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The NADPH Oxidases DUOX1 and NOX2 Play Distinct Roles in Redox Regulation of Epidermal Growth Factor Receptor Signaling*[S]

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The epidermal growth factor receptor (EGFR) plays a critical role in regulating airway epithelial homeostasis and responses to injury. Activation of EGFR is regulated by redox-dependent processes involving reversible cysteine oxidation by reactive oxygen species (ROS) and involves both ligand-dependent and -independent mechanisms, but the precise source(s) of ROS and the molecular mechanisms that control tyrosine kinase activity are incompletely understood. Here, we demonstrate that stimulation of EGFR activation by ATP in airway epithelial cells is closely associated with dynamic reversible oxidation of cysteine residues via sequential sulfenylation and *S*-glutathionylation within EGFR and the non-receptor-tyrosine kinase Src. Moreover, the intrinsic kinase activity of recombinant Src or EGFR was in both cases enhanced by H_2O_2 but not by GSSG, indicating that the intermediate sulfenylation is the activating modification. H_2O_2-induced increase in EGFR tyrosine kinase activity was not observed with the C797S variant, confirming Cys-797 as the redox-sensitive cysteine residue that regulates kinase activity. Redox-dependent regulation of EGFR activation in airway epithelial cells was found to strongly depend on activation of either the NADPH oxidase DUOX1 or the homolog NOX2, depending on the activation mechanism. Whereas DUOX1 and Src play a primary role in EGFR transactivation by wound-derived signals such as ATP, direct ligand-dependent EGFR activation primarily involves NOX2 with a secondary role for DUOX1 and Src. Collectively, our findings establish that redox-dependent EGFR kinase activation involves a dynamic and reversible cysteine oxidation mechanism and that this activation mechanism variably involves DUOX1 and NOX2.

The epidermal growth factor receptor (EGFR[2]; HER1; ErbB1) is the principal member of the human ErbB receptor-tyrosine kinase family that facilitates diverse signal transduction pathways to regulate imperative cellular functions including growth, development, differentiation, and migration (1). A transmembrane protein expressed on the cell membrane, EGFR consists of an extracellular ligand binding domain, a single transmembrane-spanning sequence, and an intracellular kinase domain containing a C-terminal peptide tail with several tyrosine residues targeted for autophosphorylation or phosphorylation by related tyrosine kinases (2). Activation of EGFR is enabled though binding of one of seven ligands to its extracellular domain, thereby promoting receptor homo- or heterodimerization and autophosphorylation of several C-terminal tail tyrosines (*e.g.* Tyr-1068) and activation of downstream signals. In addition, it has become apparent that EGFR can also be activated in response to stimulation of a large class of G-protein coupled receptors (GPCRs), which involves activation of membrane-associated EGFR ligands by matrix metalloproteases or ADAM (a disintegrin and metalloprotease)-family sheddases, a process known as "triple-membrane-passing-signaling," as well as ligand-independent mechanisms mediated by activation of cytosolic tyrosine kinases (*e.g.* Src, Pyk) that regulate EGFR activation by direct phosphorylation of its kinase domain (*e.g.* Tyr-845) (3–5). The relative involvement of ligand-dependent and -independent mechanisms in GPCR-mediated EGFR transactivation is highly cell and context-dependent, and these various activation modes variably affect EGFR phosphorylation profiles, thereby promoting diverse EGFR-dependent signaling processes (4, 6).

Tyrosine phosphorylation by kinases such as EGFR is known to strongly depend on redox-dependent mechanisms, which typically involve reversible oxidation of catalytic or regulatory protein cysteine residues by cellular production of reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2) (7–10). A well accepted paradigm in such redox-mediated regulation of tyrosine kinase signaling is the reversible inactivation of protein-tyrosine phosphatases (PTPs) by oxidative modification of their catalytic cysteine residue, resulting in amplified and/or prolonged tyrosine phosphorylation (11, 12). More recently, evidence has been emerging that tyrosine kinases are also subject to direct redox regulation through reversible oxidation of conserved non-catalytic cysteine residues located within regu-

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[2] The abbreviations used are: EGF(R), epidermal growth factor (receptor); GPCR, G-protein coupled receptor; ROS, reactive oxygen species; PTP, protein-tyrosine phosphatase; DUOX1, dual oxidase 1; MTE, mouse tracheal epithelial; HBE, human bronchial epithelial; ANOVA, analysis of variance; BioGEE, biotinylated glutathione ethyl ester; $P2Y_2R$, P2Y purinoceptor 2.

latory or kinase domains of these proteins. Indeed, a growing number of studies indicate that Src family kinases can be activated by oxidant-dependent mechanisms, depending on oxidation of one or more conserved cysteine residues (13–15). Similarly, recent studies have also indicated that EGFR kinase activity can be enhanced by reversible oxidation of a cysteine residue near the ATP binding site in the kinase domain (Cys-797) (16).

Although it is evident that oxidation of catalytic cysteines in PTPs result in loss of activity, the molecular mechanism(s) by which cysteine oxidation contributes to activation of Src and/or EGFR is less apparent, as the implicated cysteine residues are not directly involved in enzyme catalysis. H_2O_2-induced cysteine oxidation is reversible and involves initial formation of a sulfenic acid (-SOH), which can subsequently react with other reduced thiols to form disulfides (*e.g.* with glutathione to form *S*-glutathionylated proteins; -SSG) or be further oxidized to sulfinic and sulfonic acids (17). The relative importance of these specific oxidative cysteine modifications in direct regulation of tyrosine kinases is, however, poorly understood. For example, oxidative activation of Src has been attributed to formation of a disulfide between Cys-245 and Cys-485, although direct evidence for this is lacking (13), and others have suggested that oxidation of Cys-277 within the kinase domain actually inhibits activity (18). Similarly, H_2O_2 was shown to enhance EGFR kinase activity presumably due to formation of a sulfenic acid (−SOH) at Cys-797 within the kinase domain (16), although the importance of other oxidative cysteine modifications is less clear (19). Interestingly, Cys-797 is localized near the ATP binding site and is a target for recently developed irreversible EGFR inhibitors (20, 21), which indicates that regulation of EGFR kinase activity by Cys-797 modification strongly depends on the nature of the modification.

Although evidence is emerging that ROS can contribute to activation of Src or EGFR, the precise source of ROS responsible for activation of these kinases is often unclear and may be cell type-dependent. Indeed, recent studies from our group revealed that EGFR transactivation in airway epithelial cells by initial activation with ATP of purinergic GPCRs, due to epithelial injury or exposure to environmental allergens, is associated with enhanced cysteine oxidation within Src as well as EGFR, depending on activation of the NADPH oxidase homolog dual oxidase 1 (DUOX1) (22–24). Other studies indicate a comparable role for NOX1 in EGFR transactivation in vascular smooth muscle cells in response to thrombin (25). Alternatively, recent studies by Carroll and co-workers (16) indicated that EGF-dependent activation of EGFR in epidermoid carcinoma A431 cells involves cysteine oxidation within the EGFR kinase domain mediated by ROS generated from the NADPH oxidase NOX2. These diverse findings highlight the potential involvement of different redox pathways in ligand-dependent and -independent EGFR activation pathways.

The present studies were designed to address the relative importance of cysteine sulfenylation and *S*-glutathionylation in oxidant-mediated activation of Src and EGFR. Second, we also addressed the source of ROS that mediates cysteine oxidation and activation of EGFR during ligand-dependent and -independent EGFR activation in bronchial epithelial cells, focusing on DUOX1 and NOX2 as the major NOX isoforms with known roles in airway epithelial biology (26, 27). Our findings indicate that oxidant-induced activation of Src and EGFR primarily involves sulfenylation rather than *S*-glutathionylation, indicating a dynamic reversible mechanism involved in redox-dependent regulation of these kinases, and furthermore demonstrate distinct and potentially concerted roles of DUOX1 and NOX2 in promoting cysteine oxidation and activation of EGFR, depending on ligand-dependent and -independent EGFR activation mechanisms.

Results

DUOX1-dependent EGFR Activation Involves Stepwise Protein Oxidation to Sulfenic Acids and S-Glutathionylation—Several reports indicate that EGFR and Src are subject to redox regulation by cysteine oxidation within these kinases (8, 9, 28, 29), but the nature of the oxidative cysteine modification responsible for activating these kinases is unknown. Extending our recent observations of ATP-stimulated redox-dependent activation of EGFR and Src within the airway epithelium (22, 24), we assessed the relationship between cysteine oxidation and activation of Src and EGFR by determining the temporal association between phosphorylation of EGFR (at Tyr-845 and Tyr-1068) and Src (at Tyr-416) and cysteine oxidation within these proteins to either sulfenic acids (-SOH) or *S*-glutathionylation (-SSG). As expected (22), ATP stimulation of lung epithelial H292 cells rapidly increased phosphorylation of both EGFR (Fig. 1*A*) and Src (Fig. 1*B*), which was maximal at 1 min and persisted for up to 10 min before declining to baseline after 30–60 min. ATP stimulation also induced EGFR sulfenylation with highly similar kinetics and also enhanced EGFR *S*-glutathionylation, albeit in a slightly delayed manner (Fig. 1, *A* and *D*). Comparable findings were observed with respect to temporal Tyr-416 phosphorylation and cysteine oxidation of Src, although in this case sulfenylation appears to precede Src phosphorylation at Tyr-416 (Fig. 1, *B* and *E*). Similarly, ATP stimulation also resulted in sulfenylation and delayed *S*-glutathionylation of PTP1B (Fig. 1, *C* and *F*), which is expected to inactivate phosphatase activity and enhance or prolong phosphorylation of EGFR and Src (30). These findings indicate that the transient activation of Src and EGFR is dynamically associated with reversible cysteine oxidation within these kinases and appears to be most closely associated with cysteine sulfenylation of EGFR and Src as well as inactivation of PTP1B. To more directly address the relationship between sulfenylation and *S*-glutathionylation within these proteins, primary mouse tracheal epithelial (MTE) cells were preloaded with biotinylated glutathione ethyl ester (BioGEE) and subsequently stimulated with ATP in the absence or presence of dimedone (to trap intermediate sulfenylated proteins), and *S*-glutathionylation of proteins of interest was assessed after avidin purification (Fig. 2*A*). As shown in Fig. 2*B*, ATP stimulation readily induced *S*-glutathionylation of EGFR, Src, and PTP1B in MTE cells from wild-type mice, whereas these responses were largely diminished in MTE cells from DUOX1-deficient mice. Importantly, ATP-stimulated *S*-glutathionylation of each of these proteins was markedly diminished in the presence of dimedone, consistent

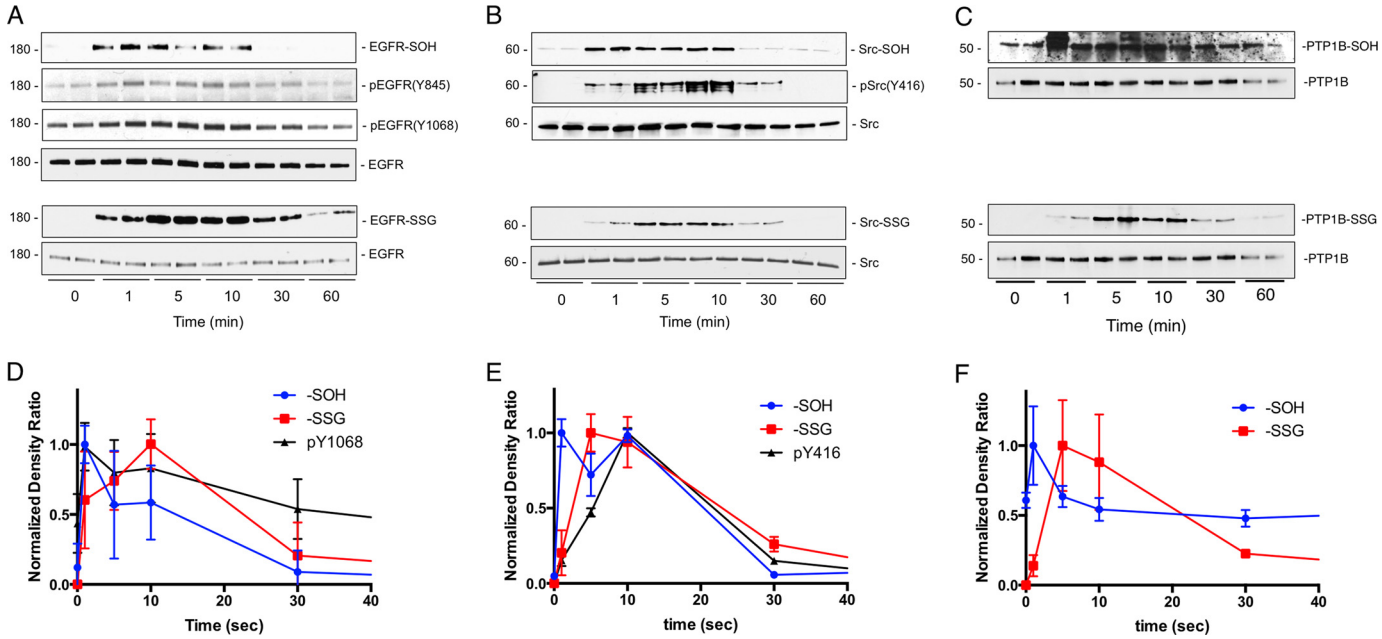

FIGURE 1. **ATP-dependent activation of EGFR and Src is temporally coupled to cysteine sulfenylation and *S*-glutathionylation.** H292 cells were stimulated with 100 μM ATP, and time-dependent changes in of EGFR phosphorylation at Tyr(P)-845 and Tyr(P)-1068 as well as EGFR sulfenylation (*-SOH*) and *S*-glutathionylation (*-SSG*) were analyzed by Western blot as detailed under "Experimental Procedures" (*A* and *D*), and similar phosphorylation (Tyr(P)-416), sulfenylation, and *S*-glutathionylation was evaluated in Src (*B* and *E*) as well as sulfenylation and *S*-glutathionylation PTP1B (*C* and *F*). *D–F*, densitometry analysis of two-three independent experiments. Data represent the mean ± S.D. (maximal band density ratios scaled to 1.0) showing the time-dependent association of phosphorylation and cysteine oxidation of Src and EGFR.

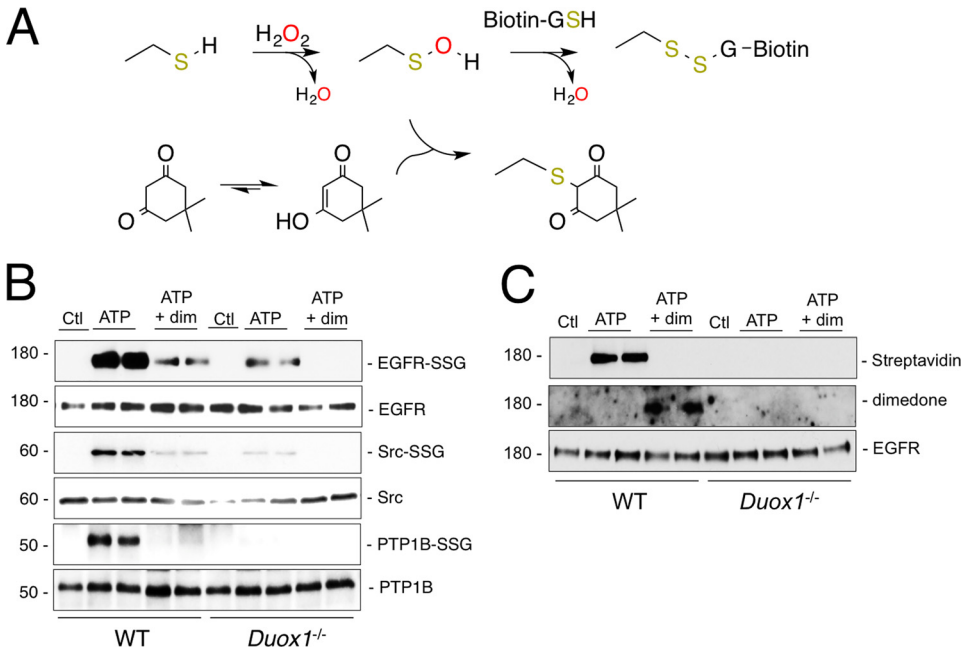

FIGURE 2. **DUOX1 activation induces sequential sulfenylation and *S*-glutathionylation of EGFR, Src, and PTP1B.** *A*, scheme illustration of experimental approach to assess sequential cysteine sulfenylation and *S*-glutathionylation. *B* and *C*, MTE cells from WT and *Duox1*$^{-/-}$ mice were preloaded with BioGEE and stimulated with ATP (100 μM; 10 min) in the absence or presence of dimedone (*dim*). *B*, Western blotting analysis of *S*-glutathionylation of EGFR, Src and PTP1B. *C*, Western blot of immunoprecipitated EGFR for streptavidin (reflecting incorporation of biotin-GSH), α-dimedone, or α-EGFR. Western blots are representative of two-three independent experiments.

with intermediate formation of sulfenic acids in these proteins (Fig. 2*B*). Analysis of immunoprecipitated EGFR from cell lysates confirmed these findings and showed marked biotin incorporation (reflecting addition of biotin-tagged GSH) in response to ATP stimulation, which was diminished in the presence of dimedone and instead revealed EGFR conjugation of dimedone (Fig. 2*C*), reflecting formation of sulfenic acid. These findings indicate a stepwise oxidative mechanism within EGFR and Src as well as PTP1B in response to ATP-mediated DUOX1 activation, with initial sulfenylation followed by *S*-glutathionylation, consistent with temporal analysis in Fig. 1.

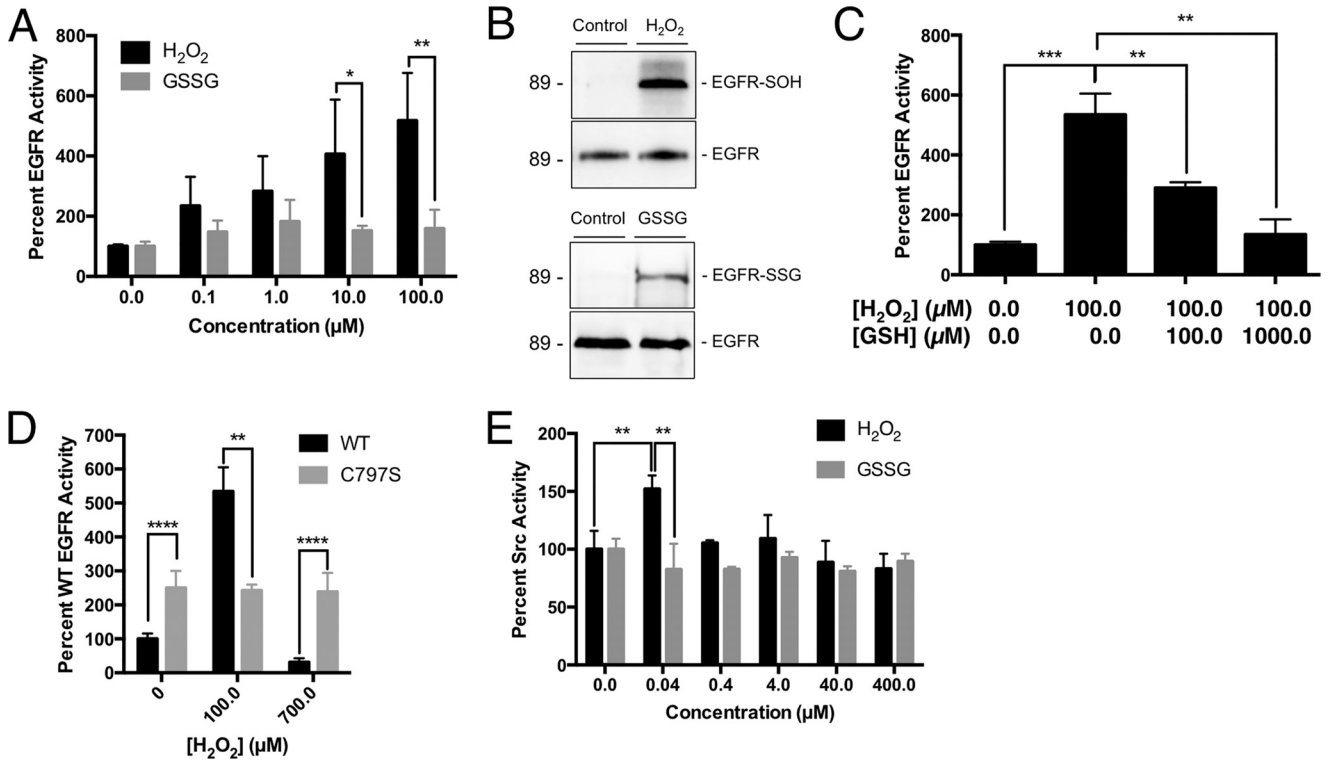

FIGURE 3. **H_2O_2 enhances EGFR and Src tyrosine kinase activity via cysteine sulfenylation.** *A*, effect of H_2O_2 or GSSG on intrinsic kinase activity of recombinant EGFR. *B*, Western blotting analysis of sulfenylation and *S*-glutathionylation of recombinant EGFR by H_2O_2 or GSSG (100 μM) using dimedone (DCP-Bio1) and α-GSH antibody, respectively. *C*, effects of GSH on H_2O_2-dependent increase in recombinant EGFR-tyrosine kinase activity. Note that listed GSH concentrations do not include GSH (333 μM) that was originally included in the EGFR storage buffer. *D*, comparison of H_2O_2-enhanced tyrosine kinase activity WT recombinant EGFR and C797S variant. *E*, effect of H_2O_2 and GSSG on intrinsic tyrosine kinase activity of recombinant Src. Data represent the mean ± S.D. of at least $n = 4$ replicates; Student's *t* test; **, $p < 0.01$; ****, $p < 0.001$. Western blots are representative of two-three independent experiments.

Protein Cysteine Sulfenylation at Cys-797 Enhances EGFR-tyrosine Kinase Activity—Although cysteine sulfenylation or *S*-glutathionylation both inactivate PTP1B (30), their relative importance in altering tyrosine kinase activity of EGFR or Src is less clear. To address this, we subjected recombinant EGFR or Src to dose-dependent treatment with H_2O_2 or GSSG, to produce sulfenylated and *S*-glutathionylated cysteines, respectively, and evaluated the effects on intrinsic tyrosine kinase activity. Consistent with findings by Paulsen *et al.* (16), H_2O_2 at concentrations between 10 and 100 μM significantly enhanced intrinsic EGFR kinase activity (Fig. 3*A*), which was associated with EGFR sulfenylation (Fig. 3*B*). Higher H_2O_2 concentrations were found to inhibit EGFR activity (Fig. 3*D*), most likely due to further cysteine oxidation to sulfinic or sulfonic acids (16). H_2O_2-induced increase in intrinsic EGFR activity was not due to inhibition of potentially co-purified phosphatases, as the addition of the tyrosine phosphatase inhibitor Na_3VO_4 did not affect EGFR activity (data not shown). In contrast, *S*-glutathionylation of EGFR induced by similar concentrations of GSSG did not significantly affect activity (Fig. 3, *A* and *B*). H_2O_2-mediated activation of EGFR-tyrosine kinase was inhibited in the presence of increasing doses of GSH (Fig. 3*C*), presumably due to reaction of GSH with intermediate sulfenic acids to form *S*-glutathionylated EGFR before analysis of kinase activity. Interestingly, due to the presence of GSH in the EGFR storage buffer (Signal Chem) our reaction mixtures contained 333 μM GSH originating from the storage buffer (in the absence of added GSH), which would suggest that the observed H_2O_2-mediated activation may in fact be an underestimation due to inhibitory effects of GSH from the storage buffer. The role of Cys-797 as the expected target involved in H_2O_2-dependent activation was tested with the C797S variant of EGFR. The C797S variant exhibited ~2.5 times greater intrinsic tyrosine kinase activity compared with WT EGFR (3.8 and 9.5 nmol ATP/min/μg of protein for WT EGFR and C797S, respectively) but was resistant to H_2O_2-dependent activation as well as inhibition of kinase activity by high H_2O_2 concentrations (Fig. 3*D*), consistent with previous findings (16) and establishing Cys-797 as the residue responsible for redox-mediated regulation of tyrosine kinase activity. Our findings additionally illustrated a dynamic oxidative mechanism that regulated EGFR kinase activity by direct Cys-797 oxidation to a sulfenic acid, which was then reversed by *S*-glutathionylation and restored to reduce EGFR by cellular reducing mechanisms. Qualitatively similar findings were observed with recombinant Src after treatment with H_2O_2 or GSSG, which showed significant activity enhancement by H_2O_2 at low μM concentrations (Fig. 3*E*) despite the fact that it was constitutively active due to its gatekeeper T341M mutation (Millipore, Temecula, CA). Again, GSSG was ineffective, implying that cysteine sulfenylation may also be the activating intermediate for Src.

DUOX1 and NOX2 Mediate Ligand-independent and Ligand-dependent EGFR Activation, Respectively—Based on previously reported involvement of either DUOX1 or NOX2 in mediating EGFR cysteine oxidation as a potential mechanism of activation (16, 24), we addressed the relative participation of

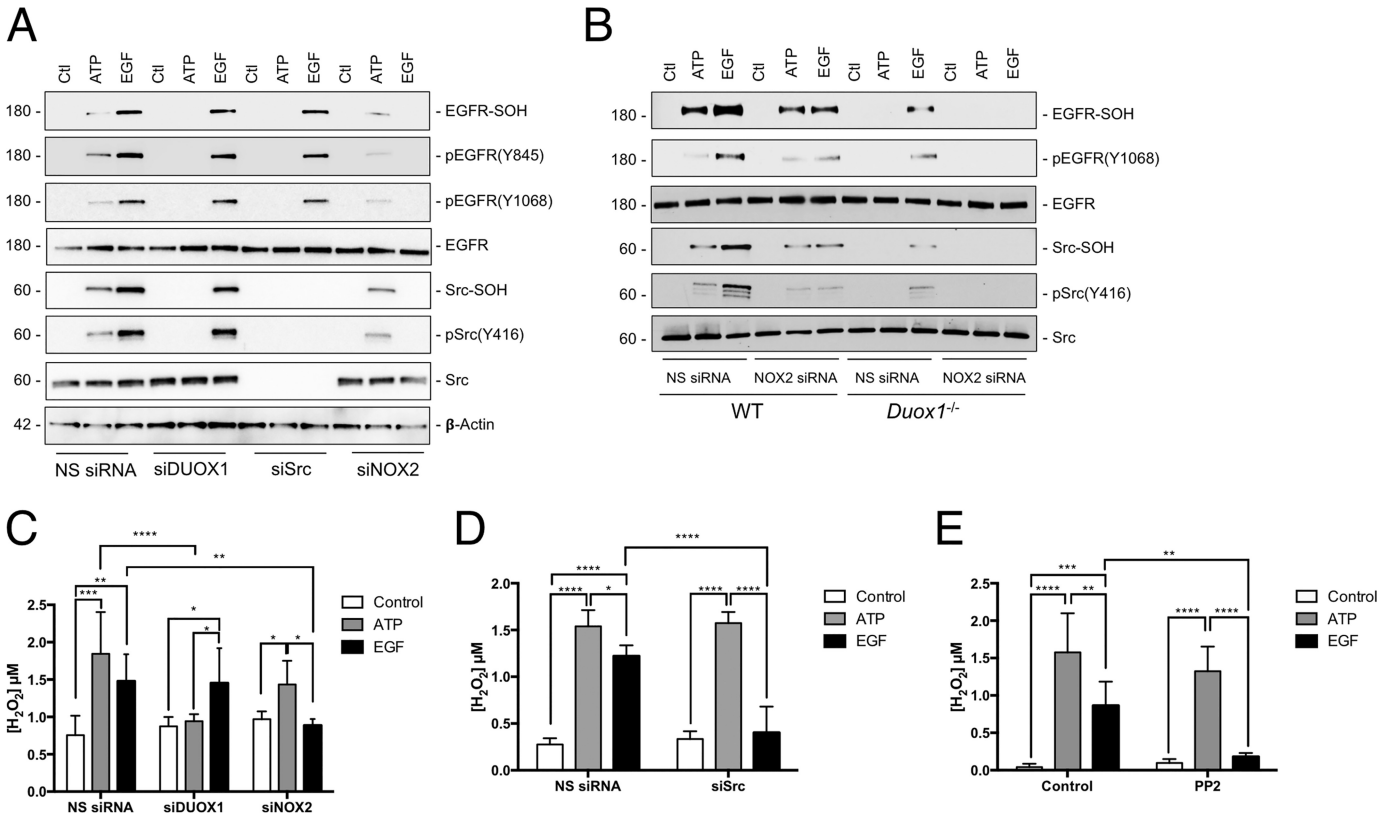

FIGURE 4. **Distinct roles for DUOX1 and NOX2 on ATP-dependent EGFR transactivation and direct ligand-dependent EGFR activation.** *A*, H292 cells were transfected with siRNA against DUOX1, Src, or NOX2 and stimulated with ATP (100 μM) or EGF (100 ng/ml), and tyrosine phosphorylation and cysteine sulfenylation of EGFR and Src was determined by Western blot. *B*, Western blotting analysis of ATP- or EGF-stimulated MTE cells from WT and *Duox1*$^{-/-}$ mice with or without siRNA silencing of NOX2 for Src and EGFR phosphorylation and sulfenylation. *C–E*, analysis of extracellular H_2O_2 production by H292 cells after siRNA-mediated silencing of DUOX1 and NOX2 (*C*), siRNA silencing (*D*), or pharmacological inhibition (*E*) of Src with PP2 (1 μM). Western blots are representative of two independent experiments. Data represent the mean ± S.D. from four-six replicates of three independent experiments. Two-way ANOVA; $n = 4–6$; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.005$, ****, $p < 0.001$. *NS*, not significant.

these NOX isoforms in EGFR activation in either human airway epithelial H292 or HBE1 cells or in MTE cells in response to either exogenous ATP (100 μM; to induce EGFR transactivation via initial $P2Y_2R$ stimulation) or direct ligand-induced EGFR activation by EGF (100 ng/ml). As expected (22), silencing of DUOX1 in H292 cells (Fig. 4*A* and supplemental Figs. S2 and S3) or HBE1 cells (supplemental Fig. S1) eliminated ATP-mediated phosphorylation of EGFR and Src and similarly attenuated cysteine sulfenylation (Cys-SOH) within these proteins (Fig. 4*A* and supplemental Figs. S2 and S3). In contrast, phosphorylation and cysteine oxidation of EGFR and Src in response to EGF stimulation was affected only marginally by DUOX1 siRNA (Fig. 4*A* and supplemental Figs. S2 and S3). Comparable findings were observed using MTE cells, which indicated that DUOX1-deficient cells showed minimal EGFR and Src phosphorylation and cysteine oxidation in response to ATP, whereas responses to EGF were only partially attenuated (Fig. 4*B* and supplemental Figs. S2 and S4). Conversely, silencing of NOX2 dramatically suppressed EGF-dependent phosphorylation and cysteine oxidation of EGFR and Src in both H292 and HBE1 cells but did not affect responses to ATP (Fig. 4*A* and supplemental Figs. S2 and S3). Similarly NOX2 suppression failed to inhibit ATP-stimulated oxidation and phosphorylation of EGFR and Src in MTE cells but significantly attenuated similar responses to EGF (Fig. 4*B* and supplemental Figs. S2 and S4). In agreement with these various findings, analysis of extracellular H_2O_2 production by H292 cells as a measure of NOX activation showed that ATP-stimulated H_2O_2 production depended primarily on DUOX1, whereas H_2O_2 production induced by EGF was largely DUOX1-independent and was instead mediated by NOX2 (Fig. 4*C*). Although EGF-mediated phosphorylation and cysteine oxidation of Src and EGFR in H292 and HBE cells were found to be almost completely dependent on NOX2 and independent of DUOX1 (Fig. 4*A* and supplemental Fig. S1), similar EGF-mediated responses in MTE cells were also partially attenuated in DUOX1-deficient MTE cells and were suppressed further by NOX2 silencing (Fig. 4*B* and supplemental Figs. S2 and S4) indicating that both DUOX1 and NOX2 are involved in redox-regulation of Src and EGFR in a concerted manner.

Ligand-dependent and -independent EGFR activation mechanisms are thought to differentially stimulate downstream signaling pathways (4, 5). Therefore, we investigated the impact of DUOX1 or NOX2 on activation of two downstream effector targets of EGFR activation, ERK1/2 and STAT3, in response to either ATP or EGF. As expected (22, 31), DUOX1 silencing suppressed ATP-mediated phosphorylation of both STAT3 (Fig. 5, *A* and *B*) and ERK1/2 (Fig. 5, *A* and *C*) but had no effect on activation of these pathways by direct EGFR stimulation with EGF. On the other hand, NOX2 silencing attenuated

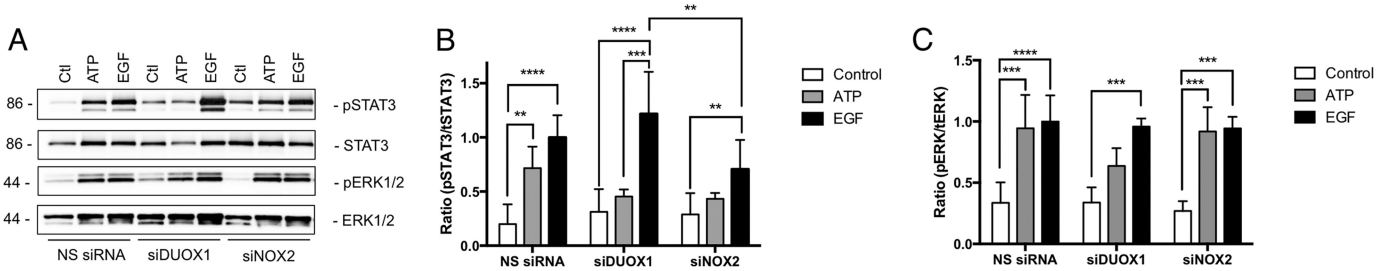

FIGURE 5. **DUOX1 and NOX2 have stimulus-dependent impact on EGFR downstream signaling.** *A*, representative Western blotting analysis of 100 μM ATP- and 100 ng/ml EGF-stimulated H292 cells of phosphorylation of STAT3 and ERK1/2 as a function of siRNA-mediated silencing of DUOX1 and NOX2. Quantification represents the mean ± S.D. of phosphorylation changes of STAT3 (*B*) and ERK1/2 (*C*) normalized ratio of phospho/total of NS siRNA EGF treatment of 1.0. **, $p < 0.01$; ***, $p < 0.005$, ****, $p < 0.001$. *NS*, not significant.

STAT3 phosphorylation in response to both ATP and EGF (Fig. 5, *A* and *B*) but did not affect ERK1/2 phosphorylation in either case (Fig. 5, *A* and *C*). These findings indicate that DUOX1 and NOX2 have a variable impact on EGFR activation and downstream signaling depending on the activating stimulus.

EGFR and Src Kinase Activities Are Required for EGF-mediated NOX2 Activation—We recently reported that ATP-dependent transactivation of EGFR is mediated by initial DUOX1-dependent activation of Src (22). Accordingly, suppression of Src by siRNA dramatically suppressed ATP-dependent phosphorylation and cysteine oxidation of both Src and EGFR (Fig. 4*A* and supplemental Figs. S2 and 3). However, Src siRNA or pharmacological Src inhibition with PP2 only partially inhibited EGFR phosphorylation and cysteine oxidation in response to direct EGF ligand stimulation (Fig. 4*A*, supplemental Figs. S2 and 3, and Fig. 6*A*) even though Src siRNA or pharmacological Src inhibition almost completely prevented EGF-induced extracellular H_2O_2 production (Fig. 4, *D* and *E*). These findings imply that EGF-dependent cysteine oxidation within EGFR cannot be fully explained by Src-dependent extracellular H_2O_2 production via NOX2 and may involve intracellular NOX2-mediated oxidative events that are independent of Src. To test the role of extracellular H_2O_2 in cysteine oxidation within EGFR or Src, cells were stimulated in the presence of catalase (2000 units/ml). Intriguingly, the presence of catalase did not perturb ATP-mediated cysteine oxidation and phosphorylation of EGFR and Src but partially suppressed phosphorylation and cysteine oxidation of EGFR and Src in response to EGF (supplemental Fig. S5*A*). Because the primary product of NOX2 is superoxide ($O_2^{\cdot-}$), similar experiments were performed in the presence of extracellular superoxide dismutase (10 units/ml), which did not alter EGFR phosphorylation and oxidation by either ATP or EGF (supplemental Fig. S5*B*). Collectively, these findings indicate that EGFR activation and cysteine oxidation by direct EGF stimulation involves both Src-dependent extracellular H_2O_2 production as well as intracellular oxidative mechanisms, which both appear to involve NOX2.

Because EGFR stimulation resulted in NOX2-dependent extracellular H_2O_2 production as well as cysteine oxidation within EGFR and Src, we wondered if these oxidative events require EGFR-tyrosine kinase activity. Indeed, although ATP-mediated extracellular H_2O_2 production originating from DUOX1 was not affected by treatment of EGFR with its known inhibitor AG1478 (32) or EGFR blockade with EGFR mAb, EGF-stimulated extracellular H_2O_2 production was markedly attenuated (Fig. 6*B* and supplemental Fig. S6). Similarly, AG1478 or the related inhibitor erlotinib markedly inhibited EGF-induced phosphorylation and cysteine oxidation of both EGFR and Src, whereas these inhibitors were less effective against EGFR phosphorylation and cysteine oxidation in response to ATP (Fig. 6*A*), further highlighting the differences between ligand-dependent and -independent EGFR activation mechanisms. The fact that NOX2 activation requires initial EGFR activation implies that NOX2 is not essential for initial EGFR activation in response to ligand stimulation but, rather, acts to sustain EGFR activation. Indeed, silencing NOX2 results in diminished duration of EGF-dependent sulfenylation and phosphorylation of EGFR and Src (supplemental Fig. S7), confirming that NOX2 functions to extend and sustain the activation of EGFR.

Discussion

The impact of redox-dependent processes on tyrosine phosphorylation is well appreciated, but the precise mechanisms involved in this are still incompletely understood. Indeed, in addition to well documented redox-dependent inactivation of protein-tyrosine phosphatases as a mechanism of enhanced tyrosine kinase activation (30), evidence is emerging that tyrosine kinases themselves can also be regulated by reversible cysteine modifications (7, 12, 33). Appropriate redox-dependent control of tyrosine phosphorylation would require tightly regulated ROS production, *e.g.* by NOX enzymes. However, the sources of ROS or the nature of cysteine modification responsible for altered kinase activity are often not addressed, and our present studies sought to address both issues.

We first addressed the nature of cysteine modifications that may be responsible for tyrosine kinase activation. Although diverse modifications of catalytic cysteines in *e.g.* tyrosine phosphatases typically result in loss of activity either through sulfenylation (30), alkylation (34), *S*-nitrosylation (35), or *S*-glutathionylation (36), because each of these would abrogate its nucleophilic function critical for hydrolysis of phosphotyrosine (12), it is much less clear how similar modifications of non-catalytic cysteines in protein kinases would enhance their activity (16, 19). Indeed, although cysteine oxidation has been linked with increased tyrosine kinase activity of both Src and EGFR, non-catalytic cysteines within their kinase domains are also targeted by irreversible inhibitors of these kinases (37–39). This applies to Cys-797 (Fig. 7), located within the kinase domain of EGFR (40), which is targeted for recently developed covalent

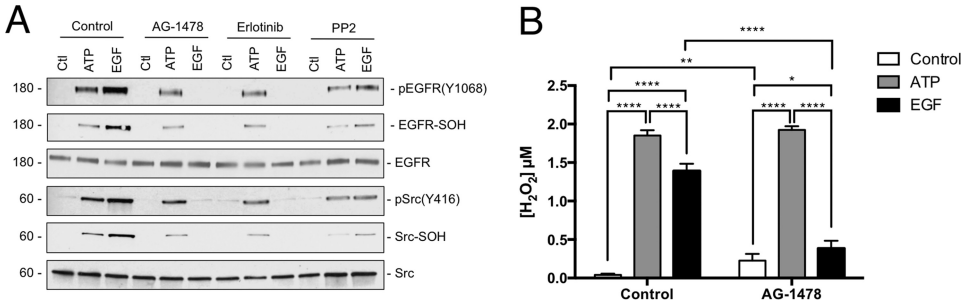

FIGURE 6. **Direct ligand-dependent activation of EGFR causes the activation of NOX2 that depends on tyrosine kinase activity.** *A*, Western blotting analysis of EGFR and Src phosphorylation and sulfenylation as a function of EGFR-tyrosine kinase inhibitors AG-1478, erlotinib, and Src inhibitor PP2 (1 μM) in MTE cells. *B*, effects of hydrogen peroxide generation from H292 cells upon inhibition of EGFR with AG-1478 (1 μM). Data represent the mean ± S.D. from four-six replicates of two-three independent experiments. Western blots are representative of two-three independent experiments. Two-way ANOVA: $n = 4-6$; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.005$; ****, $p < 0.001$.

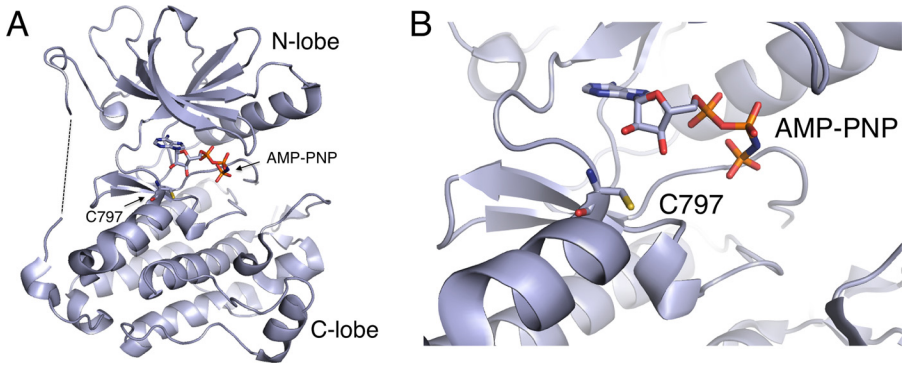

FIGURE 7. **Cysteine 797 resides within the kinase domain of EGFR near the site of ATP binding.** *A*, crystal structure of the EGFR kinase domain (PDB code 2ITX) with bound AMP-PNP (adenosine 5′-(β,γ-imino)triphosphate) highlighting cysteine 797. *B*, active site view showing proximity of Cys-797 to AMP-PNP.

tyrosine kinase inhibitors (19, 20, 41), but is also the main target for H_2O_2 in mediating increased kinase activity (Refs. 16 and 42 and this work). Because structural or electrostatic properties vary widely depending on the nature of the cysteine modification, more detailed molecular-level understanding will be required to understand their functional impact. DUOX1-dependent cysteine oxidation within EGFR and Src was found to involve sequential oxidation to sulfenic acids (-SOH) and *S*-glutathionylated proteins (-SSG), which likely has divergent impact on protein structure and function (17, 43). Interestingly, our studies indicate that H_2O_2-enhanced activation of both Src and EGFR is likely due to intermediate formation of a sulfenic acid, as protein glutathionylation induced by GSSG was incapable of enhancing kinase activity. Moreover, H_2O_2-stimulated increase in EGFR kinase activity was reversed dose-dependently by GSH, consistent with its ability to convert intermediate sulfenic acids to *S*-glutathionylated cysteines. Our findings are consistent with previous reports indicating that EGFR kinase activation can be enhanced by protein sulfenylation but not by *S*-glutathionylation (16, 19). Additionally, our findings suggest a similar oxidant-mediated activation mechanism for the Src kinase, although the cysteine residues involved are still unclear (13, 18, 44).

The present studies support the notion that EGFR and Src are directly oxidized by H_2O_2 within cells even though kinetic arguments would make this unlikely, as reaction of H_2O_2 with (protein) cysteines is typically orders of magnitude slower compared with its reaction with abundant peroxiredoxins (45, 46). However, the peroxidatic activity of peroxiredoxins is subject to phosphorylation (47) as well as hyperoxidation (48), and localized inhibition of peroxiredoxins due to these events is thought to allow for sufficient accumulation of H_2O_2 to oxidize other target proteins within a signaling complex, especially in the context of localized NOX-dependent generation of H_2O_2 (49). Recent findings that DUOX1 activation promotes protein-protein interactions with Src (22) and EGFR activation enhances interactions with NOX2 (16) further support this notion.

The apparent kinetics of protein sulfenylation and *S*-glutathionylation in Fig. 1 might be viewed as inconsistent with the expected rapid rate of deglutathionylation by *e.g.* cellular glutaredoxin(s). However, it should be pointed out that such an interpretation is not valid because of the experimental conditions in which the activating stimulus, ATP, is persistently present throughout this time period (depending on its rate of degradation). Continuous purinoceptor activation during this time period most likely involves dynamic and repeated cycles of receptor activation and inactivation/recycling. Likewise, experimental data suggest that ATP-dependent H_2O_2 production persists for up to 10–15 min (23), although this may not truly reflect the dynamics of DUOX1 activation. Therefore, the data in Fig. 1 should be viewed as snapshots of the relative extent of these oxidative events at various intermediate time points rather than a true reflection of the cellular dynamics of these processes. Nevertheless, the apparent delay in *S*-glutathionylation compared with sulfenylation (Fig. 1) is consistent with the proposed sequential oxidation mechanism.

The structural or functional consequences of protein cysteine sulfenylation are not well understood, but likely involve

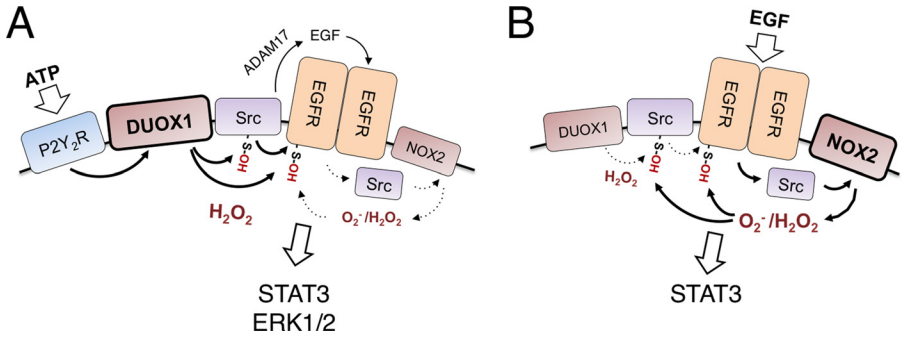

FIGURE 8. **Molecular mechanisms of ATP-dependent transactivation and EGF-dependent direct activation of EGFR.** *A*, ATP-mediated stimulation of $P2Y_2R$ activates DUOX1-dependent H_2O_2 production leading to cysteine oxidation and activation of Src, which promotes shedding EGF of ligands from the cell surface in an ADAM17-dependent fashion, and the activation of EGFR in addition to cysteine oxidation resulting in phosphorylation of ERK1/2 and STAT3. *B*, EGF-mediated direct activation of EGFR activates NOX2-dependent ROS generation through Src activation that leads to cysteine oxidation and activation of Src and EGFR and phosphorylation of STAT3.

electronic effects due to its higher polarity or increased ability to engage in hydrogen bonding compared with a cysteine thiol. In the case of EGFR Cys-797, this could potentially affect ATP hydrolysis or ATP/ADP binding dynamics, thereby altering kinase turnover, as this cysteine is located near the ATP binding site (Fig. 7). Conceivably, initial sulfenylation could result in formation of an intramolecular disulfide within either Src or EGFR, although this seems unlikely as structural data do not indicate the presence of structurally adjacent reduced cysteines in either protein (40, 50–54). Likewise, formation of disulfides between EGFR or Src monomers is unlikely, and no significant cross-linking was observed at the H_2O_2 concentrations used in this study (not shown). H_2O_2-enhanced kinase activity might also be due to more irreversible cysteine oxidation to sulfinic or sulfonic acids, but this is unlikely as H_2O_2-increased activity was readily reversed by GSH (Fig. 3*C*) or DTT (22). Finally, it is possible that increased EGFR kinase activity is in fact due to the formation of a sulfenyl amide by reaction of sulfenic acid with the neighboring backbone chain amide bond as has been observed in PTP1B (12). The resulting strained cyclic sulfenyl amide (or sulfonamide) with a five-atom ring structure induces significant conformational alterations and is also readily reversed by cellular reductants such as GSH and is thus believed to protect cysteines from irreversible overoxidation (11, 55, 56). However, computational structural biology approaches indicate that sulfenyl amide formation is not involved in all protein cysteine oxidations but preferentially affects proteins with constrained conformations, specifically a conserved β-sheet-loop-helix motif (57). Because several protein families were identified with such a constrained cysteine motif with reported roles in redox-related processes, it has been suggested that this motif may have been selected by evolution to enhance cysteine reactivity toward ROS and also to protect the cysteine from further oxidation when it is oxidized to a sulfenic acid by favoring formation of a cyclic sulfenyl amide (57). Unfortunately, the specific involvement of a cyclic sulfenyl amide in enhancing kinase activity of EGFR or similar kinases cannot be proven experimentally at present, as biochemical reagents to selectively probe for this modification are not available, although sulfenyl amide may also be reactive toward dimedone (58).

It is important to note the technical limitations involved in the detection of sulfenic acids using dimedone-based approaches (59, 60). Because reaction kinetics of sulfenic acids with dimedone are slow compared with reaction with other thiols to form *e.g. S*-glutathionylated adducts, these probes do not allow for adequate stoichiometric analysis of protein cysteine sulfenylation in intact cells, although it has been estimated that up to 20% of EGFR might be sulfenylated at Cys-797 in cancer cells (42). Current efforts to develop novel probes for sulfenic acids with more favorable reaction kinetics (61, 62) will likely help address this issue. Nevertheless, our present findings and earlier studies (24) strongly support the biological relevance of cysteine sulfenylation (or related sulfenyl amide formation) within EGFR and/or Src in promoting their kinase activity.

Whereas previous studies with diverse cell models have revealed involvement of different NOX isoforms in regulating EGFR activation (16, 22), our studies are the first to systematically address the contribution of different NOX enzymes in EGFR activation in one cell model and demonstrate that redox-dependent activation mechanisms of EGFR-tyrosine kinase in airway epithelial cells strongly depend on the activating stimulus. Indeed, EGFR transactivation by GPCR stimulation (such as by exogenous ATP) in airway epithelial cells was found to largely depend on activation of DUOX1 (Fig. 8*A*), consistent with previous reports (22, 24), but direct EGFR activation by its cognate ligand (EGF) depended primarily on a different NADPH oxidase, NOX2 (Fig. 8*B*). Moreover, kinase activation in both cases corresponded closely with cysteine sulfenylation within EGFR and Src. Therefore, our studies demonstrate that redox-dependent EGFR activation is highly context-dependent involving multiple NADPH oxidases as the source ROS, even within the same cell type. This picture is further complicated by the fact that EGFR signaling involves redundant pathways with several feedback mechanisms. For example, EGFR transactivation by GPCR stimulation is mediated by initial activation of Src, which promotes EGFR activation by both ligand-dependent and -independent mechanisms. However, Src can also be activated in response to EGFR stimulation, thus activating a positive feedback loop. Interestingly, although Src activation by ATP critically depends on DUOX1, EGF-mediated Src activation was found to be largely DUOX1-independent. Therefore, the diverse roles of NOX isoforms in this complex signaling pathway likely depend on the stage of EGFR activation or on

specific subcellular events after EGFR/Src internalization or trafficking localization. Moreover, the relative role(s) of DUOX1 or other NOX isoforms may also depend on the cell type, highlighted by previous studies showing a role for NOX1 in Src-dependent transactivation of EGFR in vascular smooth muscle cells (25). Nevertheless, our findings indicate that EGFR activation in airway epithelial cells is associated with combined oxidative mechanisms initiated by activation of both DUOX1 and NOX2, which both contribute to cysteine oxidation within EGFR and Src. Intriguingly, recent studies in keratinocytes indicated that EGFR activation can contribute to DUOX1 activation (63), which further highlights the existence of complex feedback mechanisms and diverse involvement of different NOX isoforms in this overall signaling pathway.

Collaborative function of DUOX1 and NOX2 in activating airway epithelial EGFR, depending on initial activating stimuli, indicate distinctive roles for these NOX isozymes in modulating these complicated signaling events and are likely related to direct interactions of these NOX isoforms with either EGFR or Src (16, 22). ATP-dependent activation of DUOX1 and involves Ca^{2+} and protein kinase C (64, 65), it does not require assembly of co-factors, whereas activation of NOX2 involves phosphorylation of cytosolic cofactors (p47phox) and their recruitment to the (plasma) membrane (66, 67). Therefore, the ability of EGFR activation to promote NOX2 activation (*e.g.* Ref. 16) likely involves initial activation of EGFR-tyrosine kinase activity, although the mechanism(s) by which EGFR activation results in NOX2 activation is still unclear. Inhibition of EGFR activity did not inhibit DUOX1-dependent H_2O_2 generation by ATP but markedly suppressed EGF-stimulated activation of NOX2-dependent H_2O_2 generation (Fig. 6*B*). Indeed, silencing of NOX2 resulted in abbreviated activation of EGFR and Src (supplemental Fig. S7). Therefore, activation of NOX2 by initial EGFR activation serves to enhance or prolong EGFR activation in a positive feedback role, whereas DUOX1 plays a more critical role in initiating ATP-dependent activation of Src and subsequent EGFR transactivation. Similarly, DUOX1 and NOX2 also play diverse roles in activation of STAT3 and ERK1/2 as downstream signaling events that are initiated by Src/EGFR activation. For example, NOX2 appears to regulate STAT3 activation by either ATP or EGF but has no effect on activation of ERK1/2, which may be related to a prominent role of Src in STAT3 activation (68). In contrast, DUOX1 regulates activation of ERK1/2 and STAT3 in response to ATP but does not affect these pathways when stimulated by EGF. This further supports a role for DUOX1 in initiating EGFR transactivation, whereas NOX2 functions in a more secondary feedback mechanism to enhance EGFR activation as well as STAT3. Our findings also indicate a role for Src in the activation of NOX2, consistent with previous observations (69, 70). However, although Src inhibition strongly inhibited EGF-induced and NOX2-dependent H_2O_2 production, it had minimal effect on oxidation and phosphorylation of EGFR even though this also depended strongly on NOX2. This would suggest diverse modes or stages of NOX2 activation in response to EGFR activation, which are either Src-dependent or independent and have a divergent impact on extracellular H_2O_2 production and on EGFR oxidation. Additionally, observations that extracellular catalase was capable of attenuating EGF-dependent but not ATP-dependent cysteine oxidation and phosphorylation of EGFR further indicate the variable involvement of oxidative events at the cell surface or in intracellular compartments. Collectively, our observations indicate distinct but also concerted roles of DUOX1 and NOX2 at different stages of EGFR activation either during initial Src-dependent transactivation as part of downstream mechanisms involved in amplification or extension of EGFR activity. More detailed future studies aimed at evaluating these redox-specific events within distinct subcellular regions (*i.e.* after internalization or translocation to other cell organelles) as well as their consequences for specific downstream signaling pathways will be required to more fully clarify the specific roles of specific NOX isoforms in regulating this important signaling mechanism.

In summary, our studies indicate that redox-dependent regulation of the tyrosine kinases Src and EGFR involve a dynamic coupling of sulfenylation and *S*-glutathionylation within these kinases, which directly enhance catalytic activity of these kinases, in addition to the previously demonstrated redox-dependent inactivation of protein-tyrosine phosphatases. Our findings furthermore establish specific and concerted roles for DUOX1 and NOX2 in the redox-regulation of EGFR signaling within the airway epithelium, which critically depends on cellular context and activating mechanisms. The precise consequences of these various NOX-specific actions for specific actions of EGFR on diverse aspects of airway epithelial biology (proliferation, wound responses, mucus production) remain to be more fully elucidated.

Experimental Procedures

Cell Culture and Treatments—Primary MTE cells were isolated from C57BL6/J mice or DUOX1-deficient mice (generated by Lexicon Pharmaceuticals; Ref. 71) as previously described (24) by overnight incubation of tracheas with 0.1% protease 14 (Sigma) and culture of MTE cells on rat tail collagen I gel (BD Biosciences) in DMEM/F-12 media (Invitrogen) supplemented with 20 ng/ml cholera toxin (List Biological Laboratories, Campbell, CA), 10 ng/ml transferrin (Sigma), 100 nmol/liter dexamethasone (Sigma), 5 μg/ml insulin (Sigma), 15 μg/ml bovine pituitary extract (Invitrogen), 2 mmol/liter L-glutamine, and 50 units/50 μg/ml penicillin/streptomycin (Invitrogen). MTE cells were used at passages 2 to 3 for experiments. Human pulmonary mucoepidermoid NCI-H292 cells (American Type Culture Collection) were grown in RPMI 1640 medium containing 10% fetal bovine serum and 1% penicillin/streptomycin at 37 °C and 5% CO_2. Immortalized human bronchial epithelial (HBE) cells, originally provided by Yankaskas and Wu (72), were cultured in DMEM-F-12 supplemented with insulin (5 μg/ml), transferrin (5 μg/ml), EGF (10 ng/ml), dexamethasone (0.1 μM), cholera toxin (10 ng/ml), bovine serum albumin (0.5 mg/ml), and bovine hypothalamus extract (15 μg/ml). For experimentation, cells were seeded at 100,000 cells/well in 24-well plates (Corning), which were precoated with collagen (Type 1 rat tail; BD Biosciences, 50 μg/ml) in the case of MTE cells. In appropriate cases, cells were transfected with siRNA targeted against DUOX1, NOX2, or Src, as detailed below. Upon reaching confluence, cells were cultured over-

night in the absence of serum (H292) or EGF (MTE, HBE1) and placed in fresh serum/EGF-free medium for 1–2 h before stimulation with either exogenous ATP (Sigma; 100 μM) or EGF (Calbiochem; 100 ng/ml). Where indicated, 2000 units/ml catalase (bovine liver; Worthington) and superoxide dismutase 10 units/ml (Bovine, Sigma) were added 30 min before cell stimulation. At the indicated time points, cells were collected for extraction of mRNA or protein lysates for various analyses described below.

Small Interfering RNA Silencing—H292, HBE, and MTE cells were seeded at 70% confluence for siRNA transfection in serum-free medium. Silencing in MTE cells was performed by transfection with the siRNA reagents for NOX2: ON-TARGETplus Mouse Cybb (13058) siRNA-SMARTpool or with Silencer Negative Control #1 siRNA (Ambion). After overnight transfection, medium was replaced with full DMEM/F-12 media (full contents mention above) the following day, and cells were grown for 48–72 h until experimentation. Silencing in H292 and HBE cells was performed by transfection with the siRNA reagents for NOX2 (ON-TARGETplus human CYBB (1536) siRNA-SMARTpool), DUOX1 (Silencer Predesigned siRNA DUOX1 (Ambion): sense (GCUAUGCAGAUGGCGUGUAtt) and antisense (UACACGCCAUCUGCAUAGCtg)), and Src (siGENOME human Src (6714) siRNA-SMARTpool with Silencer Negative Control #1 siRNA (Ambion)). After overnight transfection, medium was replaced with full RPMI 1640 medium (10% FBS and 1% penicillin/streptomycin) for H292 cells and full DMEM-F-12 for HBE cells the following day, and cells were grown for 48–72 h until experimentation. Efficacy of siRNA-mediated silencing was verified by RT-PCR (supplemental Fig. S1).

Western Blotting—Cell lysates were collected by placing cells on ice in 100 μl of Western solubilization lysis buffer (50 mM HEPES, 250 mM NaCl, 1.5 mM $MgCl_2$, 1% Triton-X100, 10% glycerol, 1 mmol/liter EDTA, 1 mM phenylmethylsulfonyl fluoride, 2 mM Na_3VO_4, 10 mg/ml aprotinin, and 10 mg/ml leupeptin (pH 7.4)) per well for 30 min. Lysates were collected by scraping, briefly sonicated, and cleared of insoluble material by centrifugation (14,000 rpm, 5 min) for analysis. Lysates containing equal amounts of protein (15–35 μg, measured using BCA protein assay kit; Pierce) were loaded on 10% SDS-PAGE gels and transferred to nitrocellulose membranes and probed using antibodies against EGFR (C74B9; 1:1,000), pEGFR Tyr-845 (1:1,000), pEGFR Tyr-1068 (D7A5; 1:1,000), p-Src Tyr-416 (1:1,000), Src (L4A1; 1:1,000), p-STAT3 Tyr-705 (1:1,000), STAT3 (1:1,000), pERK1/2 Thr-202/Tyr-204 (1:1,000), ERK1/2 (1:1,000), β-actin (1:1,000; Sigma), PTP1B (1:200; R&D Systems), streptavidin peroxidase polymer ultrasensitive (1:10,000), and anti-cysteine sulfenic acid (1:1,000). Primarily antibodies were probed with rabbit or mouse-specific secondary antibodies conjugated with HRP (Cell Signaling) and detected by enhanced chemiluminescence (Pierce). Western blot band densities were quantified using the ImageJ (1.48v) or ImageQuant TL (v8.1.0.0).

RT-PCR—Total cell RNA was extracted using TRIzol (Invitrogen) and RNeasy Mini Kit (Qiagen, Germantown, MD) and reverse-transcribed using Moloney murine leukemia virus reverse transcriptase and Oligo(dT)12–16 primer. Expression of genes of interest was analyzed by quantitative PCR relative to GAPDH using the $\Delta\Delta C_T$ method using SYBR Green PCR Supermix (Bio-Rad) and the following primers: mouse NOX2 (forward, GAA TCA GCC TTA GTG TCA CAG G; reverse, ATT CCG GTA TGC GTC CAG C); human DUOX1 (forward, AAC AGA ACA TTG CGA TGT ATG AG; reverse, AGA ATG GAC GGT ATC CTG GA); NOX2 (forward, AAT CAT CCA TGC CAC CAT TT; reverse, TCA AAA TCT GCT GTC CTT CC); Src (forward, GGG TGA TGT TTG ACC TTC AG; reverse, TAG GCA CTC TTT TCC CTC CT) and normalized to GAPDH.

In Vitro Tyrosine Kinase Assays—Tyrosine kinase activity of recombinant active EGFR kinase domain (Signal Chem; sequence 695-end) or active EGFR C797S variant (Signal Chem) was analyzed using the ADP-Glo (Promega) assay kit according to the manufacturer's protocol with the exception of excluding DTT from in the kinase reaction buffers. EGFR (~50 ng) was pretreated at room temperature with hydrogen peroxide (H_2O_2; Thermo) and oxidized glutathione (GSSG; Sigma) for 15 min before initiating catalysis with substrate (poly[4Glu:Tyr]; Sigma) and ATP (Promega) for 60 min at room temperature within the linear kinetic range where changes in percent tyrosine kinase activity reflect difference in initial catalytic velocities. Reactions were quenched with ADP Glo reagent for 40 min, further incubated with detection reagent for 30 min, and measured for luminescence. Identical incubation conditions were employed for experiments with recombinant EGFR to detect sulfenylation and *S*-glutathionylation (see below). Additional experiments included L-glutathione (GSH; Sigma) added to EGFR 10 min before pretreatment with H_2O_2. Recombinant active Src T341M (Millipore) was assayed for tyrosine kinase activity using the antibody beacon tyrosine kinase kit (Thermo). Src (~40 ng) was pretreated at room temperature with hydrogen peroxide and oxidized glutathione incubated for 15 min before initiating catalysis with substrate (poly[4Glu:Tyr]; Thermo) and ATP (Thermo) in the presence of anti-phosphotyrosine antibody (Thermo) and Oregon Green 448 ligand (Thermo) for 10 min at 37 °C within the linear kinetic range.

Analysis of Protein Cysteine Oxidation—For analysis of protein sulfenylation (-SOH), cells were lysed in Western solubilization buffer containing 1 mM DCP-bio1 (Kerafast, Boston, MA), 200 units/ml catalase (Worthington, Lakewood, NJ), and 10 mM *N*-ethylmaleimide (Sigma) and incubated for 1 h on ice. Protein *S*-glutathionylation was determined after cell loading with BioGEE, which was prepared by reacting 0.5 M glutathione ethyl ester (Sigma) with 0.5 M EZ-link sulfo-NHS-biotin (Pierce) in 50 mM $NaHCO_3$ (pH 8.5) and added to MTE or H292 cells at a final concentration of 250 μM for 1 h before cell treatment. After derivatization, excess DCP-bio1 reagent was removed by 6 successive washes with 20 mM Tris-HCl (pH 7.4) on Amicon Ultra-0.5 Centrifugal Filter Devices (Millipore). Similarly, excess BioGEE was removed using G25 columns (GE Healthcare). Biotin-tagged proteins were subsequently collected with high capacity NeutrAvidin-agarose beads (50 μl of a 50/50 slurry; Pierce). To address intermediate sulfenylation in protein *S*-glutathionylation, Bio-GEE-loaded cells were pretreated with 1.0 mM 5,5′-dimethyl-1,3-cyclohexanedione (dimedone) to trap sulfenic acids (60, 73), which were detected

using α-dimedone antibody (EMD Millipore). Detection of *S*-glutathionylation of recombinant EGFR used immunoprecipitation with anti-glutathione antibody (ViroGen) and A/G-agarose beads (Santa Cruz Biotechnology) and detection with α-EGFR antibody.

Detection of Extracellular H_2O_2 Production—H292 cells were seeded at 100,000 cells/well in 24 plates, and the medium was replaced with 200 μl of Hanks' balanced salt solution (HBSS) for cell stimulation with ATP or EGF for 15 min, after which HBSS was removed and mixed with 10 μg/ml lactoperoxidase (Sigma) and 1 mM tyrosine for 15 min. Reactions were terminated by the addition of 5% TCA for analysis of dityrosine production by HPLC (72).

Statistical Analysis—Statistical differences were analyzed using two-way ANOVA or Student's *t* test in GraphPad Prism 6.0, and differences were considered significant at $p < 0.05$.

Author Contributions—A. v. d. V. and D. E. H. conceived and designed the experiments. M. H., D. E. H., C. M. D, K. D., and A. H. performed the experiments. A. v. d. V., D. E. H., M. H., and C. M. D. analyzed the data. D. E. H. and A. v. d. V. wrote the paper.

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