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Assessment of Redox Changes to Hydrogen Peroxide-Sensitive Proteins During EGF Signaling

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Abstract

Hydrogen peroxide acts as a second messenger in growth factor signaling where it can oxidize and modify the function of redox-sensitive proteins. While selective thiol oxidation has been measured, there has been no global assessment of protein oxidation following growth factor activation. Significant changes to the abundant and widely distributed redox sensitive thiol proteins were observed in A431 epidermoid carcinoma cells exposed to hydrogen peroxide, but no changes were observed following treatment with epidermal growth factor (EGF). This included members of the peroxiredoxin family, which were also monitored in the presence of the thioredoxin reductase inhibitor auranofin to limit their capacity to recycle to the reduced form. We conclude that widespread thiol oxidation does not occur in cells during EGF signaling, and that hydrogen peroxide must act in a highly localized or selective manner. *Antioxid. Redox Signal.* 15, 167–174.

Introduction

Signaling through the epidermal growth factor receptor (EGFR) is a key regulator of cell survival and proliferation. Various growth factor signaling models invoke hydrogen peroxide as a second messenger, which acts via oxidation of redox-sensitive signaling proteins. For example, the phosphatases PTEN and PTP1B contain an invariant cysteine residue essential for activity, and have been shown to undergo partial oxidation in cells treated with growth factors (23, 25, 27, 29, 32) . Such oxidative inactivation should promote phosphorylation and provides a mechanism for signal transduction. The most likely oxidant source is membrane-associated NADPH oxidases (NOX). This family of enzymes catalyzes the one electron reduction of molecular oxygen to form superoxide which can then dismutate to hydrogen peroxide (5). Increased generation of reactive oxygen species has been reported in multiple cell lines stimulated with peptide growth factors (1, 2, 7, 8, 13, 14, 20, 38, 41).

Hydrogen peroxide reacts with the cysteine thiolate anion, and selectivity for specific cysteines can be achieved in part through the local protein microenvironment lowering the pKa of the target cysteine (47). However, the rate constants for reaction of hydrogen peroxide with low pKa thiol proteins such as protein tyrosine phosphatases (PTPs) are still low relative to specialized and abundant hydrogen peroxide scavengers such as the glutathione peroxidases and peroxiredoxins (Prxs) (44), and it is unresolved how these less reactive proteins become oxidized during growth factor signaling. Possible explanations include a combination of a localized site of oxidant action and modification of Prxs by hyperoxidation or phosphorylation (33, 45, 46).

Although small changes in the redox state of thiol proteins such as PTPs (23, 25, 29), Trx 1 (16, 18), and the selenoprotein TrxR (40) have been detected during growth factor signaling, studies have not been carried out to determine how these relate to more global changes in thiol protein redox state. A selection of methods is available to visualize global redox changes in cells (3, 24, 26), and some thiol proteins, including Prxs and GAPDH, have been shown to be sensitive to small amounts of exogenous hydrogen peroxide (4, 10, 19, 28, 35, 39). These would be expected to be among the most sensitive to change if low levels of hydrogen peroxide were generated freely within the cell. We have therefore investigated whether oxidation of hydrogen peroxide-sensitive proteins is detectable in A431 epidermoid carcinoma cells after exposure to epidermal growth factor (EGF), using a gel-based proteomic approach and a Western blotting method to investigate peroxiredoxins.

Materials and Methods

Materials

EGF, urea, thiourea, anti-rabbit HRP and anti-Prx 2 antibody were from Sigma Chemical Company (St. Louis, MO). Tris HCl, Plus One urea, Plus One glycine, Plus One bromophenol blue, and anti-mouse HRP was from GE Healthcare (Buckinghamshire, UK). Anti-Prx 1 was from Abcam (Cambridge, UK). Anti-Prx 3 and anti-Trx 1 were from AbFrontier

Free Radical Research Group and National Research Centre for Growth and Development, Department of Pathology, University of Otago, Christchurch, New Zealand.

(Seoul, Korea). Anti-phosphotyrosine (clone PY20) was from Zymed Laboratories (San Francisco, CA). Dichlorodihydrofluorescein (DCF) diacetate and 5-iodoacetamidofluorescein (IAF) were from Invitrogen (Carlsbad, CA). Micro Bio-Spin 6 desalting columns, 3–10 ampholytes, 11 cm 3–10 IPG strips, and 18% Criterion gels were from Bio-Rad (Hercules, CA). Complete Protease Inhibitor and PhosStop phosphatase inhibitor were from Roche (Mannheim, Germany). The EGFR kinase inhibitor PD158780 was from Calbiochem (La Jolla, CA). Hydrogen peroxide was from LabServ (Victoria, Australia). Auranofin was from ICN Biomedicals Inc. (Costa Mesa, CA). Mal-PEG (α-methoxy-ω-ethyl-maleinimide poly(ethylene glycol)) (PEG1147) was from Iris Biotech (Marktredwitz, Germany).

Cell culture

The A431 epidermoid carcinoma cell line was obtained from the American Tissue Culture Collection (Rockville, MD) and maintained in high glucose DMEM (#12430, Invitrogen) supplemented with 10% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin. Media was replaced on cells at 70%–80% confluence an hour prior to treatment with EGF.

DCF assay

A431 cells at 70%–80% confluence in 6-well plates were preloaded with 10 μM DCF-diacetate for 10 min, in the presence or absence of 100 n*M* PD158780. Cells were treated with 500 ng/ml EGF for 30 min, washed with phosphate buffered saline (PBS; 140 m*M* NaCl, 13 m*M* KCl in 10 m*M* sodium phosphate buffer, pH 7.4) and lysed with 300 μl RIPA buffer (50 m*M* Tris-HCl pH 7.4, 150 m*M* NaCl, 2 m*M* EDTA, 1% sodium deoxycholate, 1% Triton X-100, 0.1% sodium dodecyl sulfate, and Complete™ Protease Inhibitors). 250 μl of whole cell lysate was transferred to a 96-well black fluorescence plate and the fluorescence read using a POLARstar fluorescent plate reader (BMG Labtechnologies Pty. Ltd. Mt Eliza, Australia). Cells were also examined by visualization of fluorescence microscopy images.

Glutathione assay

Reduced glutathione was assayed using monobromobimane as described previously (9).

2D SDS-PAGE

Oxidized thiol proteins were monitored as described previously (3, 12). Briefly, A431 cells were lysed in NEM buffer (40 m*M* HEPES, 50 m*M* NaCl, 1 m*M* EDTA, 1 m*M* EGTA, pH 7.4, 1% Triton X-100, Complete ™ Protease Inhibitors) containing 100 m*M* *N*-ethyl maleimide (NEM). Excess NEM was removed using a desalting column, and oxidized thiols were reduced with 1 m*M* dithiothreitol (DTT) for 10 min. Newly reduced thiols were labeled with 200 μM IAF for 10 min. It has been established that this procedure is effective at blocking intracellular thiol proteins and preventing oxidation on lysis of the cells (3), and that labeling with IAF is effective in the presence of DTT (15).

For 1D SDS-PAGE, excess IAF was removed by acetone precipitation and resultant proteins were separated using 18% Criterion gels, and imaged using a Bio-Rad Molecular Imager® FX (Bio-Rad Laboratories) at excitation 488 nm/emission 530 nm. For 2DE, excess IAF was removed using a desalting column with buffer exchange into rehydration buffer and the resultant protein was rehydrated overnight onto an 11 cm 3–10 immobilized pH Gradient (IPG) strip in 200 μl rehydration buffer (7 *M* urea, 2 *M* thiourea, 10 m*M* DTT, 1% Triton X-100, 0.2% Biolytes). The strip was focused for 45 kVh and then separated on an 18% Criterion gel and scanned as described above. Gel images of resolved IAF-labeled thiol proteins were analyzed with PDQuest™ version 8.0 build 035 2-D electrophoresis gel analysis software (Bio-Rad Laboratories).

Redox Western analysis of thioredoxin 1

The redox state of Trx 1 was assessed using the cysteine-reactive probe Mal-PEG (17, 30) with minor modifications. Briefly, A341 cells were grown and treated in 6-well plates, then lysed in 100 μl NEM buffer. Samples were clarified by centrifugation and excess NEM was removed by passing 80 μl through a desalting spin column pre-equilibrated with EB-Tx (40 m*M* HEPES, 50 m*M* NaCl, 1 m*M* EDTA, 1 m*M* EGTA, pH 7.4, 1% Triton X-100, Complete ™ Protease Inhibitors). Samples were reduced using 1 m*M* DTT, and newly reduced thiols labeled using 25 m*M* Mal-PEG. The samples were then acetone-precipitated to remove excess Mal-PEG and proteins were separated by SDS-PAGE. Gels were transferred to PVDF, probed with an anti-Trx 1 rabbit polyclonal antibody, and quantified using ECL detection.

Detection of the redox state of cellular peroxiredoxins

The redox state of the Prxs 1 and 2 was assessed as described previously (11). The 2-Cys Prxs have three detectable redox states: reduced monomer, disulfide-linked dimer, and hyperoxidized monomer. These can be monitored via two types of Western blot. Oxidizedimer can be separated from reduced and hyperoxidized monomer by nonreducing SDS-PAGE after lysis in the presence of NEM. Hyperoxidized monomer can be quantified when cells are lysed in the absence of NEM, which results in conversion of the reduced monomer to the dimer.

Following treatment, A431 cells were washed with PBS, then lysed in EB-Tx buffer containing PhosStop phosphatase inhibitor, with or without NEM, and incubated for 15 min. Protein extracts were combined with nonreducing sample loading buffer, resolved by SDS-PAGE, and transferred to PVDF membrane. Immunoblot analysis was then performed using polyclonal antibodies against either phosphotyrosine (1:3000 in 3% BSA containing 1mM sodium orthovanadate (phosphatase inhibitor)), Prx 1, or Prx 2 (both 1:10,000 in 3% nonfat dry milk). Immunoreactivity was visualized using a peroxidase system with enhanced chemiluminescence (ECL) (GE Healthcare). Quantity One® software (BioRad) was used to quantify the densitometry of scanned images.

Results

EGF-triggered tyrosine phosphorylation and oxidant production in A431 cells

The epidermoid carcinoma cell line A431 is enriched with EGF receptor (EGFR) and triggers phosphorylation cascades when stimulated with EGF, the amplitude of which is both dose and time dependent. To establish that our system gave

the expected responses to EGF, we first measured phosphorylation and changes in the redox environment. Dose-dependent tyrosine phosphorylation of multiple bands was observed (Fig. 1A). This occurred within 1 min and decreased after 10 min (Fig. 1B). Phosphorylation was specific to signaling via the EGF receptor (EGFR) as it was completely inhibited by the EGFR kinase inhibitor PD158780 (Fig. 1C). The redox environment was monitored by preloading the cells with DCF diacetate, which hydrolyses and becomes fluorescent when oxidized. There was a 1.7 fold increase in the oxidation of DCF after stimulation with 500 ng/mL EGF for 30 min (Fig. 2), as visualized by fluorescence microscopy and quantified by fluorescence spectroscopy ($p < 0.001$). The EGFR kinase inhibitor PD158780 inhibited DCF oxidation (Fig. 2). DCF oxidation involves the formation of an intermediate radical that can be quenched by thiols (42, 43). To determine if increased DCF oxidation could be due to loss of cellular thiols we monitored reduced glutathione levels 30 min after EGF treatment. No significant difference was observed (EGF 97 ± 4% of control).

Monitoring redox-sensitive thiol proteins in EGF-treated cells

Previous studies have shown that the thiol-reactive probe IAF can be used to visualize subtle changes in reversibly oxidized thiol proteins following treatment of cells with hydrogen peroxide (4). We used this technique with A431 cells in which reduced thiols had been blocked with the alkylating agent NEM. Reversibly oxidized proteins were reduced and labeled with IAF. Initially, hydrogen peroxide sensitive proteins that could be detected in the < 50 kD region of 1D SDS-PAGE were visualized by treating the cells with 0–500 μM hydrogen peroxide for 5 min. As shown in Figure 3, each band represents a protein containing an oxidized protein thiol. Several changes were noted (open arrows), even with 50 μM hydrogen peroxide, including a band in the position of glyceraldehyde-3-phosphate dehydrogenase (GAPDH; solid arrow), which is known to be particularly sensitive to hydrogen peroxide (identified in Ref. 4). The changes became more evident at higher concentrations. In contrast, there were no detectable changes in intensity of these bands following EGF treatment for up to 60 min (Fig. 3).

In order to observe a greater range of sensitive thiol proteins, the lysates were separated by 2D electrophoresis. Approximately 450 protein spots were detected in each sample, comparable to that previously reported in Jurkat T-lymphoma cell lysates (3). A high hydrogen peroxide concentration was used as reference to show clearly where peroxide-mediated changes might be expected. Multiple changes in spot intensity are evident (Supplementary Fig. S1; Supplementary Data are available at www.liebertonline.com/ars). A 5 min treatment with EGF was selected to coincide with maximum tyrosine phosphorylation and capture thiol oxidation before any transient changes could be reversed. There were no obvious changes to any of spots that run in the GAPDH or Prx regions, or indeed any of the other proteins that changed with hydrogen peroxide (Fig. 4A and Supplementary Fig. S2). PDQuest software was used to determine if there were statistically significant changes in any spots on three replicate sets of gels from independent treatments of the cells. With hydrogen peroxide, over a quarter of the spots changed in fluorescence intensity. In comparison, only one spot changed consistently between control and EGF-treated cells (spot A), with a 1.7 fold increase in intensity ($p = 0.027$, (Figs. 4B and 4C). Spot A also increased in intensity with hydrogen peroxide. It was excised from the gel and identified as Trx 1 by mass spectrometry (Fig. 5A).

Thioredoxin redox changes

Western blotting confirmed that Trx 1 was present at the identical site that spot A was excised. Trx 1 protein was present as two IAF-labeled spots (Fig. 5B), of which spot A was only a small proportion in control and EGF-treated cells (Fig. 4). Increased spot intensity could be due to increased IAF incorporation (oxidation) or a post-translational modification that caused a shift from a different region of the gel. To determine if Trx 1 oxidation occurred during EGF signaling in A431 cells, we used redox Western blotting to analyze its oxidation states. In this method, oxidized thiols are reduced and labeled with Mal-PEG, which adds 2 kDa in molecular weight to each cysteine. Reduced Trx (position 0) runs towards the bottom of the gel, with increasing levels of oxidation laddering up the gel. A431 cells treated with 2 mM diamide gave much higher proportions of the slower migrating bands (position 4), indicating extensive Trx oxidation, while 1 mM hydrogen peroxide increased the oxidation state of Trx 1 from primarily the 0 position to an even split between the +2 and +3 position. In contrast, there was no observable change following EGF treatment (Fig. 5C), suggesting that any Trx oxidation would involve a minor fraction of the total protein.

Peroxiredoxin redox changes

As peroxiredoxins have been shown to be highly sensitive to oxidation in cells treated with hydrogen peroxide (10, 39), we looked specifically at the oxidation state of Prx 1 and Prx

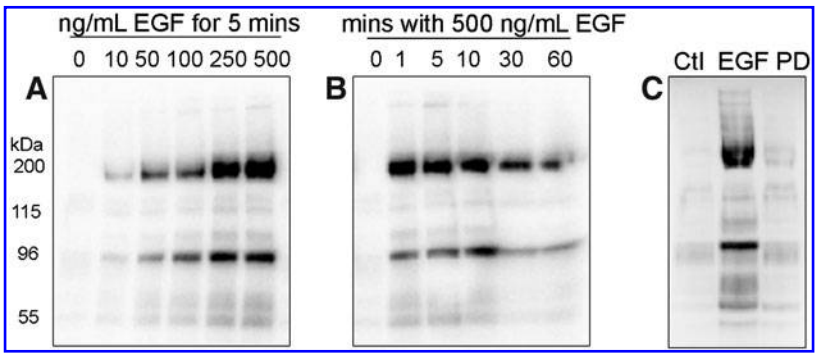

FIG. 1. Epidermal growth factor-induced tyrosine phosphorylation in A431 cells. (A) A431 cells were stimulated with 0–500 ng/ml EGF for 5 min, then Western blotted for phosphotyrosine. **(B)** A431 cells were stimulated with 500 ng/mL EGF for 0–60 min, then Western blotted for phosphotyrosine. **(C)** A431 cells were stimulated with 500 ng/mL EGF for 5 min, or pretreated with 100 nM EGFR kinase inhibitor PD158780 for 10 min and then stimulated with 500 ng/ml EGF for 5 min and Western blotted for phosphotyrosine.

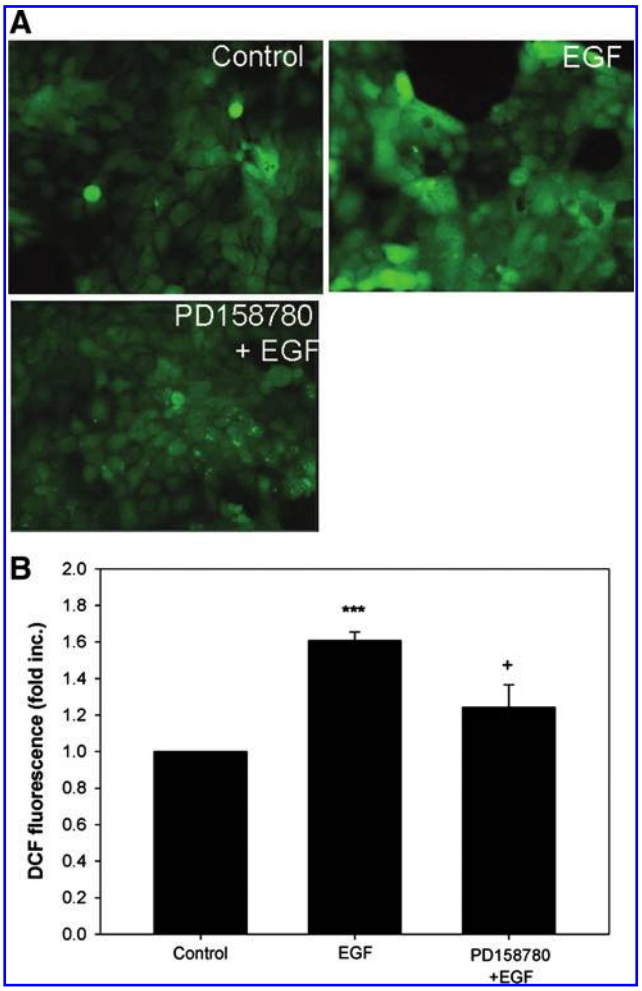

FIG. 2. Increased hydrogen peroxide after EGF treatment of A431 cells. A431 cells were pretreated with 10 μM DCF-DA in the presence or absence of the EGFR kinase inhibitor PD158780 (100 n*M*) for 10 min, then treated with 500 ng/ml EGF for 30 min and fluorescence intensity measured by **(A)** fluorescence microscopy, or **(B)** fluorescent plate reader. (***Control vs. EGF, $p < 0.001$; + Ctrl vs. PD158780 and EGF, p = n.s., ANOVA with Holm–Sidak, $n = 7$–9). (To see this illustration in color the reader is referred to the web version of this article at www.liebertonline.com/ars).

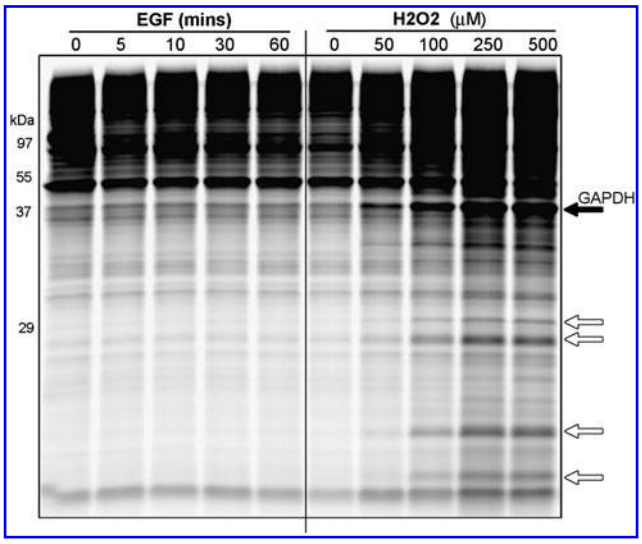

FIG. 3. 1D redox proteomics of EGF signaling and sensitivity to hydrogen peroxide. A431 cells were treated with either 500 ng/ml EGF for 0–60 min, or 0–500 μM hydrogen peroxide for 5 min. Cells were then lysed in NEM buffer, oxidized protein thiols labeled with IAF, separated by SDS-PAGE, and imaged. Increasing band intensity indicates increasing protein thiol oxidation (*solid arrow* = the peroxide sensitive protein GAPDH, *white arrows* = other hydrogen peroxide sensitive proteins).

2 in EGF-treated A431 cells. These are cytoplasmic typical 2-Cys Prxs that undergo oxidation to disulfide-linked dimers or to hyperoxidized sulfinic acid monomers. These changes can be followed as molecular weight shifts by nonreducing SDS-PAGE and Western blotting (11). In many cell types dimers do not accumulate and hyperoxidation is seen. Hyperoxidation of specific Prxs can be observed by preparing cell extracts in the absence of an alkylating agent, resulting in oxidation of all reduced Prxs to a dimer, and retention of hyperoxidized Prxs as monomers (11). Hyperoxidation of Prx 1 and Prx 2 was evident in A431 cells treated with 10–25 μM hydrogen peroxide (Fig. 6). This was detected by Western blotting under conditions where only the hyperoxidized form ran as a monomer (Fig. 6A), and with lower sensitivity using an antibody against the hyperoxidized form (Fig. 6B). However, treatment with EGF caused no detectable hyperoxidation of Prx 1 or Prx 2 either at 500 ng/ml for 5 min (Fig. 6C) or at other times up to 60 min (data not shown).

There was also no increase in Prx 1 or Prx 2 dimer formation after EGF treatment (Fig. 6C). As shown for Prx 1 (Fig. 6D), this was the case even when the cells were treated with auranofin to inhibit thioredoxin reductase, and impede reduction of Prx dimers by Trx. Although dimerization was not seen with hydrogen peroxide alone (Figs, 6C and 6D), dimers did accumulate when recycling was inhibited with auranofin (Fig. 6D) Auranofin itself did not cause oxidation under these conditions.

Discussion

The transient oxidation of a limited number of proteins has been reported during growth factor signaling, but no global assessment of thiol protein oxidation patterns has been undertaken. In this study, we explored oxidation in A431 cells after treatment with EGF, and related these changes to those detectable with hydrogen peroxide. Consistent with other studies, we observed rapid protein phosphorylation and DCF oxidation following EGF stimulation. However, this did not correspond with significant changes to hydrogen peroxide-sensitive thiol proteins, including GAPDH and Prxs. The thioredoxin reductase inhibitor auranofin was used to limit the cycling capacity of Prxs, but even then there was no increase in Prx oxidation. This is consistent with the absence of Prx hyperoxidation in cells treated with PDGF (8, 45). These results support the hypothesis that thiol protein oxidation following EGF stimulation is a localized process that does not spread throughout the cell.

Our conclusions are limited to the A431/EGF model and we can not dismiss the possibility that differences may be observable with other growth factors or in different cell types.

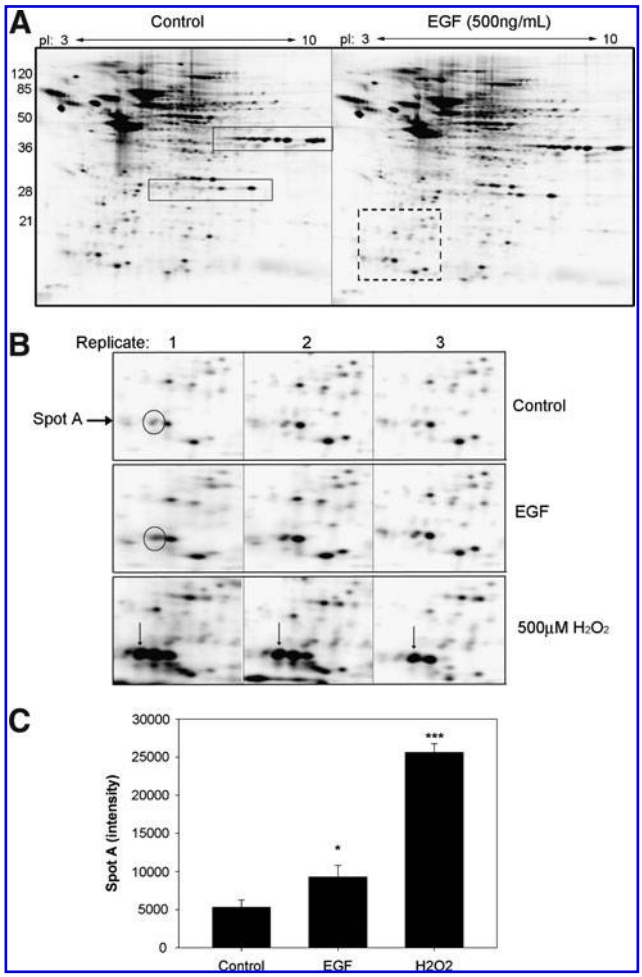

FIG. 4. 2D redox proteomics of EGF signaling. (A) A431 cells were treated with 500 ng/ml EGF for 5 min, then lysed in alkylating buffer and prepared for redox 2D-PAGE (as described in *Materials and Methods*. *Solid line boxes*, hydrogen peroxide-sensitive proteins GAPDH and Prxs; *dashed line box*, Spot A. Overlay is shown in Supplementary Fig. S2. **(B)** Enlargement of Spot A (*circled*) in replicate control, EGF, and hydrogen peroxide gels. **(C)** Quantification of IAF fluorescence of Spot A between control, EGF, and hydrogen peroxide treated cells. ($p = 0.027$, One-way repeated measures ANOVA with Holm–Sidak, $n = 3$).

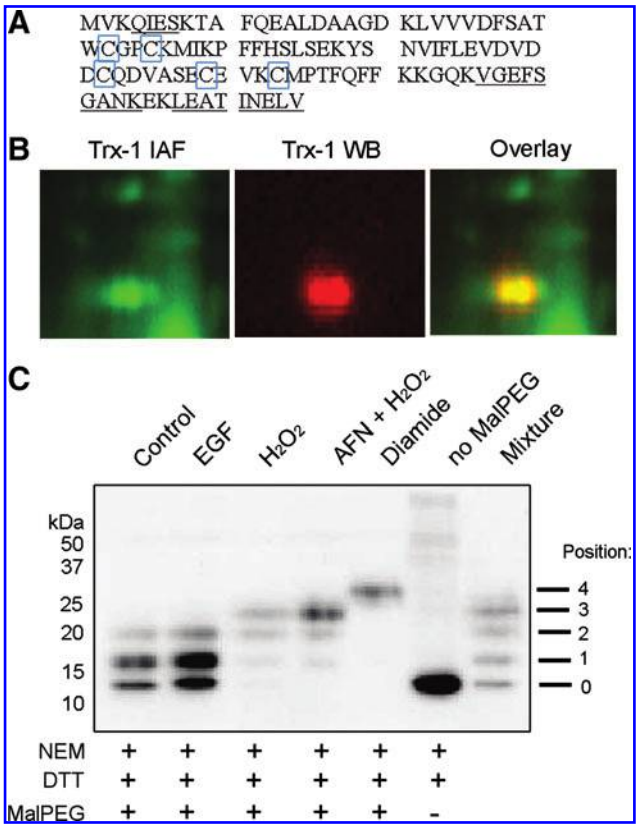

FIG. 5. Identification of Spot A as thioredoxin 1. (A) Identification of spot A by mass spectrometry. Spot A was excised from redox 2D gels, trypsinized, and protein fragments identified by mass spectrometry. Sequences identified are underlined. Cysteine residues are outlined in *blue*. **(B)** A431 cells were prepared as for redox 2D-PAGE (*left panel, green*) then Western blotted (*middle panel, red*) for Trx 1, and the images superimposed (*right panel*). **(C)** A431 cells treated with EGF, 1 m*M* hydrogen peroxide, 2.5 μM auranofin, followed by 1 m*M* hydrogen peroxide or 2 m*M* diamide to cause extensive oxidation, were blocked with NEM, then oxidized thiols were labeled with 2 kDa Mal-PEG and Western blotted for Trx 1. Band 0 is fully reduced protein, bands 1 contains one oxidized cysteine, band 2 contains 2 oxidized cysteines, and so on. The "No MalPEG" lane confirms the position of the fully reduced and unlabeled protein. The "Mixture" lane is mixture of control and peroxide-treated samples such that a ladder of possible positions is created. (To see this illustration in color the reader is referred to the web version of this article at www.liebertonline.com/ars).

A431 cells display a high number of EGF receptors, and have been used extensively to illustrate oxidant generation following stimulation (1, 21). Despite the degree of receptor activation it is expected that the amount of hydrogen peroxide generated during growth factor signaling is low. NOX enzymes were initially discovered through their capacity to generate large amounts of superoxide in the phagosomes of neutrophils. While NOX activation is reported in nonphagocytic cells upon exposure to a variety of receptor-ligand interactions, including growth factors (7, 22, 31, 36, 37, 48), it is very difficult to detect either superoxide or the dismutation product hydrogen peroxide. Increased DCF fluorescence is commonly reported (1, 2, 8, 34, 38, 41), and we used the assay to illustrate our A431 cells were responding in a similar manner to previous reports. We also observed that the amount of DCF oxidation following EGF treatment was greater than that observed with 100 μM hydrogen peroxide (not shown). However, it is not possible to use this assay to estimate the amount of hydrogen peroxide generated in different systems. DCF oxidation is influenced by multiple factors including probe loading, catalyst availability and activity, and amplification and quenching of radical intermediates in the oxidation of DCF (44). Since alteration of any of these factors can alter fluorescence, it does not provide quantitative information on the levels of hydrogen peroxide generation following growth factor stimulation. Rather than use hydrogen peroxide to mimic what level cells may be exposed to following growth factor activation, we have used it to illustrate the most sensitive thiol proteins.

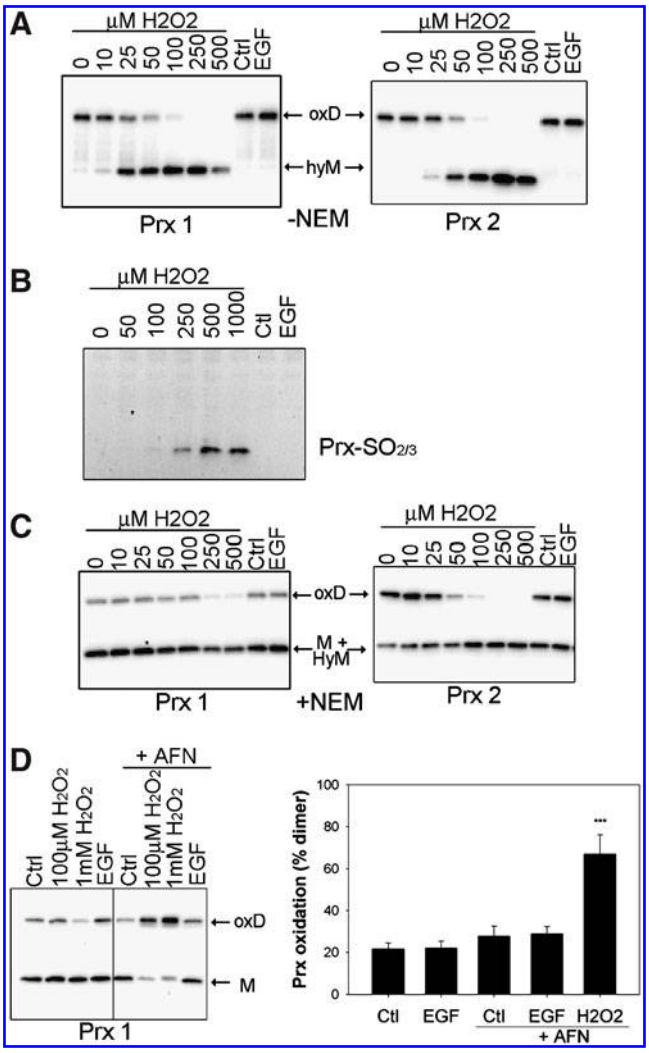

FIG. 6. Oxidation of 2-Cys peroxiredoxins after treatment with EGF. (A) A431 cells were treated with 0–500 μM hydrogen peroxide for 5 min, or 500 ng/mL EGF for 5 min. Cells were lysed in the absence of the alkylating agent NEM, then separated by SDS-PAGE, and Western blotted for Prx 1 (*left panel*), or Prx 2 (*right panel*) (hyM, hyperoxidized monomer; oxD, dimer). **(B)** A431 cells were treated with 0–1000 μM hydrogen peroxide for 5 min, or 500 ng/m*L* EGF for 5 min, separated by SDS-PAGE and Western blotted for hyperoxidized Prxs (Prx-$SO_{2/3}$). **(C)** A431 cells were treated with 0–500 μM hydrogen peroxide for 5 min, or 500 ng/ml EGF for 5 min. Cells were lysed in the presence of the alkylating agent NEM, then separated by SDS-PAGE, and Western blotted for Prx 1 (*left panel*) or Prx 2 (*right panel*). (M + HyM, reduced and hyperoxidized monomer; oxD = dimer). **(D)** A431 cells were pretreated for 10 min with 2.5 μM auranofin to inhibit thioredoxin reductase, then treated with either 500 ng/ml EGF, or 100 μM or 1 mM hydrogen peroxide for 5 min. Cells were lysed in the presence of NEM, and the increase in oxidized Prx 1 dimer monitored. *Right panel:* quantification of oxidized dimer (hydrogen peroxide, 1 mM) ($p < 0.0001$, One way ANOVA, $n = 7$–9).

The only change detected by the 2D-IAF technique was a 1.7-fold increase in labeling of the lower abundance acidic isoform of Trx 1. However, a specific redox blotting technique failed to measure significant Trx 1 oxidation, indicating that only a small proportion of the total Trx 1 was affected. It is possible that the fluorescence increase could have been due to a post-translational modification such as phosphorylation increasing the amount of protein running at this position. This result contrasts with two reports of Trx 1 oxidation following EGF treatment, which, interestingly, both used the immortalized human keratinocyte cell line HaCaT (16, 18).

The model of growth factor signaling proposes that hydrogen peroxide acts to transiently inactivate thiol-dependent phosphatases, thereby facilitating protein phosphorylation. There is considerable evidence to support this model (23, 25, 27, 29, 32). One unresolved issue is that hydrogen peroxide has to evade specialist scavengers such as the Prxs and oxidize low abundance thiol proteins that react relatively slowly. These observations have led to models of localized action whereby membrane-associated Prxs are inactivated by hyperoxidation or phosphorylation, allowing less reactive targets the opportunity to be oxidized (45, 46). This is consistent with the low level of protein phosphatase inactivation previously reported in EGF and PDGF signaling (6, 23). However, this mechanism fails to sufficiently account for the diffusability of hydrogen peroxide. Prxs within the same compartment will still protect less reactive proteins from oxidation.

Another mechanism of NOX-mediated signaling is emerging that places emphasis on the compartmentalization of oxidant generation. NOX orientation means that superoxide is generated on the extracellular surface of the plasma membrane, which also corresponds to the interior of an endosome following internalization of the EGFR complex. While hydrogen peroxide is able to diffuse across membranes, extracellular catalase is unable to inhibit EGF signaling (Cuddihy, unpublished observation) compared to that seen with electroporated enzyme (1), supporting an endosome-based model. Such a model also offers the possibility of channeling oxidizing potential to specific targets and signaling pathways. Alternatively, substrate channeling that limits free diffusion of hydrogen peroxide may be possible, particularly when colocalization of Nox4 and PTP1B has been observed (7).

We conclude that widespread thiol oxidation does not occur during EGF signaling, and any effects of hydrogen peroxide are likely to be selective and localized. Recent evidence using targeted redox sensitive probes provide some support for this proposal (33). Further investigation with these probes, combined with improved methods for detecting protein modifications, are needed to discern the relationship between hydrogen peroxide and the localized oxidation of cellular thiol proteins.

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Author Disclosure Statement

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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Abbreviations Used

DCF = dichlorodihydrofluoroscein
DTT = dithiothreitol
ECL = enhanced chemiluminescence
EGF = epidermal growth factor
EGFR = epidermal growth factor receptor
GAPDH = glyceraldehyde-3-phosphate dehydrogenase
IAF = 5-iodoacetamidofluorescein
IPG = immobilized pH gradient
Mal-PEG = α-methoxy-ω-ethyl-maleinimide poly(ethylene glycol)
NEM = *N*-ethyl maleimide
NOX = NADPH oxidase
PBS = phosphate buffered saline
Prx = peroxiredoxin
PTP = protein tyrosine phosphatase

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