Regulation of Hyperoxia-induced NADPH Oxidase Activation in Human Lung Endothelial Cells by the Actin Cytoskeleton and Cortactin*

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Although the actin cytoskeleton has been implicated in the control of NADPH oxidase in phagocytosis, very little is known about the cytoskeletal regulation of endothelial NADPH oxidase assembly and activation. Here, we report a role for cortactin and the tyrosine phosphorylation of cortactin in hyperoxia-induced NADPH oxidase activation and ROS production in human pulmonary artery ECs (HPAECs). Exposure of HPAECs to hyperoxia for 3 h induced NADPH oxidase activation, as demonstrated by enhanced superoxide production. Hyperoxia also caused a thickening of the subcortical dense peripheral F-actin band and increased the localization of cortactin in the cortical regions and lamellipodia at cell-cell borders that protruded under neighboring cells. Pretreatment of HPAECs with the actin-stabilizing agent phallacidin attenuated hyperoxia-induced cortical actin thickening and ROS production, whereas cytochalasin D and latrunculin A enhanced basal and hyperoxia-induced ROS formation. In HPAECs, a 3-h hyperoxic exposure enhanced the tyrosine phosphorylation of cortactin and interaction between cortactin and p47phox, a subcomponent of the EC NADPH oxidase, when compared with normoxic cells. Furthermore, transfection of HPAECs with cortactin small interfering RNA or myristoylated cortactin Src homology domain 3 blocking peptide attenuated ROS production and the hyperoxia-induced translocation of p47phox to the cell periphery. Similarly, down-regulation of Src with Src small interfering RNA attenuated the hyperoxia-mediated phosphorylation of cortactin tyrosines and blocked the association of cortactin with actin and p47phox. In addition, the hyperoxia-induced generation of ROS was significantly lower in ECs expressing a tyrosine-deficient mutant of cortactin than in vector control or wild-type cells. These data demonstrate a novel function for cortactin and actin in hyperoxia-induced activation of NADPH oxidase and ROS generation in human lung endothelial cells.

An emerging theme in vascular physiology and pathophysiology is the generation and involvement of reactive oxygen species (ROS), such as superoxide anion (O2·−) and hydrogen peroxide (H2O2), as second messengers in altering key cellular responses (1–3). As one of many potential sources of ROS, vascular NADPH oxidase plays a significant role in basal as well as agonist-mediated ROS/O2·− generation (4–6). Activation of phagocytic NADPH oxidase requires the assembly of the cytosolic p47phox, p67phox, p40phox, and Rac2 with membrane-associated cytochrome b558 reductase, which consists of p22phox and gp91phox (Nox2) (7, 8). Vascular cells express most of the subcomponents of phagocytic NADPH oxidase subunits, including Rac1 (1, 9), and recent studies have indicated that several novel Nox family isofoms, in addition to Nox2 (gp91phox), are highly expressed in vascular endothelial and smooth muscle cells (1, 10–13). We have recently demonstrated that exposure of human pulmonary artery endothelial cells (HPAECs) to hyperoxia (95% O2) increases the ROS/O2·− production that is dependent on NADPH oxidase activation and independent of the mitochondrial electron transport or xanthine/xanthine oxidase systems (10).

The mechanisms of NADPH oxidase activation are complex. In phagocytes, activation of NADPH oxidase requires serine phosphorylation of the cytosolic p47phox, p67phox, and p40phox components, assembly of the phosphorylated subunits with Rac2, and translocation to the phagosomes for association with cytochrome b558. Here, one-electron reduction of molecular O2 to O2·− occurs with NADPH as the electron donor (7). In leukocytes, formyl-Met-Leu-Phe-OH or phorbol ester stimulates phosphorylation of p47phox at multiple serine residues through reactions involving several protein kinases such as protein kinase C, protein kinase A, and mitogen-activated protein kinases (14–17). In HPAECs, tumor necrosis factor-α-mediated activation of NADPH oxidase involved p47phox phosphorylation that was dependent on PKC-δ and independent of PKC-α or PKC-βII.

2 The abbreviations used are: ROS, reactive oxygen species; EC, endothelial cell; HPAEC, human pulmonary artery endothelial cell; HUVEC, human umbilical vein endothelial cell; siRNA, silencing RNA; Nox, NADPH oxidases; O2·−, superoxide; SH3, Src homology 2; CBP, cortactin SH3 domain blocking peptide; EBM, endothelial cell basal medium; DCFDA, 6-carboxyfluorescein diacetate; HO, hyperoxia; PBS, phosphate-buffered saline; TBST, Tris-buffered saline with 0.1% Tween 20; GFP, green fluorescent protein; PRR, proline-rich regions; FBS, fetal bovine serum; MAPK, mitogen-activated protein kinase; FMLP, formylmethionylleucylphenylalanine; EGM, endothelial cell growth medium.

† To whom correspondence should be addressed: Center for Integrative Science Bldg., Rm. 408B, 929 East 57th St., Chicago, IL 60637. Tel.: 773-834-2687; E-mail: vnataraj@medicine.bsd.uchicago.edu.
lated NADPH oxidase activation and generation of O$_2^-$ is regulated by phosphatidylinositol 3-kinase (18) and protein kinase C$_\zeta$ (18, 19), and inhibition of protein kinase C$_\zeta$ attenuates tumor necrosis factor-α-mediated serine phosphorylation of p47$^{phox}$ and its interaction with gp91$^{phox}$ at the cell periphery (19). In neutrophils and macrophages, FcyR-mediated activation of NADPH oxidase and ROS production is regulated by the Vav guanine exchange factor and the phosphatidylinositol 3-kinase-dependent phosphorylation of p40$^{phox}$ (20). Hyperoxia-dependent activation of lung endothelial cell (EC) NADPH oxidase is also partly regulated by extracellular signal-regulated kinase (ERK) and p38 MAPK, suggesting the involvement of serine/threonine phosphorylation of the subcomponents of the enzyme in regulating ROS production (10). Furthermore, in HPAECs, the activation of NADPH oxidase is regulated by Src-dependent tyrosine phosphorylation of p47$^{phox}$ and the association of p47$^{phox}$, but not p67$^{phox}$, with Src (21). However, angiotensin II-mediated activation of Src in vascular smooth muscle cells results in serine phosphorylation of p47$^{phox}$, translocation from the cytosol to the membrane, and increased ROS production (22). Thus, serine or tyrosine phosphorylation of p47$^{phox}$ in response to a stimulus seems to be an important post-translational modification that regulates the assembly translocation and activation of NADPH oxidase in vascular cells (10, 21, 22). However, the mechanisms of assembly and translocation of p47$^{phox}$ with the membrane components of NADPH oxidase are yet to be completely defined.

Actin cytoskeleton and other cytoskeletal proteins may play a role in phagocytic and non-phagocytic assembly and the activation of NADPH oxidase (23–27). In phorbol ester-stimulated neutrophils, oxidase activity has been shown to co-sediment with the heavy plasma membrane fraction that contains actin and fodrin; furthermore, the labile oxidase can be stabilized by chemical cross-linking and is not extractable by Triton X-100, but p67$^{phox}$, with Src (21). However, angiotensin II-mediated activation of Src in vascular smooth muscle cells results in serine phosphorylation of p47$^{phox}$, translocation from the cytosol to the membrane, and increased ROS production (22). Thus, serine or tyrosine phosphorylation of p47$^{phox}$ in response to a stimulus seems to be an important post-translational modification that regulates the assembly translocation and activation of NADPH oxidase in vascular cells (10, 21, 22). However, the mechanisms of assembly and translocation of p47$^{phox}$ with the membrane components of NADPH oxidase are yet to be completely defined.

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In the present study, we have evaluated the role of the actin cytoskeleton and the actin-binding protein cortactin in hyperoxia-induced translocation of p47$^{phox}$ to cell periphery, activation of NADPH oxidase, and generation of ROS/O$_2^-$ in response to hyperoxia in HPAECs.

**EXPERIMENTAL PROCEDURES**

**Materials**—HPAECs, endothelial cell basal medium-2 (EBM-2), and Bullet kits were obtained from Cambrex (Walkervile, MD). Phosphate-buffered saline (PBS) was obtained from Biofluids Inc. (Rockville, MD). Ampicillin, fetal bovine serum (FBS), trypsin, MgCl$_2$, EGTA, Tris-HCl, Triton X-100, sodium orthovanadate, aprotinin, Tween 20, phallacidin, cytochalasin D, latrunculin A and Me$_2$SO were all obtained from Sigma. Dihydroethidium (hydroethidine), 6-carboxy-2',7'-dichlorodihydrofluorescein diacetate (DCFDA), Alexa Fluor 568 phallacidin, and Alexa Fluor 488 or 568 mouse, rabbit, or goat secondary antibodies were purchased from Molecular Probes (Eugene, OR). The enhanced chemiluminescence (ECL) kit was from Amersham Biosciences. SMART pool® siRNA duplex oligonucleotides targeting cortactin, gp91$^{phox}$ (Nox-2), and Src were purchased from Dharmacon, Inc. (Lafayette, CO). Polyclonal goat anti-p47$^{phox}$ antibody, p47$^{phox}$ cDNA in pGEX-C2 vector, and recombinant p47$^{phox}$ protein from baculovirus were provided by Dr. Thomas L. Leto (National Institutes of Health, Bethesda, MD). Monoclonal antibody to Src, cortactin, and phosphocortactin, and polyclonal antibody to gp91$^{phox}$ (Nox-2) were obtained from Upstate Biotechnology (Lake Placid, NY); polyclonal anti-Src and protein A/G plus-agarose were procured from Santa Cruz Biotechnology (Santa Cruz, CA). Polyclonal rabbit phospho-Src antibody was purchased from BIOSOURCE (Camarillo, CA). Glass bottom microwell dishes were obtained from MatTek Corporation (Ashland, MA). The myristoylated VDKPPVPPKKMKPV sequence comprising the cortactin SH3 domain blocking peptide (CBP) and scrambled control peptide were synthesized by Multiple Peptide Systems (San Diego, CA). HUVECs stably expressing wild-type cortactin-GFP and cortactin$^{Y421F/Y66F/Y482F}$ mutants were generated as described previously (34).

**Endothelial Cell Culture**—HPAECs at passage 5–8 in endothelial cell growth medium-2 (EGM-2) with 10% FBS, 100 units/ml penicillin, and streptomycin were grown to contact-inhibited monolayers with a typical cobblestone morphology in complete EGM-2 medium. Cells were detached from T-75 flasks with 0.05% trypsin and resuspended in fresh complete medium, then cultured in 35- or 60-mm dishes or on glass coverslips for immunofluorescence studies. All cells were starved overnight in EGM-2 medium containing 1% FBS prior to exposure to normoxia or hyperoxia.

**Exposure of Cells to Hyperoxia**—HPAECs (at ~90% confluence) in complete EGM-2 medium were placed in a humidity-controlled airtight modulator incubator chamber (Billups-Rothenberg, Del Mar, CA) and flushed continuously with 95% O$_2$, 5% CO$_2$, 95% air atmosphere as described previously (10, 21). Cells were detached from T-75 flasks with 0.05% trypsin and resuspended in fresh complete medium, then cultured in 35- or 60-mm dishes or on glass coverslips for immunofluorescence studies. All cells were starved overnight in EGM-2 medium containing 1% FBS prior to exposure to normoxia or hyperoxia.

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1. The recruitment of Src to the cell periphery by hyperoxia and the actin cytoskeleton is essential for the generation of ROS.
2. Src plays a role in the activation of NADPH oxidase by hyperoxia.
3. The interaction between cortactin, actin, and p47$^{phox}$ is essential for the generation of ROS.
4. The association between cortactin and p47$^{phox}$ is essential for the generation of ROS.

These results suggest that cortactin may function as a scaffold protein to link and organize the assembly of NADPH oxidase components with the actin cytoskeleton during the generation of ROS in response to hyperoxia in HPAECs.
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digital oxygen monitor. The buffering capacity of the cell culture medium did not change significantly during the period of hyperoxic exposure and was maintained at pH 7.4.

Transfection and Infection of HPAECs—To optimize conditions for efficient transfection, HPAECs were transfected with Fl-luciferase GL2 duplex siRNA (target sequence: 5′-CGTACGGGAATCTGCGGA-3′, Dharmacon) as a positive control. HPAECs grown to ~60–70% confluency in 35-mm dishes were transfected with Gene Silence® (Gene Therapy System, Inc., San Diego, CA) transfecting agent plus cortactin or Src siRNA (100 nM) or Nox-2 siRNA (50 nM) in serum-free EBM-2 medium according to the manufacturer’s recommendations. At 3 h post-transfection, 1 ml of fresh complete EGM-2 medium containing 10% FBS was added, and the cells were cultured for an additional 48 h for analysis of Nox-2 protein expression or 72 h for analysis of cortactin or Src protein expression by Western blotting. For experiments using scrambled control and myristoylated peptides, HPAECs (35-mm dishes; ~50% confluence) were incubated with 10 μg/ml CBP or control peptide for 45 min (35) prior to exposure to hyperoxia. HPAECs were infected with retroviral GFP-tagged wild-type cortactin or tyrosine-deficient triple mutant cortactin for 48 h as previously described (34, 36). In some experiments, HPAECs grown to ~50% confluence on glass coverslips were transfected with 1 μg of plasmid DNA/ml of p47phox-GFP and FuGENE 6 (3 μg/ml) for 3 h in serum-free EGM-2 medium according to the manufacturer’s recommendations. The medium was replaced by complete EBM-2, and the cells were incubated for 24 h post-transfection.

Determination of Hyperoxia-induced Production of O2− and Total ROS—Hyperoxia-induced O2− release by HPAECs was measured by hydroethidine fluorescence as described (10, 21). Total ROS production in HPAECs exposed to either normoxia or hyperoxia was determined by the DCFDA fluorescence method (21). In brief, HPAECs (~90% confluent in 35-mm dishes) were loaded with 10 μM DCFDA in EBM-2 basal medium and incubated at 37 °C for 30 min. Fluorescence of oxidized DCFDA in cell lysates, an index of ROS formation, was measured with an Aminco Bowman series 2 spectrophotometer with excitation and emission set at 490 and 520 nm, respectively, and appropriate blanks. The extent of ROS formation was expressed as % of the normoxic control.

ROS/O2− Detection and Quantification in Cells by Epifluorescence Microscopy—Hyperoxia-induced formation of ROS/O2− was also quantified by fluorescence microscopy (21). HPAECs (~90% confluent) in 35-mm dishes or glass bottom microwell dishes were loaded with DCFDA or hydroethidine (10 μM) in EBM-2 basal medium for 30 min at 37 °C in a 95% air, 5% CO2 environment. After 30 min of loading, the medium containing DCFDA or hydroethidine was aspirated and the cells were rinsed once with EGM-2 complete medium, then preincubated with agents for the indicated time periods, followed by exposure to either normoxia (95% air, 5% CO2) or hyperoxia (95% O2, 5% CO2) for 1–3 h. At the end of the incubation, the cells were washed twice with PBS at room temperature and examined under a Nikon Eclipse TE 2000-S fluorescence microscope with a Hamamatsu digital CCD camera (Japan), using a ×20 or ×60 objective lens and MetaVue software (Universal Imaging Corp., PA).

Immunofluorescence Microscopy—HPAECs grown on gelatinized 9-mm coverslips to ~95% confluence were exposed to either normoxia or hyperoxia for 1–3 h. Coverslips were washed twice with PBS at room temperature, permeabilized for 2 min in PBS containing 0.25% Triton X-100 and 3.7% formaldehyde, rinsed once with PBS, and fixed in 3.7% formaldehyde and 3% paraformaldehyde for 20 min at room temperature. The cells were rinsed three times in PBS and incubated for 30 min at room temperature in TBST blocking buffer containing 1% bovine serum albumin. Cells were incubated with primary antibodies (1:200 dilution in blocking buffer, 1 h), then thoroughly rinsed with TBST and stained with Alexa Fluor secondary antibodies (1:200 dilution in blocking buffer for 1 h). Cells were examined with a Nikon Eclipse TE 2000-S fluorescence microscope and Hamamatsu digital camera (Japan), using a ×60 oil immersion objective lens (10, 21, 37). Some images were captured with a Coolsnap HQ CCD (Roper, Duluth, GA) and Openlab software (Improvision, Lexington, MA).

Preparation of Cell Lysates, Immunoprecipitation, and Western Blotting—HPAECs grown on 100-mm dishes (to ~90% confluence) were serum-deprived for ~16–18 h in EBM-2 containing 1% FBS; all subsequent incubations were carried out in serum-free minimal essential medium. Cells were scraped into 1 ml of modified lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 0.25% sodium deoxycholate, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM Na3VO4, 1 mM NaF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 μg/ml pepstatin), sonicated on ice with a probe sonicator (3 times for 15 s), and centrifuged at 5,000 × g in a microcentrifuge (4 °C for 5 min). Protein concentrations of the supernatants were determined using a Pierce protein assay kit. Equal volumes of the supernatants, adjusted to 1 mg of protein/ml (cell lysates), were denatured by boiling in 6× SDS sample buffer for 5 min, and samples were separated on 10% SDS-PAGE gels and analyzed by Western blotting. For immunoprecipitation, cell lysates (0.5–1 mg of protein) were incubated overnight with anti-Src or anti-cortactin antibody conjugated to agarose for 2 h at 4 °C, then centrifuged at 5,000 × g in a microcentrifuge, dissociated by boiling in 2× SDS sample buffer for 5 min, and separated on 10% SDS-PAGE precast gels (21, 37). Protein bands were transferred overnight (24 V, 4 °C) on a polyvinylidene difluoride membrane (Millipore), probed with primary and secondary antibodies according to the manufacturer’s protocol, and detected using the Enhanced Chemiluminescence kit (Amersham Biosciences). The blots were scanned (UMAX Power Lock II) and quantified by an automated digitizing system UN-SCAN-IT GEL (Silk Scientific Corporation).

Statistics—Analysis of variance and Student-Newman-Keul’s test were used to compare means of two or more different treatment groups. The level of significance was set to p < 0.05 unless otherwise stated. Data are expressed as mean ± S.E.
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RESULTS

Hyperoxia-induced ROS Production Is Dependent on Nox-2 (gp91phox), but Not Mitochondrial Electron Transport, in HPAECs—In our recent studies we have shown that antisense to p22phox (10) or p47phox siRNA (21) attenuated hyperoxia-induced ROS generation in HPAECs. Furthermore, we have demonstrated that the hyperoxia-induced ROS production was insensitive to allopurinol or rotenone (10), suggesting non-involvement of xanthine/xanthine oxidase or mitochondrial electron transport as a source of hyperoxia-induced ROS in ECs. To further address the role of Nox-2 (gp91phox) and mitochondrial electron transport as potential sources of ROS, we used Nox-2 siRNA and specific inhibitors of complex II (rotenone) and complex III (stigmatellin) of the mitochondrial electron transport. Transfection of HPAECs with 50 nm Nox-2 siRNA, but not scrambled siRNA, for 72 h resulted in a significant downregulation of Nox-2 protein and mRNA expression (Fig. 1, A and B). The effect of Nox-2 siRNA was specific as it had no effect on either Nox-1 or Nox-3 mRNA expression (data not shown). Furthermore, under similar experimental conditions, Nox-2 siRNA, but not scrambled siRNA, reduced hyperoxia-induced (3 h) ROS/O2 production in HPAECs as measured by DCFDA/hydroethidine oxidation (Fig. 1, C and D). To test the importance of mitochondria as a potential source for ROS, HPAECs were pretreated for 1 h with mitochondrial electron transport complex I inhibitor rotenone (2 μM) or complex III inhibitor stigmatellin (10 μM). As shown in Fig. 1E, both inhibitors had no effect on either the basal or hyperoxia-induced ROS/O2 generation. These data are consistent with a role of Nox-2, but not mitochondrial electron transport chain, in hyperoxia-induced ROS production in human lung ECs.

Hyperoxia-induced ROS Production Is Regulated by Actin Cytoskeleton Rearrangement in HPAECs—To establish a role for actin cytoskeleton in hyperoxia-mediated NADPH oxidase activation and ROS generation, HPAECs were exposed to hyperoxia for 3 h. Staining of cell monolayers with phalloidin showed marked differences in F-actin distribution under conditions of normoxia and hyperoxia (Fig. 2A). In normoxia, cells revealed typical faint F-actin staining with a few stress fibers in the central area of the cell; however, exposure to hyperoxia (3 h) led to increased F-actin reorganization and stress fiber formation in the center of the cell and thickening of the actin staining near cell periphery (Fig. 2A).

We then used actin-stabilizing and -disrupting agents to determine whether the actin cytoskeleton plays a functional role in hyperoxia-induced ROS/O2 production. Hyperoxia (3 h) increased the assembly of F-actin near the cell periphery, and pretreatment of HPAECs for 60 min with 1 μM phallacin, an actin filament-stabilizing agent (38), significantly reduced hyperoxia-induced accumulation of F-actin near the cell periphery (Fig. 2A). In addition, phallacin pretreatment blocked hyperoxia-induced ROS generation (Fig. 2B). These results suggest a role for actin reorganization in hyperoxia-induced ROS production in HPAECs. The actin depolymerizing agent cytochalasin D (1 μM) (39) decreased net actin polymerization, as evidenced by a reduction in the number of phalloidin-stained stress fibers (Fig. 2A). However, the enhanced basal ROS production and effect of cytochalasin D on ROS formation...
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FIGURE 2. Effects of phallacidin, cytochalasin D, and latrunculin A on hyperoxia-induced actin reorganization and ROS production. HPAECs grown in 8-well glass slide chambers (A) or on 35-mm dishes (B) were pretreated with 1 μM phallacidin, cytochalasin D, or latrunculin A for 60 min prior to exposure to either normoxia or hyperoxia for 3 h. A, cells were fixed and immunostained for actin with phalloidin. B, cells were pretreated, then loaded with DCFDA (10 μM) for 30 min prior to normoxic or hyperoxic (3 h) exposure. Oxidation of DCFDA was analyzed by epifluorescence microscopy, and fluorescent intensity was calculated as described under “Experimental Procedures.” Values for ROS production are mean ± S.D. from three independent experiments done in triplicate. *, significantly different from normoxia (p < 0.05); **, significantly different from untreated hyperoxia (p < 0.01).

were additive with hyperoxia (Fig. 2B). Furthermore, destabilization of actin filaments by 1 μM latrunculin A (40) increased ROS production in HPAECs under both normoxic and hyperoxic conditions (Fig. 2B). Thus, these results suggest a role for the microfilament cytoskeleton in hyperoxia-induced ROS production in HPAECs.

Cortactin Depletion Attenuates Hyperoxia-induced Actin Rearrangement and ROS Production in HPAECs. Cortactin, an actin-binding protein, is involved in the regulation of cortical actin rearrangement, and growth factor stimulation results in its translocation and localization to lamellipodia and membrane ruffles (41–43). We next examined the effect of hyperoxia on the organization of cortactin and explored the role of cortactin in ROS production in HPAECs. Cells under conditions of normoxia showed a diffused actin and cortactin distribution, with little localization near the cell periphery (Fig. 3, A and B). Exposure to hyperoxia (3 h) induced actin stress fibers (Fig. 3D), and translocation of cortactin to regions near the cell periphery and areas of membrane ruffle (Fig. 3E). Furthermore, hyperoxia enhanced the interaction between actin filaments and cortactin in membrane ruffles as evidenced by immunofluorescence co-localization of actin and cortactin (Fig. 3F). These results indicate that both actin and cortactin undergo reorganization and targeting to lamellipodia and membrane ruffles in response to hyperoxia in HPAECs.

Our earlier results showed that hyperoxia-mediated ROS/O$_2^-$ generation in HPAECs is dependent upon NADPH oxidase activation, rather than mitochondrial electron transport or xanthine/xanthine oxidase (10). To analyze the functional role of cortactin in hyperoxia-induced ROS/O$_2^-$ generation, we developed molecular strategies to down-regulate or block cortactin action. HPAECs were pretreated for 72 h with cortactin siRNA, which down-regulated >90% of cortactin protein expression (Fig. 4C). This cortactin down-regulation reduced hyperoxia-induced oxidation of DCFDA fluorescence (ROS production) and hydroethidine fluorescence (O$_2^-$ production) by ~2-fold below normoxic conditions (Fig. 4, A and B). Also, cortactin siRNA attenuated hyperoxia-induced F-actin reorganization and thickening of the peripheral actin band (Fig. 5). Semi-quantitation of the co-localization of cortactin with actin, using an image analyzer, showed a ~2.2-fold increase in the intensity of yellow color in scrambled cells exposed to hyperoxia as compared with cortactin siRNA-transfected cells (scrambled siRNA: normoxia, 100 ± 10%; hyperoxia, 229 ± 12%; cortactin siRNA: normoxia, 100 ± 15%; hyperoxia, 89 ± 14%). Taken together, these results suggest that cortactin regulates hyperoxia-dependent ROS production and actin rearrangement in HPAECs.

Tyrosine Phosphorylation of Cortactin Is Essential for Hyperoxia-induced ROS Production—Src-dependent tyrosine phosphorylation of human cortactin at Tyr$^{421}$, Tyr$^{466}$, and Tyr$^{482}$ has been implicated in agonist-induced changes in the cytoskeleton and cell morphology (44), cell migration (45), and endothelial barrier enhancement (35). We have previously demonstrated that hyperoxia stimulates Src and Src-dependent activation of NADPH oxidase and ROS/O$_2^-$ production in HPAECs (21). However, very little information is available concerning the hyperoxia-induced tyrosine phosphorylation of cortactin and activation of NADPH oxidase/ROS production. We characterized the hyperoxia-mediated tyrosine phosphorylation of cortactin by Src and the role of this tyrosine phosphorylation of cortactin in ROS/O$_2^-$ generation. HPAECs were exposed to either normoxia or hyperoxia for varying time periods (0.5–3 h), and total cell lysates were immunoprecipitated with anti-cortactin antibody. Immunoblot analysis revealed that hyperoxia increased the tyrosine phosphorylation of cortactin in a time-dependent manner (Fig. 6A). This was done by

![Figure 3](image-url) Actin and cortactin co-localize at the cell periphery after hyperoxia. HPAECs grown on glass slides were exposed to normoxia (A–C) or hyperoxia (D–F) (3 h), which resulted in the redistribution of actin and cortactin to the cell periphery. Co-localization of actin (green) (A and D) and cortactin (red) (B and E) is indicated by the merged images (C and F) by yellow color at the cell periphery and in membrane ruffles. An representative images from one of several independent experiments is shown. Scale bar, 20 μm.
immunoblotting the immunoprecipitates with anti-phospho-
tyrosine antibody, and the total cell lysates using anti-phospho-
cortactin antibody revealed that hyperoxia increased the tyro-
sine phosphorylation of cortactin in a time-dependent manner
(Fig. 6A). To query the role of Src in hyperoxia-induced tyrosine
phosphorylation of cortactin, we transfected HPAECs with Src siRNA, which down-regulated Src protein expression
by 80% (Fig. 6B). The specificity of this reaction was indicated
by the fact that Src siRNA had no effect on the expression of
Yes, a member of the Src family (Fig. 6C). As shown in Fig. 6B,
cells pretreated with Src siRNA showed a decreased level of
hyperoxia-induced cortactin tyrosine phosphorylation than
hyperoxia-exposed cells that were treated with scrambled

FIGURE 4. Cortactin siRNA attenuates hyperoxia-induced ROS/O₂° produc-
tion. HPAECs grown on 35-mm dishes were transfected with scrambled siRNA
(scRNA) or cortactin siRNA (100 nM) for 72 h. Cells were loaded with 10 μM DCFDA
(A) or hydroethidine (B) for 30 min and washed once with basal medium. Cells in
A or B were exposed to normoxia (N) or hyperoxia (HO) for 3 h. At the end of the
exposure, the formation of total ROS (A) or superoxide (O₂°) was measured as
described under “Experimental Procedures.” The formation of total ROS (A) and
production of O₂° (B) in cells were visualized under the fluorescence microscope
and quantified. Values are mean ± S.D. from three independent experiments
done in triplicate and expressed as % control. *, significantly different from nor-
moxia (p < 0.05); **, significantly different from scRNA-treated hyperoxia.

FIGURE 5. Inhibition by cortactin siRNA of hyperoxia-induced actin-cortactin
co-localization at the cell periphery. HPAECs grown on glass coverslips were
transfected with scrambled siRNA or cortactin siRNA for 72 h as described under
“Experimental Procedures.” Cells were exposed to normoxia (N) or hyperoxia
(HO) for 3 h, washed, fixed, permeabilized, and probed with anti-cortactin (green)
antibody or phalloidin (red) for actin. Under normoxic conditions, cortactin is
distributed diffusely in the cytosol, and the peripheral band of actin is thin. After
exposure to hyperoxia, both cortactin (green) and actin (red) are partially redis-
tributed to the cell periphery and sites of membrane ruffles, where they appear to
be co-localized (yellow overlap on merged image). This localization was blocked
by cortactin siRNA. A representative immunofluorescence micrograph from
three independent experiments is shown.

FIGURE 6. Inhibition of hyperoxia-mediated tyrosine phosphorylation of
cortactin by cortactin siRNA. A, HPAECs grown on 35-mm dishes were
exposed to normoxia or hyperoxia (3 h) for varying time periods, cell lysates
were subjected to immunoprecipitation with anti-cortactin antibody, sepa-
rated by SDS-PAGE, and Western blotted with antibodies against phospho-
Src, phospho-cortactin, and cortactin antibodies. B, HPAECs grown on 35-mm
dishes were transfected with scrambled or Src siRNA (100 nM) for 72 h as
described under “Experimental Procedures,” then exposed to normoxia or
hyperoxia (1–3 h). After lysis, cell lysates were immunoprecipitated with anti-
cortactin antibodies and analyzed by SDS-PAGE and Western blotting with
anti-phosphocortactin or cortactin antibodies. Total cell lysates were also
analyzed for the expression of Src and Yes after SDS-PAGE and Western blot-
ting with anti-Src or Yes antibodies. Representative blots from three inde-
pendent experiments are shown for each of the experiments in this figure.

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siRNA. These data suggest that hyperoxia-induced tyrosine phosphorylation of cortactin is Src-dependent in HPAECs.

To further characterize the role of tyrosine cortactin in hyperoxia-induced ROS generation, we then transiently overexpressed a cortactin-F-GFP mutant in HPAECs in which tyrosine residues Tyr421, Tyr466, and Tyr482 have been replaced by phenylalanine (34, 35). After 72 h of retroviral infection, HPAECs expressed the cortactin-GFP mutant, and showed decreased hydroethidine fluorescence in living cells, as demonstrated by epifluorescence microscopy (Fig. 7A). Expression of the Y-F cortactin mutant attenuated hyperoxia-induced superoxide formation (vector with normoxia = 100%; vector with hyperoxia = 240%; cortactin mutant with normoxia = 90%; cortactin mutant with hyperoxia = 128%; Fig. 7B). Similarly, HUVECs stably expressing the cortactin-F mutant showed decreased ROS production in response to hyperoxia (Fig. 7C). These results indicate that tyrosine phosphorylation of cortactin is important for hyperoxia-induced ROS production in HPAECs.

**Hyperoxia Enhances the Association of p47phox with Cortactin**—We have previously demonstrated that hyperoxia enhances the migration and association of p47phox with Src (21). Based on our observation that Src is involved in the hyperoxia-mediated tyrosine phosphorylation of cortactin, we investigated the possibility that NADPH oxidase could be activated through an enhanced interaction between cortactin and oxidase components in HPAECs. For this purpose, we transfected HPAECs with a control GFP-vector or GFP-p47phox plasmid for 48 h, exposed the cells to normoxia or hyperoxia for 3 h, and analyzed the localization of cortactin and p47phox using immunofluorescence microscopy. As shown in Fig. 8A, most of the GFP-p47phox was localized in the cytoplasm and perinuclear region in normoxic cells. Hyperoxia caused the redistribution of GFP-p47phox from the cytoplasm to the cell periphery (green), where it was concentrated in lamellipodia with a somewhat punctate appearance (Fig. 8D). Similarly, hyperoxia induced the translocation of cortactin (red) to the cell periphery (Fig. 8E), in contrast to the faint and diffuse distribution in normoxic cells (Fig. 8B). Furthermore, p47phox co-localized (green) and cortactin (red) co-localized (yellow in merged images, Fig. 8F) at the cell periphery under conditions of hyperoxia. The role of cortactin in hyperoxia-induced translocation of p47phox to the cell periphery was then investigated using cortactin siRNA. When compared with scrambled siRNA, down-regulation of cortactin with cortactin siRNA decreased the translocation of cortactin and p47phox to the cell periphery and areas of membrane ruffles and the co-localization of cortactin with p47phox in these ruffles (Fig. 9). Further semi-quantitation of the cortactin-p47phox co-localization in Fig. 9 by an image analyzer revealed an ~4.2-fold increase in the intensity of the yellow color in scrambled cells exposed to...
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FIGURE 8. Cortactin and GFP-p47phox co-localize at the cell periphery after hyperoxia. HPAECs grown to ~50% confluence on glass coverslips were transfected with 1 μg of plasmid DNA/ml of p47phox-GFP. 24 h later, cells were exposed to either normoxia (A–C) or hyperoxia (D–F) (3 h), and prepared for immunofluorescence analysis of cortactin (green) and p47phox-GFP localization (red). In normoxic conditions, cortactin and p47phox-GFP were distributed diffusely in the cytosol and perinuclear areas, respectively. Hyperoxia induced redistribution of both proteins to the cell periphery, where they appear to co-localize (yellow in F). A representative image from several independent experiments is shown. Scale bar, 20 μm.

FIGURE 9. Down-regulation of cortactin with cortactin siRNA prevents hyperoxia-induced translocation of p47phox and association with cortactin. HPAECs grown to ~50% confluence on glass coverslips were transfected with scrambled siRNA or cortactin siRNA for 72 h as described under “Experimental Procedures.” Cells were exposed to normoxia or hyperoxia (3 h), washed, fixed, permeabilized, probed with anti-cortactin or anti-p47phox antibodies, and examined by immunofluorescence microscopy using a ×60 oil objective. The cortactin (green) and p47phox (red) images show matched cell fields for each condition. Exposure of cells to hyperoxia resulted in redistribution of cortactin and p47phox to the cell periphery, whereas cortactin siRNA blocked the p47phox redistribution and co-localization (yellow in merged images). A representative image from several independent experiments is shown.

Western blotting. p47phox and Src were co-immunoprecipitated with cortactin under normoxic conditions, and exposure to hyperoxia further increased this association (Fig. 10A). In addition, Western blotting also revealed the presence of cortactin and p47phox in the Src immunoprecipitates under normoxic conditions, and this was increased by hyperoxia (Fig. 10B). Taken together, these results indicate that hyperoxia increases the association between Src, cortactin, and p47phox in HPAECs.

Tyrosine Phosphorylation of Cortactin Is Essential for Hyperoxia-induced Association of Cortactin and p47phox—Having established a role for the tyrosine phosphorylation of cortactin in hyperoxia-induced ROS production, we next asked whether hyperoxia-induced phosphorylation of cortactin on tyrosines is critical for its interaction with p47phox. The role of Src was investigated first. Transfection of HPAECs with Src siRNA for 72 h abolished the hyperoxia-induced association of p47phox with cortactin (Fig. 11A), suggesting that tyrosine phosphorylation of cortactin is an essential component of its translocation and co-localization at the cell periphery. Semiquantitation of the co-localization of cortactin with p47phox from Fig. 11A, using an image analyzer, showed ~3-fold increase in the intensity of yellow color in scrambled cells exposed to hyperoxia as compared with Src siRNA-transfected cells (scrambled siRNA: normoxia, 100 ± 11%; hyperoxia, 302 ± 13%; Src siRNA: normoxia, 100 ± 24%; hyperoxia, 145 ± 14%). Next, we evaluated Src siRNA effects on actin association with cortactin in membrane ruffles following hyperoxia. As shown in Fig. 11B, pretreatment with Src siRNA blocked increased interaction between actin and cortactin following hyperoxia (3 h). Analysis of the co-localization of cortactin with actin from Fig. 11B, using an image analyzer, showed an ~2-fold increase in the intensity of yellow color in scrambled cells exposed to hyperoxia as compared with Src siRNA-transfected cells (scrambled siRNA: normoxia: 100 ± 9%; hyperoxia, 198 ± 8%; Src siRNA: normoxia: 100 ± 8%; hyperoxia, 108 ± 7%).

Next, we used HUVECs stably expressing GFP-tagged wild-type cortactin or the Cortactin-F mutant to assess the role of tyrosine phosphorylation of cortactin in the hyperoxia-enhanced peripheral translocation and co-localization of cortactin and p47phox (34). Both wild-type GFP-cortactin and native p47phox were mainly dispersed throughout the cytoplasm (Fig. 12); however, 3 h of hyperoxia resulted in the translocation of hyperoxia as compared with cortactin siRNA-transfected cells (scrambled siRNA: normoxia, 100 ± 4%; hyperoxia, 424 ± 13%; cortactin siRNA: normoxia, 100 ± 6%; hyperoxia, 146 ± 11%).

Interactions between cortactin, p47phox, and Src were further investigated by co-immunoprecipitation studies. HPAECs were exposed to either normoxia or hyperoxia (3 h), and cell lysates (500 μg of protein) were subjected to immunoprecipitation with anti-cortactin or anti-Src antibodies and analyzed by
both wild-type GFP-cortactin and p47phox to the cell periphery and merging of the immunofluorescence images of wild-type GFP-cortactin (green) and p47phox (red) at the cell periphery. However, in HUVECs stably expressing cortactin-F-GFP (triple mutation at Tyr421, Tyr466, and Tyr482), exposure to hyperoxia did not induce a similar translocation and co-localization of GFP-cortactin and p47phox (Fig. 12). Taken together, these data show that the increased tyrosine phosphorylation of cortactin at Tyr421, Tyr466, and Tyr482 that is mediated by hyperoxia regulates the peripheral translocation and intracellular interaction of cortactin with p47phox in HPAECs.

**Interactions Involving the Cortactin SH3 Domain Mediate the Hyperoxia-induced Association with p47phox and ROS Generation**—Cortactin contains a C-terminal SH3 domain that is required for interaction with cytoskeleton-associated proteins such as CortBP-1, dynamin, ZO-1, WIP, and EC MLCK (46–49). To assess the functional importance of the cortactin SH3 domain in hyperoxia-induced translocation of p47phox to the cell periphery, the association with cortactin, and ROS production, we effectively blocked interactions at this site by pre-incubating HPAECs for 45 min with a cell-permeable myristoylated peptide derived from CortBP-1 that mimics the optimal binding sequence for the cortactin SH3 domain (35). Pretreatment of cells with a CBP (10 μM) for 45 min effectively prevented hyperoxia-mediated translocation of p47phox to the cell periphery and co-localization of p47phox with cortactin, when compared with cells treated with a control peptide (Fig. 13A). However, the relocalization of cortactin to membrane ruffles and areas of lamellipodia was not altered by CBP. Furthermore, cells treated with the cortactin-blocking peptide showed reduced hyperoxia-induced ROS production (by ~1.5-fold) when compared with the cells treated with the control peptide (Fig. 13B). This suggests that interactions occurring via the cortactin SH3 domain mediate the translocation of p47phox, the association of p47phox with cortactin, and the increase in ROS production following hyperoxia.

**DISCUSSION**

In the present study, we provide novel data that support a role for cortactin in the hyperoxia-induced translocation of p47phox to the cell periphery and its generation of ROS/O₂⁻ in
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human lung ECs. Our results also suggest that the interaction between cortactin and p47phox, which facilitates NADPH oxidase activation and ROS production, is dependent on Src and cortactin association with cortactin. Whereas the exact mechanisms governing the hyperoxia-mediated association of cortactin, p47phox, and Src in NADPH oxidase assembly, activation, and ROS generation remain unclear, we have previously demonstrated that hyperoxia enhances the association of p47phox, but not p67phox, with Src and ROS production in HPAECs (21).

Accumulating evidence supports a link between the actin cytoskeleton and actin-binding proteins in the activation of phagocytic and non-phagocytic NADPH oxidase (23–27). Stimulation of neutrophils with phorbol ester induces translocation of p47phox to the cytoskeleton without altering the distribution of either p40phox or p67phox and increases the oxidase activity that co-sediments with a heavy plasma membrane fraction consisting of actin and fodrin (28, 29). A similar association of p47phox with actin has been demonstrated in vascular smooth muscle and endothelial cells (31, 33, 51). Earlier studies have suggested that disruption of actin fibers at the plasma membrane and phagosomes enhances the fMLP-triggered oxidative burst in phagocytes (26). These observations are further supported in the present study of lung ECs by our demonstration that disruption or depolymerization of actin filaments by cytochalasin D or latrunculin A markedly increased basal and hyperoxia-mediated ROS production, whereas stabilization of actin filaments with phalloidin blocked the oxidative response. The molecular mechanisms of cytochalasin D- and latrunculin A-mediated modulation of ROS production are unclear; however, in neutrophils cytochalasin B is known to potentiate the fMLP-induced accumulation of diacylglycerol, which could activate NADPH oxidase via protein kinase C (26, 39). In the present study, we did not evaluate diacylglycerol levels after pretreatment of HPAECs with cytochalasin D, but we observed that cytochalasin D activated Rac1, a key regulator and activator of NADPH oxidase in non-phagocytic cells (data not shown). Thus, activation of both phagocytic and non-phagocytic NADPH oxidase by actin-destabilizing agents such as cytochalasin D may involve multiple signaling pathways associated with actin cytoskeletal reorganization.

In addition to actin, actin-binding proteins such as coronin and cortactin are known to interact with NADPH oxidase subcomponents and participate in the regulation of oxidase-dependent ROS production. In neutrophils, p67phox has been shown to co-purify with a coronin (57 kDa), and binding studies have revealed that coronin interacts with the C-terminal half of p47phox (52). Furthermore, stimulation of macrophages with opsonized zymosan leads to recruitment of coronin with phosphatidylinositol 3-kinase to membranes of nascent and early phagosomes that co-localize with actin cytoskeleton (53). It is particularly interesting that the interaction between coronin and F-actin in adherent neutrophils is markedly diminished in cells from patients lacking p47phox or p67phox, suggesting malfunctioning of the cytoskeleton in various genetic forms of chronic granulomatous disease (52). However, the role of coronin in regulating phorbol ester-dependent NADPH oxidase activity was not evaluated in that study.

Another interesting and novel observation in the present study was the hyperoxia-enhanced association between p47phox, cortactin, and actin, as evidenced by co-immunoprecipitation and immunofluorescence microscopy studies. Down-regulation of cortactin with cortactin siRNA blocked hyperoxia-dependent translocation of p47phox to the cell periphery as well as ROS/O2 formation, indicating a role for cortactin in NADPH oxidase assembly and activation. Although an earlier report by Touyz et al. (33) has shown that angiotensin II stimulates the p47phox/actin interaction through cortactin in human resistance and coronary artery smooth muscle cells, our current study is the first to demonstrate that cortactin can regulate NADPH oxidase activity and ROS production in human lung ECs. We have previously demonstrated that hyperoxia activates Src and Src-dependent tyrosine phosphorylation of p47phox, that it enhances the association of Src with p47phox, and that this association between Src and p47phox is critical to ROS/O2 production in HPAECs (21). Given that cortactin is a substrate for Src kinase, we have shown in the present study that hyperoxia-stimulated tyrosine phosphorylation of cortactin involves Src and that Src siRNA can attenuate...
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hyperoxia-mediated co-localization of cortactin with p47^{phox} and of cortactin with actin at the cell perimeter. These results suggest that the Src-mediated tyrosine phosphorylation of cortactin serves as a mechanism to regulate the translocation of p47^{phox} to the cell periphery, the activation of NADPH oxidase, and the generation of ROS.

Cortactin is involved in many aspects of cytoskeleton-mediated cellular function, such as cell motility, changes in shape (34), and barrier enhancement (35). It is also targeted to sites of actin polymerization and rearrangement (44, 54, 55). Translocation of cortactin from the cytosol to the periphery of the cell is observed in several types of mammalian cells in response to a variety of stimuli (43). In HPAECs, sphingosine 1-phosphate induces translocation of cortactin to the cell periphery and to membrane ruffles (35), whereas shear stress acts through Rac GTPase to cause the translocation of cortactin to the periphery in ECs (56). In the present study, we have provided evidence for cortactin rearrangement to the cell periphery in response to hyperoxia. Furthermore, the data presented here suggest that the cortactin-p47^{phox} interaction acts as an important platform at the EC periphery during hyperoxia-induced activation of NADPH oxidase and generation of ROS. It is interesting that although our co-immunoprecipitation and immunofluorescence microscopy data suggest that Src enhances the association between cortactin and p47^{phox} after exposure of HPAECs to hyperoxia, we did not see any enhanced association of cortactin with p67^{phox} under these conditions (data not shown).

Human cortactin, a target of Src, is tyrosine-phosphorylated at Tyr^{231}, Tyr^{466}, and Tyr^{282} (57). As demonstrated earlier, exposure of HPAECs to hyperoxia activates Src, and blocking Src with Src siRNA attenuates hyperoxia-induced tyrosine phosphorylation of p47^{phox} and NADPH oxidase-dependent ROS production (21). Our present data involving HPAECs and HUVECs transfected with a vector control or with wild-type or mutant cortactin indicate that hyperoxia-induced tyrosine phosphorylation of cortactin is essential for its interaction with p47^{phox} and with actin as well as ROS production. These results are in agreement with the reported role of cortactin tyrosine phosphorylation in hydrogen peroxide-induced shape changes and migration of NIH 3T3 cells (34). In contrast, the cortactin-dependent pulmonary EC barrier enhancement by sphingosine 1-phosphate is not affected by blocking Src with PP2, suggesting that tyrosine phosphorylation of cortactin is not involved in this process (35). It is also unclear whether tyrosine phosphorylation is important for the interaction of cortactin with p47^{phox} that occurs after human vascular smooth muscle cells are stimulated with angiotensin II (33).

Although the mechanisms that govern the interactions between cortactin and p47^{phox} are as yet unclear, it appears that the SH3 and proline-rich regions (PRR) of cortactin (57) and the PRR, PX, and SH3 domains of p47^{phox} (51) may be involved in the enhanced association that is stimulated by hyperoxia. We found that incubation of HPAECs with a myristoylated peptide that blocks the cortactin SH3 binding site caused an attenuation of the hyperoxia-induced production of ROS, translocation of p47^{phox} to the cell periphery, and co-localization with cortactin. However, the SH3 binding peptide did not affect the hyperoxia-induced relocation of cortactin to the cell periphery.

In addition to PRR and two tandem SH3 domains, p47^{phox} contains a novel PX domain, which is responsible for its association with the actin cytoskeleton and subsequent translocation to the plasma membrane (51). Studies to further characterize the role of the SH3, PRR, and PX domains that regulate interactions between cortactin, p47^{phox}, and NADPH oxidase are ongoing.

Whereas professional phagocytes generate excess O_2^- by activation of phagocytic NADPH oxidase, recent studies suggest that the vascular endothelial and smooth muscle cells are fully capable of ROS/O_2^- production (4–6). Furthermore, ROS generated by the vascular cells have emerged as important second messengers modulating signal transduction pathways regulating vascular growth, cell motility, cytoskeletal reorganization, and barrier function (1–3). Although mitochondria seems to play a major role in 4-hydroxynonenal- or hypoxia/reoxygenation-induced ROS formation in vascular smooth muscle cells, and human umbilical vein ECs (58, 59), the present results suggest that mitochondria not be involved in hyperoxia-induced ROS generation in HPAECs. However, we have earlier demonstrated that 4-hydroxynonenal modulated ROS production in bovine microvascular lung ECs via mitochondrial electron transport (37), suggesting stimuli-dependent stimulation of either NADPH oxidase or mitochondria in endothelial ROS production. Furthermore, our results with Nox-2 siRNA clearly show that activation of non-phagocytic Nox-2 produces ROS/O_2^- by hyperoxia in HPAECs. A recent study has implicated recruitment of Nox-2 into the endosomal compartment for interleukin-1β-stimulated O_2^- formation in mammary epithelial MCF-7 cells (50). However, exposure of HPAECs to hyperoxia (3 h) did not induce ROS/O_2^- generation in the endosomes as determined by in vivo co-localization of early endosomes and ROS using OxyBURST Green dihydro-2',4,5,6,7',8'-dichlorofluorescein diacetate (38, 39). It is also unclear whether tyrosine phosphorylation of cortactin is involved in hyperoxia-induced ROS generation in HPAECs.
hexafluorofluorescein. Therefore, it appears that hyperoxia-induced ROS/O2 formation is not in the endosomes and partly dependent on Nox-2 in HPAECs.

In summary, the data presented in this study demonstrate an essential and critical role for cortactin and Src-dependent tyrosine phosphorylation of cortactin in the hyperoxia-induced translocation of p47phox to the EC periphery and in the generation of ROS (Fig. 14). The interactions between cortactin, actin, and p47phox may form the basis of a protein platform for the assembly of NADPH oxidase components and the generation of ROS in the endothelium.

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