mean H_2O_2 generation by EGF–sEGFR compared to sEGFR alone (one-tailed t test, $p < 0.01$). (B) Percentage increase in H_2O_2 production by EGF–sEGFR or GM-CSF–sEGFR compared to sEGFR alone, based on reaction conditions in (A). (*) Statistically significant difference in mean percentage increase in H_2O_2 generation by EGF–sEGFR compared to GM-CSF–sEGFR (one-tailed t test, $p < 0.01$). (C) Activity of PTP1B (1×10^{-2} U) incubated with 3×10^3 U/mL catalase, 500 nM H_2O_2, or 200 nM each EGF and sEGFR for 45 min at 37 °C. (*) Statistically significant decrease in mean PTP1B phosphatase activity by treatment with EGF–sEGFR compared to untreated control (one-tailed t test, $p < 0.001$). (#) Statistically significant rescue of mean phosphatase activity of PTP1B with the addition of catalase to EGF–sEGFR compared to treatment with EGF–sEGFR alone (one-tailed t test, $p < 0.0001$). (D) Percentage inhibition of PTP1B activity by receptor–ligand interaction in the presence or absence of catalase, based on reaction conditions in (C). (*) Statistically significant difference in mean percentage inhibition of PTP1B activity by treatment with EGF–sEGFR compared to treatment with EGF–sEGFR and catalase (one-tailed t test, $p < 0.0001$).

transduction [10,14,24]. Using a fluorescence-based tyrosine phosphatase assay kit, we found that EGFR–ligand interaction was sufficient to significantly inhibit PTP1B activity in a cell-free system (Fig. 1C). Furthermore, under this condition, PTP1B activity was partially rescued when catalase was included in the reaction, thereby confirming a role of H_2O_2 in the inactivation of the phosphatase (Fig. 1C). As a control, the addition of catalase to PTP1B did not alter its phosphatase activity, whereas 500 nM H_2O_2 inhibited 60% of PTP1B activity (Fig. 1C). After 45 min, incubation with EGF and sEGFR resulted in a 42% inhibition of PTP1B activity as compared to untreated PTP1B (Fig. 1D). The addition of catalase to EGF and sEGFR resulted in a partial rescue of PTP1B activity (Fig. 1D). The levels of phosphatase inhibition by EGF–sEGFR correlate with the quantities of H_2O_2 generated by the receptor–ligand interaction in vitro, as described above.

To extend our in vitro findings with purified proteins, we transiently transfected 293T cells with the cDNA encoding the full-length EGFR. Treatment with EGF ligand resulted in a rapid phosphorylation of the receptor, which could be markedly inhibited by pre-incubation with exogenously added catalase (Fig. 2A). As catalase does not cross the plasma membrane, this strongly implicates a role of extracellular hydrogen peroxide in the early steps in EGFR-mediated signal transduction. We next sought to determine whether the receptor–ligand-generated extracellular H_2O_2 was capable of inhibiting phosphatases associating with the EGFR. Cells (293T) transiently transfected with EGFR cDNA were treated with EGF ligand and/or exogenously added catalase, and cell lysates were immunoprecipitated with anti-EGFR antibody. Using the RediPlate in vitro assay system for the detection of PTP activity, protein G beads incubated with immunoprecipitated EGFR-associated proteins were added to 96-well RediPlate plates coated with phosphate-releasing substrate and tyrosine phosphatase activity quantitated by fluo-

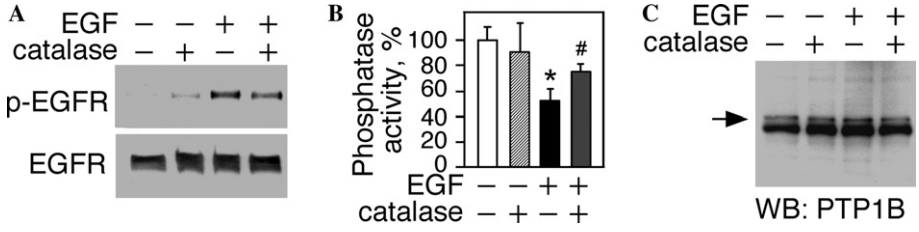

Fig. 2. Extracellular H_2O_2 generated by EGF treatment in 293T cells overexpressing EGFR is involved in early EGFR-dependent signaling events. (A) 293T cells expressing EGFR were pre-incubated with or without catalase (3×10^3 U/mL) for 10 min at 37 °C, then treated for 5 min with or without EGF (1 μg/mL). Phosphorylated (p-EGFR, upper panel) and total EGFR (EGFR, lower panel) levels in cell lysates were detected by immunoblotting. (B) Lysates prepared following treatment as in (A) were immunoprecipitated overnight with anti-EGFR antibody and bound to protein G beads. Tyrosine phosphatase activity associated with protein G beads was quantitated by fluorescence spectroscopy following incubation for 45 min at 37 °C. (*) Statistically significant decrease in mean tyrosine phosphatase activity by treatment with EGF compared to untreated control. (#) Statistically significant rescue of mean tyrosine phosphatase activity by treatment with EGF and catalase compared to treatment with EGF alone (one-tailed t tests, $p < 0.05$). (C) Levels of PTP1B associated with EGFR in cell lysates prepared following treatment as in (A) were detected by immunoblotting following immunoprecipitation with anti-EGFR antibody.

rescence spectroscopy. As shown in Fig. 2B, treatment of cells with EGF resulted in nearly 50% reduction in EGFR-associated phosphatase activity as compared to untreated control. The reduction in EGFR-associated phosphatase activity could be rescued by pre-incubation with exogenously added extracellular catalase (Fig. 2B). Immunoblotting was performed with antibody against PTP1B protein following immunoprecipitation with anti-EGFR antibody. We detected an approximately 50 kDa protein consistent with published data for the molecular weight of PTP1B [14], which confirms that PTP1B is associated with the EGFR and that its levels are not altered by ligand addition or by pre-incubation with exogenous catalase (Fig. 2C). Since catalase does not permeate the cell, these results indicate that extracellular H_2O_2 plays a role in the inactivation of receptor-associated tyrosine phosphatases. Taken together, our findings from in vitro and cell-based assays support the thesis that H_2O_2 generated extracellularly by EGF–EGFR interaction permeates the cell leading to the inactivation of EGFR-associated tyrosine phosphatases that may include PTP1B.

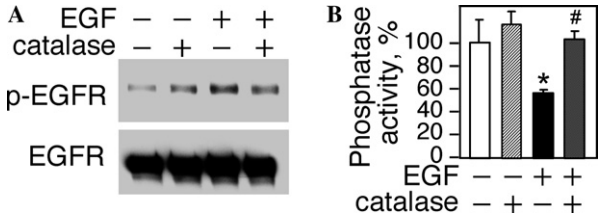

Fig. 3. Extracellular H_2O_2 generated by EGF treatment in A431 cells participates in early EGFR-dependent signaling events. (A) A431 cells were pre-incubated with or without catalase (3×10^3 U/mL) for 10 min at 37 °C, and then treated for 5 min with or without EGF (1 μg/mL). Phosphorylated (p-EGFR, upper panel) and total EGFR (EGFR, lower panel) levels in cell lysates were detected by immunoblotting. (B) Lysates prepared following treatment as in (A) were immunoprecipitated overnight with anti-EGFR antibody and bound to protein G beads. Tyrosine phosphatase activity associated with protein G beads was quantitated by fluorescence spectroscopy following incubation for 2.5 h at 37 °C. Phosphatase activity was normalized based on total phosphatases. (*) Statistically significant decrease in mean tyrosine phosphatase activity by treatment with EGF compared to untreated control. (#) Statistically significant rescue of mean tyrosine phosphatase activity by treatment with EGF and catalase compared to treatment with EGF alone (one-tailed t tests, $p < 0.05$).

We next sought to extend our findings with EGFR-transfected 293T cells to the A431 lung epidermoid carcinoma cell line that highly expresses the EGFR. Similar to our results for 293T cells, treatment of A431 cells with EGF resulted in the phosphorylation of the EGFR, which could be inhibited by exogenous catalase (Fig. 3A). We immunoprecipitated EGFR-associated phosphatases and found that incubation with EGF resulted in an approximate 40% decrease in phosphatase activity by EGFR-associated phosphatases (Fig. 3B). Incubation with exogenously added catalase prior to EGF treatment prevented the inhibitory activity of EGF incubation. In order to control for the levels of phosphatase in the various treatment groups, we measured total phosphatase activity by incubating the reactions in reaction buffer containing 20 mM DTT.

As hydrogen peroxide has been shown to inactivate phosphatases by modifying conserved active site cysteine residues, co-incubation with DTT results in a strong reducing environment that restores phosphatase activity to baseline levels. We found that pre-incubation with exogenously added catalase restored phosphatase activi-

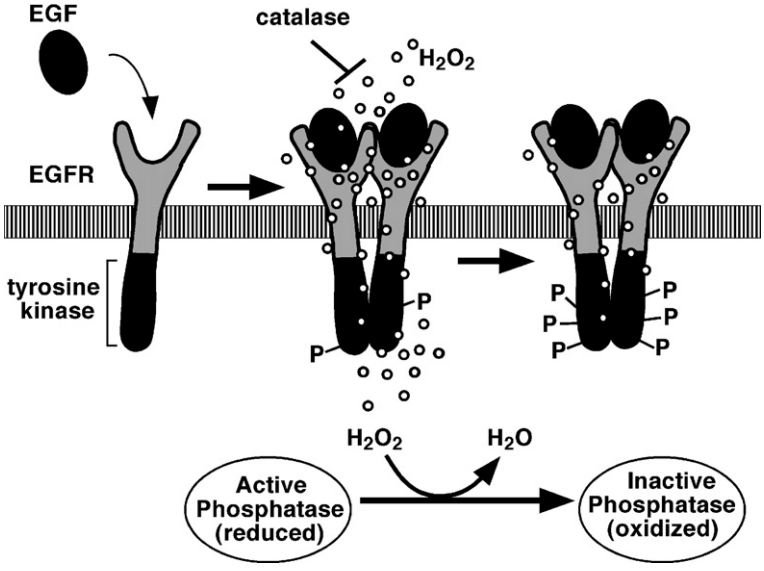

Fig. 4. Proposed schematic model of the role of extracellular H_2O_2 in early signaling events mediated by EGFR. EGF–EGFR interaction generates H_2O_2 that diffuses across the plasma membrane and inactivates EGFR-associated phosphatases, thereby modulating EGFR-dependent signal transduction cascades.

ty to baseline levels. Since catalase cannot internalize into the cell through the cell membrane, based on our results, we postulate that H_2O_2 is generated extracellularly.

Taken together, our findings support the thesis that H_2O_2 generated extracellularly by receptor–ligand interaction, may regulate EGFR-mediated downstream signaling events. The mechanism by which extracellularly generated H_2O_2 influences signaling may involve, at least in part, the inactivation of PTPs that associate with the receptor. These data further support the concept that receptor–ligand interaction generates extracellular H_2O_2 that crosses the plasma membrane where it locally inactivates receptor-associated phosphatases, thereby modulating cell signaling (Fig. 4).

Acknowledgments

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