Src-mediated Tyrosine Phosphorylation of p47\textsuperscript{phox} in Hyperoxia-induced Activation of NADPH Oxidase and Generation of Reactive Oxygen Species in Lung Endothelial Cells*

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Superoxide (O\textsubscript{2}) production by nonphagocytes, similar to phagocytes, is by activation of the NADPH oxidase multicomponent system. Although activation of neutrophil NADPH oxidase involves extensive serine phosphorylation of p47\textsuperscript{phox}, the role of tyrosine phosphorylation of p47\textsuperscript{phox} in NADPH oxidase-dependent O\textsubscript{2}\textsuperscript{–} production is unclear. We have shown recently that hyperoxia-induced NADPH oxidase activation in human pulmonary artery endothelial cells (HPAECs) is regulated by mitogen-activated protein kinase signal transduction. Here we provided evidence on the role of nonreceptor tyrosine kinase, Src, in hyperoxia-induced tyrosine phosphorylation of p47\textsuperscript{phox} and NADPH oxidase activation in HPAECs. Exposure of HPAECs to hyperoxia for 1 h resulted in increased O\textsubscript{2}\textsuperscript{–} and reactive oxygen species (ROS) production and enhanced tyrosine phosphorylation of Src as determined by Western blotting with phospho-Src antibodies. Pretreatment of HPAECs with the Src kinase inhibitor PP2 (1 \textmu M) or transient expression of a dominant-negative mutant of Src attenuated hyperoxia-induced tyrosine phosphorylation of Src and ROS production. Furthermore, exposure of cells to hyperoxia enhanced tyrosine phosphorylation of p47\textsuperscript{phox} and its translocation to cell peripheries that were attenuated by PP2. In vitro, Src phosphorylated recombinant p47\textsuperscript{phox} in a time-dependent manner. Src immunoprecipitates of cell lysates from control cells revealed the presence of immunodetectable p47\textsuperscript{phox} and p67\textsuperscript{phox}, suggesting the association of oxidase components with Src under basal conditions. Moreover, exposure of HPAECs to hyperoxia for 1 h enhanced the association of p47\textsuperscript{phox}, but not p67\textsuperscript{phox}, with Src. These results indicated that Src-dependent tyrosine phosphorylation of p47\textsuperscript{phox} regulates hyperoxia-induced NADPH oxidase activation and ROS production in HPAECs.

Oxygen therapy often rescues and reduces the mortality resulting from acute respiratory distress syndrome, chronic obstructive pulmonary diseases, exposure to toxic fumes, and drowning. However, prolonged exposure to supra-physiological concentrations of oxygen, referred to as hyperoxia, causes extensive damage to the alveolar-capillary barrier resulting in increased permeability and decreased lung function (2). Although the molecular mechanisms of hyperoxia-induced lung injury and cell death are complex, recent studies suggest that the generation of excessive reactive oxygen species (ROS), loss of antioxidant defense pathways, cytokine-mediated inflammation, and modulation of signal transduction may regulate pulmonary edema and apoptosis/necrosis of endothelial and epithelial cells (3). The vascular endothelium has long been recognized to generate superoxide (O\textsubscript{2})\textsuperscript{–}, hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}), hydroxyl radical (\textit{OH})\textsuperscript{–}, and nitric oxide (NO) via enzymatic and nonenzymatic reactions. In endothelial cells (ECs), in addition to the mitochondrial electron transport, other potential enzymatic pathways of ROS production include cytochrome/cytochrome oxidase/lipoxygenase, cytochrome P450, xanthine oxidase, NADPH oxidase, NO synthase, and peroxidase. In the lung, the vascular NADPH oxidase seems to play an important role in excessive production of O\textsubscript{2} in atherosclerosis, ischemic lung, pulmonary hypertension, and ventilator-associated lung injury (4–9).

NADPH oxidase catalyzes the one-electron reduction of molecular oxygen to O\textsubscript{2} by using NADPH or NADH as an electron donor (9). Activated NADPH oxidase is a multimeric protein complex consisting of at least three cytosolic subunits of p47\textsuperscript{phox}, p67\textsuperscript{phox}, and p40\textsuperscript{phox}, a regulatory small molecular weight G-protein of either Rac1 or Rac2 and a membrane-associated cytochrome b\textsubscript{558} reductase made up of p22\textsuperscript{phox} and gp91\textsuperscript{phox}. We and others (10, 11) have shown that most of the subcomponents of phagocytic NADPH oxidase are expressed in vascular ECs. ECs exhibit a low output in of O\textsubscript{2} production under basal conditions, and stimulation by TNF-\alpha, pulsatile stretch, hypoxia reoxygenation, and phorbol ester enhanced the production of ROS in ECs.

1 The abbreviations used are: ROS, reactive oxygen species; DCFDA, 6-carboxy-2'7'-dichlorodihydrofluorescein diacetate diethylcarbamomethyl ester; EBM, endothelial basal medium; EC, endothelial cell; EGNI, endothelial growth medium; ERK, extracellular signal-regulated kinase; HPAECs, human pulmonary artery endothelial cells; H\textsubscript{2}O\textsubscript{2}, hydrogen peroxide; MAPK, mitogen-activated protein kinase; NO, nitric oxide; phox, phagocytic oxidase; PP2, 4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine; PKC, protein kinase C; PVDF, polyvinylidene difluoride; siRNA, silencing ribonucleic acid; TP, tumor necrosis factor; VSMC, vascular smooth muscle cell; WT, wild type; pfu, plaque-forming units; MOPS, 4-morpholino propane sulfonic acid; FBS, fetal bovine serum; GFP, green fluorescent protein; RT, reverse transcription; PBS, phosphate-buffered saline; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.
ROS generation (12). A role for PKCα and -ζ in TNF-α-mediated activation of NADPH oxidase activation and generation of ROS has been documented in vascular ECs (13). Evidence is emerging that activation of phagocytic and nonphagocytic NADPH oxidase involves serine phosphorylation of p47^phox that initiates assembly of the cytoplasmic components and translocation to the membrane for complete association with cytochrome b$_{558}$ and functioning of the oxidase. In neutrophils, stimulation with formyl-Met-Leu-Phe-OH or phorbol ester results in phosphorylation of p47^phox and in the assembly and activation of NADPH oxidase (14, 15). Phosphorylation of p47^phox occurs at multiple serine residues suggesting potential involvement of different protein kinases such as PKC, protein kinase A, and mitogen-activated protein kinases (16). Although several reports have been made on the agonist-induced phosphorylation of p47^phox in phagocytes, only a few studies show phosphorylation of p47^phox and activation of NADPH oxidase in nonphagocytic cells (17–19). In human pulmonary artery endothelial cells (HPAECs), TNF-α-mediated NADPH oxidase activation and O$_2^-$ production were regulated by PKC$_{ζ}$, and inhibition of PKC$_{ζ}$ blocked TNF-α-dependent phosphorylation and targeting to the plasma membrane of p47^phox in human HPAECs (13). Activation of Src kinase by angiotension II in vascular smooth muscle cells (VSMCs) resulted in serine phosphorylation and translocation from the cytosol to the membrane of p47^phox with increased O$_2^-$ generation; however, Src-dependent tyrosine phosphorylation of p47^phox was not evaluated (18).

We have shown recently that exposure of HPAECs to hyperoxia increases O$_2$ and ROS production that was mediated by activation of NADPH oxidase and partly regulated by ERK and p38 MAPK (10). However, very little is known regarding tyrosine phosphorylation of p47^phox, activation of NADPH oxidase, and ROS generation in vascular cells. The Src family kinases are nonreceptor tyrosine kinases that are involved in a variety of cellular responses such as motility, adhesion, carcinogenesis, barrier function, and volume regulation (20, 21). Of the nine nonreceptor tyrosine kinases that are involved in a variety of processes (22). In the present study, we addressed the role of the nonreceptor tyrosine kinase, Src, in mediating tyrosine phosphorylation of p47^phox and regulation of NADPH oxidase activation in ECs exposed to hyperoxia. By using HPAECs, we demonstrate that hyperoxia activates C-Src but not Yes or Fyn and activation of C-Src regulates NADPH oxidase-mediated O$_2^-$ production via p47^phox tyrosine phosphorylation. Inhibition of Src activation by PP2 or expression of a dominant-negative mutant of Src prevented hyperoxia-mediated phosphorylation of C-Src, translocation of p47^phox to the cell periphery, and generation of ROS mediated by NADPH oxidase. PP3, the inactive analog of PP2, had no effect on hyperoxia-induced C-Src activation, translocation of phospho-src and p47^phox to the cell periphery, and ROS production. Furthermore, hyperoxia increased association between Src and p47^phox that was attenuated by PP2 but not by PP3 in HPAECs. These results provide a novel mechanism of hyperoxia-mediated activation of NADPH oxidase involving Src-dependent tyrosine phosphorylation of p47^phox and its translocation to the membrane for assembly of the oxidase components.

**EXPERIMENTAL PROCEDURES**

**Materials**—HPAECs, EBM-2 basal media, and Bullet kit were obtained from Clonetics (San Diego, CA). Phosphate-buffered saline (PBS) was from Biofluids Inc. (Rockville, MD). Ampicillin, fetal bovine serum (FBS), trypsin, MgCl$_2$, EGTA, Tris-HCl, Triton X-100, sodium orthovanadate, aprotonin, Tween 20, ferricytochrome c, H$_2$O$_2$, lucigenin, and Me$_3$SO were all obtained from Sigma. Dihydroethidine (hydroethidine), 6-carboxy-2',7'-dichlorodihydrofluorescein diacetate, di(acectoxyethyl) ester (DCFDA), and Amplex Red hydrogen peroxide/ peroxidase assay kit were purchased from Molecular Probes (Eugene, OR). ECL kit was from Amersham Biosciences. 4-Aminophenyl-7-(t-butyl) pyrazol[3, 4-d] pyrimidine (PP2) was obtained from Calbiochem. SMART Pool® small interfering RNA duplex oligonucleotides targeting p47^phox were purchased from Dr. Thomas L. Leto (National Institutes of Health, Bethesda). Src cDNA (plasmid), p47^phox, and p38 MAPK cDNA plasmids were purchased from the Vector Centre, Cambridge, MA. Anti-Src and anti-p38 MAPK antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Polyclonal rabbit phospho-Src antibody was purchased from BIOSOURCE. Silica Gel 60 TLC plastic sheets were obtained from EMD Chemicals Inc. (Gibbs-town, NJ). [γ-32P]ATP in 10 mM Tricine buffer (specific activity 6,000 Ci/mmol) was purchased from PerkinElmer Life Sciences.

**Endothelial Cell Culture**—HPAECs, passages between 5 and 8, were grown to contact-inhibited monolayers with typical cobblestone morphology. Of the nine nonreceptor tyrosine kinases that are involved in a variety of processes (22). In the present study, we addressed the role of the nonreceptor tyrosine kinase, Src, in mediating tyrosine phosphorylation of p47^phox and regulation of NADPH oxidase activation in ECs exposed to hyperoxia. By using HPAECs, we demonstrate that hyperoxia activates C-Src but not Yes or Fyn and activation of C-Src regulates NADPH oxidase-mediated O$_2^-$ production via p47^phox tyrosine phosphorylation. Inhibition of Src activation by PP2 or expression of a dominant-negative mutant of Src prevented hyperoxia-mediated phosphorylation of C-Src, translocation of p47^phox to the cell periphery, and generation of ROS mediated by NADPH oxidase. PP3, the inactive analog of PP2, had no effect on hyperoxia-induced C-Src activation, translocation of phospho-src and p47^phox to the cell periphery, and ROS production. Furthermore, hyperoxia increased association between Src and p47^phox that was attenuated by PP2 but not by PP3 in HPAECs. These results provide a novel mechanism of hyperoxia-mediated activation of NADPH oxidase involving Src-dependent tyrosine phosphorylation of p47^phox and its translocation to the membrane for assembly of the oxidase components.

**Transfection and Transient Expression of p47^phox-GFP**—Constitutively Active and Dominant-negative Src in HPAECs—A plasmid encoding human p47^phox was cloned into the pEGFP-N1 expression vector to give C-terminally GFP-tagged p47^phox (p47^phox-GFP). HPAECs (~50–60% confluency) in 35-mm dishes (70% confluence) in 35-mm dishes were transfected with vector control, p47^phox-GFP, wild type (WT), or Src dominant-negative (Lys-to-Arg substitution at position 296 and Tyr-to-Phe substitution at position 558) cDNA plasmids (1.0 g of DNA/well) with FuGENE 6 (3 μl) according to the manufacturer’s recommendation (Roche Applied Science). After 4 h of transfection, the media were aspirated and replaced by EGM-2 complete media, and cells were incubated in a humidified 37 °C incubator under 5% CO$_2$ and 95% air atmosphere and grown to contact-inhibited monolayers with typical cobblestone morphology. Generation of purified virus (~1 × 10$^{10}$ plaque-forming units (pfu/ml) was performed by the University of Iowa Gene Transfer Vector Core facility. After 24 h, the virus-containing medium was replaced with fresh complete medium for an additional 24 h. Vector control or infected cells were exposed to normoxia or hyperoxia for 1–3 h followed by measurement of intracellular ROS production and for p47^phox protein expression by Western blotting.
RNA Isolation and Real Time RT-PCR—Total RNA was isolated from HPAECs grown on 35-mm dishes using TRIzol® reagent according to the manufacturer's instruction. One-step RT-PCR was performed in the manufacturer's recommendation. After 3 h post-transfection, 1 ml of complete EGM-2 medium containing 10% FBS was added, and cells were cultured for an additional 24–72 h for analysis of p47phox mRNA by real time PCR and protein expression by Western blotting.

ROS Detection in Cells by Fluorescence Microscopy—Hyperoxia-induced ROS formation in cells was also quantified by fluorescence microscopy (27). The radioactive bands corresponding to standard p47phox were excised from PVDF membrane and subjected to acid hydrolysis in 1 ml of 6N HCl for 20 h at 110°C in Teflon-coated glass tubes under N2 atmosphere. The hydrolysates were evaporated by N2 gas, and the residues were taken up in ethanol/water (1:1 v/v). Portions of the acid-hydrolyzed extracts were mixed with authentic mixture of phosphoserine, phosphothreonine, and phosphotyrosine and analyzed by thin layer chromatography in absolute ethanol, 25% ammonia solution (3:5:2:2 v/v) for 120 min at room temperature; the plates were air-dried, and development was repeated once more in the same solvent, and labeled phosphoamino acids were visualized by autoradiography and standards by spraying the plates with 0.5% ninhydrin in acetone (27).

Immunochemical Analysis—Preparation of Cell Lysates, Immunoprecipitation, and Western Blotting—HCAECs grown on 100-mm dishes (~90% confluence) were serum-deprived for 18–16 h in EBM-2 containing 1% FBS, and all subsequent incubations were carried out in serum-free MEM. Cells were treated with either PP2 or PP3 (1 μM) for 1 h, and cells were rinsed twice with ice-cold PBS containing 1 mM orthovanadate. Cells were scraped into 1 ml of modified lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 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 (three times for 15 s), and centrifuged at 5000 × g in a microcentrifuge (4°C for 5 min), and protein concentrations of the supernatants were determined using Pierce protein assay kit. The supernatants, adjusted to 1 mg of protein/ml (cell lysates) were denatured in Laemmli buffer for 5 min, and samples were separated on 10% SDS-polyacrylamide gels and analyzed by Western blotting. For immunoprecipitation, cell lysates (0.5–1 mg of protein) were incubated overnight with monoclonal anti-Src conjugated to agarose or with anti-p47phox or anti-Yes or anti-Fyn or anti-Lyn antibodies (1:200 dilution in blocking buffer, 1 h), and slides were incubated for 30 min at room temperature in TBST blocking buffer containing 1% bovine serum albumin. The cells were then incubated with primary antibodies (1:200 dilution in blocking buffer, 1 h), thoroughly rinsed with TBST, and stained with Alexa Fluor 488 or 568 secondary antibodies (1:200 dilution in blocking buffer, 1 h), and slides were prepared with mounting medium containing 4,6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA). Cells were viewed on an inverted Nikon Eclipse TE 2000-S fluorescence microscope with Hamamatsu digital camera (Japan) using a 60× oil immersion objective and MetaVue software (Universal Imaging Corp., PA). Images were analyzed and adjusted using Adobe Photoshop 7 and ImageJ (National Institutes of Health).

In Vivo Phosphorylation of p47phox by [32P]Orthophosphate—HPAECs (~90% confluence in 35-mm dishes) were labeled with [32P]Orthophosphate (50 μCi/ml) in phosphate-free Dulbecco's modified Eagle's medium for 3 h. The radioactive medium was aspirated, and cells were pretreated with complete EGM-2 alone or EGM-2 containing PP2 (1 μM) for 30 min prior to exposure to either normoxia or hyperoxia for 2–3 h. Total cell lysates (~500 μg of protein) were subjected to immunoprecipitation with polyclonal goat anti-p47phox antibody (26) for 18 h, and immunoprecipitates were separated by SDS-PAGE, transferred to PVDF membrane, and analyzed by autoradiography.

Hydrolysis of Total RNA (target gene) or 0.03 μg of total RNA (18 S rRNA), 10 μl of QuantiTech RT Mix, 1.5 μl of target primers, or 1 μl 18 S RNA primers, in a total volume of 20 μl. For all samples, reverse transcription was carried out at 50 °C for 20 min, followed by cycling to 95 °C for 15 min to inactivate the RT enzyme and activate the Taq polymerase. The primers used for p47phox were sense, 5'-AGTCCTGACGAGACGAAGA-3' and antisense, 5'-GGAACGAAGAGGATCTGGA-3'. Amplion expression in each sample was normalized to its 18 S rRNA content. The relative abundance of target mRNA in each sample was calculated as 2 raised to the negative of its threshold cycle (2ΔΔCT) and expressed as a fold change in the amount of target mRNA relative to the calibrator sample. The 18 S rRNA content was measured as the 2 raised to the negative of its threshold cycle (2ΔCT) and used as a housekeeping gene to normalize the reaction.

In Vivo Phosphorylation of p47phox by Src Kinase—In vitro phosphorylation of recombinant p47phox (28) by Src kinase was performed in a reaction volume of 100 μl that contained the following: 20 mM HEPES buffer, pH 7.4, 1 mM ATP, 3.4 μCi of [γ-32P]ATP, 10 mM MgCl2, 1 mM CaCl2, 1 μg orthovanadate, 1 μg of either recombinant p47phox and 2 units of Src kinase for 30 min at 37 °C in a shaking water bath. The reaction was terminated by the addition of 20 μl of 6x SDS sample buffer and samples were subjected to SDS-PAGE on 10% Tris-glycine precast gels (Invitrogen) and transferred to PVDF membrane as indicated above. [32P]-Labeled proteins were detected by autoradiography and excised from the membrane, and radioactivity was measured in a Packard scintillation counter.
buffer, pH 7.2, 75 mM MgCl₂, 1 mM Na₃VO₄, 25 mM β-glycerophosphate, 5 mM EGTA, and 1 mM dithiothreitol). The kinase reaction contained 20 µl of immunoprecipitates in kinase buffer, 10 µM ATP, 1 µg of Src family kinase peptide [Lys-19]cdc2-(6–20)-NH₂ substrate, and 10 µCi of [γ-³²P]ATP in a final volume of 100 µl. Reactions were carried out for 10 min at 30 °C and terminated by the addition of trichloroacetic acid to give a final concentration of 10%. After centrifugation at 10,000 × g for 10 min, aliquots were spotted on to P81 filter paper, washed three times with 50 ml of 0.75% phosphoric acid, and counted in a scintillation counter. The total radioactivity incorporated into the peptide substrate was normalized to total Src family kinases present in the immunoprecipitates detected by Western blotting.

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 up at p < 0.05 unless otherwise stated. Data are expressed as mean ± S.E.

RESULTS

Expression of Src Family Kinases in HPAECs—As little information is available on the expression of different Src family kinases in human lung ECs, we assessed the relative protein expression of c-Src, Yes, Fyn, and Lyn in HPAECs by Western blotting. As shown in Fig. 1A, c-Src, Yes, Fyn, and Lyn were expressed, but the level of expression was different. Indeed, expression of c-Src, Fyn, and Lyn were higher compared with Yes as evidenced by Western blotting of total cell lysates and immunoprecipitates (Fig. 1, A and B). Next, we determined which of the Src family kinases were activated by hyperoxia. HPAECs were exposed to either normoxia or hyperoxia for 2 h, and cell lysates (500 µg of protein) were subjected to immunoprecipitation with c-Src or Fyn or Yes or Lyn antibodies, and the immunoprecipitates were analyzed for kinase activity using a synthetic Src peptide substrate and [γ-³²P]ATP according to the manufacturer’s instruction. As shown in Fig. 1C, of the four kinases, only c-Src was activated (#3-fold increase in tyrosine phosphorylation of the synthetic peptide) after exposure of cells to hyperoxia. These data suggest that hyperoxia activates c-Src but not Yes, Fyn, or Lyn, and therefore, we have investigated the role of c-Src in hyperoxia-induced NADPH oxidase activation and ROS production in HPAECs.

Hyperoxia Increases Tyrosine Phosphorylation of Src—We showed previously that exogenously added H₂O₂ or diperoxovanadate increased tyrosine phosphorylation of Src in HPAECs (29). Because hyperoxia enhanced O₂⁻ROS production in HPAECs (10), we investigated whether hyperoxia modulated tyrosine phosphorylation status of Src. Exposure of HPAECs to hyperoxia (15–180 min) induced Src phosphorylation in a time-dependent manner as evidenced by Western blotting with anti-phospho-Src (Y418) antibody (Fig. 1A). Increased tyrosine phosphorylation of Src was observed as early as 60 min, peaked at 120 min after hyperoxia, and declined to near basal levels at 180 min of hyperoxia (Fig. 1A). Phosphorylation of cortactin, a substrate for Src, was assessed to confirm the functional significance of Src activation by hyperoxia. Hyperoxia stimulated tyrosine phosphorylation of cortactin in a time frame similar to Src phosphorylation (data not shown). Furthermore, pretreatment of cells with PP2 (1 µM), a specific inhibitor of the Src family of nonreceptor tyrosine kinases (24–26), but not the inactive analog PP3, almost completely blocked hyperoxia-induced Src phosphorylation as evidenced by Western blotting and immunofluorescence microscopy (Fig. 2, B and C). These results suggest that hyperoxia activates Src in HPAECs.

Inhibition of Src Prevents Hyperoxia-induced Generation of O₂⁻/ROS—We demonstrated earlier that hyperoxia-mediated O₂⁻/ROS production in HPAECs was produced by activation of NADPH oxidase and not by mitochondrial electron transport or xanthine oxidase (10). To evaluate whether hyperoxia-induced O₂⁻/ROS production was dependent on Src activation, HPAECs were pretreated with either PP2 or PP3 (1 µM) for 60 min prior to a 1-h exposure to normoxia or hyperoxia. As shown in Fig. 3, A and B, hyperoxia increased intracellular fluorescence because of formation of oxidized DCFDA (ROS production) or hydroethidine fluorescence (O₂⁻ production) by ~1.5–2.0-fold compared with normoxia. In PP2 pretreated cells, hyperoxia-induced DCFDA fluorescence (Fig. 3A) or hydroethidine fluorescence (Fig. 3B) was significantly reduced to near basal values of normoxia. The inactive analog, PP3, had no effect on hyperoxia-induced ROS or O₂⁻ production (Fig. 3, A and B). We next used molecular strategies of transient expression of WT and dominant-negative Src plasmids to further characterize the role of Src in hyperoxia-induced ROS production. As shown in Fig. 4A, transfection of HPAECs with the plasmids in mammalian expression system...
**FIG. 2.** PP2 attenuates hyperoxia-induced tyrosine phosphorylation of Src in HPAECs. A, HPAECs grown on 35-mm dishes (~90% confluent) were exposed to either normoxia (N) or hyperoxia (HO) for varying time periods of 15, 30, 60, 120, and 180 min. At the end of each time point, cells were rinsed with ice-cold PBS containing sodium orthovanadate (1 mM), and lysates (20 µg of protein) were subjected to 10% SDS-PAGE and probed with anti-phospho-Src or anti-Src antibody (1:1000 dilution). B, HPAECs grown to ~90% confluence in 35-mm dishes were pretreated with EGM-2 complete medium or EGM-2 plus PP2 or PP3 (1 µM) for 1 h and then exposed to either normoxia or hyperoxia for an additional 1 h. Cell lysates (20 µg of protein) were subjected to 10% SDS-PAGE and Western blotting with anti-phospho-Src or anti-Src antibody (1:1000 dilution). Shown are representative blots from three different experiments in triplicate. Fold change in phospho-Src/Src was calculated from the respective Western blots (IB) by image analysis, and data were normalized to total Src. C, HPAECs grown to ~90% confluence on glass coverslips were pretreated with medium alone or medium containing PP2 or PP3 (1 µM) for 30 min prior to exposure to either normoxia (N) or hyperoxia (HO) for 2 h. Cells were washed, fixed, permeabilized, probed with anti-phospho-Src antibody, stained with Alexa Fluor 568 secondary antibody, and examined by immunofluorescence microscopy using a 60X oil objective. Results are representative of three independent experiments. Arrow depicts particularly strong cell periphery staining. *, significantly different from normoxia (p < 0.01); **, significantly different in cells exposed to hyperoxia after PP2 treatment (p < 0.05); ***, statistically not significant from cells exposed to hyperoxia (p > 0.05).

**FIG. 3.** Effect of PP2 and PP3 on hyperoxia-mediated ROS production in HPAECs. HPAECs grown to ~90% confluence in 35-mm dishes were loaded with either DCFDA (10 µM) (A) or hydroethidine (10 µM) (B) for 30 min and washed once with basal medium. DCFDA- or hydroethidine-loaded cells were pretreated with EGM-2 medium alone or medium containing PP2 or PP3 (1 µM) for 30 min prior to exposure to either normoxia (N) or hyperoxia (HO) for 1 h. At the end of exposure, formation of total ROS or O₂⁻ was measured as described under “Experimental Procedures.” A, formation of total ROS in cells was visualized under fluorescence microscope and quantified. B, formation of O₂⁻ (released into the medium) was determined fluorimetrically as described under “Experimental Procedures.” Values are means ± S.D. from three independent experiments in triplicate and expressed as % control. *, significantly different compared with normoxic controls (p < 0.05); **, significantly different compared with cells exposed to hyperoxia without PP2 pretreatment (p < 0.01); ***, statistically not significant from cells exposed to hyperoxia (p > 0.05).
for 48 h significantly increased expression of Src as determined by Western blotting with anti-Src antibody. Although overexpression of Src WT increased ROS production by hyperoxia, expression of the dominant-negative mutant of Src attenuated hyperoxia-induced ROS production (vector, hyperoxia = 165%; Src WT, 240%; Src dominant-negative, 90%) (Fig. 4B). Most interestingly, overexpression of either Src WT or dominant-negative Src had a significant increase on basal ROS production compared with vector control (Fig. 4B). Taken together, these data show that Src in HPAECs regulates hyperoxia-mediated production of $O_2^\bullet$ ROS.

Src Activation by Hyperoxia Increases Tyrosine Phosphorylation of p47phox—Phosphorylation of p47phox at serine residues, in response to stimuli, has been demonstrated as a prerequisite for NADPH oxidase activation in phagocytic and nonphagocytic cells (33). As very little information is available on the role of tyrosine phosphorylation of p47phox and activation of NADPH oxidase, we investigated whether hyperoxia induces tyrosine phosphorylation of p47phox and the possible involvement of Src in tyrosine phosphorylation of p47phox. HPAECs were exposed to either normoxia or hyperoxia for 3 h, and total cell lysates (equal protein) were subjected to immunoprecipitation with anti-p47phox antibody. Analysis of the immunoprecipitates by immunoblotting revealed that hyperoxia increased the phosphorylation of p47phox in HPAECs labeled with $[^{32}\text{P}]$orthophosphate for 3 h prior to pretreatment with PP2 for 48 h. Cells were exposed to either normoxia or hyperoxia for an additional 1 h. Cell lysates were subjected to immunoprecipitation with anti-phosphotyrosine antibody conjugated to agarose and analyzed by Western blotting with p47phox antibody. As shown in Fig. 5C, hyperoxia enhanced tyrosine phosphorylation of p47phox in control, vector control, and p47phox-overexpressing cells, and pretreatment of cells with PP2 blocked hyperoxia-induced tyrosine phosphorylation of p47phox. Additionally, the role of Src in hyperoxia-induced phosphorylation of p47phox was determined in HPAECs labeled with $[^{32}\text{P}]$orthophosphate for 3 h prior to pretreatment with PP2 for 30 min and exposure to normoxia or hyperoxia. Cell lysates were immunoprecipitated with anti-p47phox antibody, separated by SDS-PAGE, and subjected to autoradiography. Inhibition of Src by PP2 attenuated hyperoxia-induced incorporation of the radioactivity in p47phox immunoprecipitates (Fig. 5D). These results show that Src activation is involved in the tyrosine phosphorylation of p47phox in HPAECs.

Phosphoamino Acids Analysis of $[^{32}\text{P}]$-Labeled p47phox—To characterize further the amino acid residues (serine/threonine and tyrosine) of p47phox phosphorylated by hyperoxia, p47phox immunoprecipitates from $[^{32}\text{P}]$orthophosphate-labeled cell lysates of normoxic and hyperoxic cells with or without PP2 treatment were separated by SDS-PAGE, and p47phox was visualized by autoradiography. Bands corresponding to p47phox were excised and hydrolyzed with 6 N HCl for 4 h. The acid hydrolysates were dried under N$_2$, reconstituted in 100 μl of ethanol/water containing phosphoserine, phosphothreonine, and phosphotyrosine standards, and spotted onto Silica Gel 60 plastic thin layer plates. Separation of the HCl digests by thin layer chromatography revealed that hyperoxia increased the incorporation of $[^{32}\text{P}]$orthophosphate into tyrosine but not serine and threonine residues of p47phox (Fig. 6). Furthermore, pretreatment of cells with PP2 attenuated hyperoxia-induced tyrosine phosphorylation of p47phox (Fig. 6). These results confirm that hyperoxia increased phosphorylation of p47phox only on the tyrosine residues and not on serine and/or threonine residues in HPAECs.

In Vitro Phosphorylation of Recombinant p47phox by Src Kinase—We next addressed if p47phox is a substrate for Src in vitro. To test this, purified recombinant p47phox protein was incubated with activated Src kinase in the presence of $[^{32}\text{P}]$ATP for varying time periods. As shown in Fig. 7, the recombinant p47phox protein was phosphorylated by Src kinase in a time-dependent manner. In the absence of added Src, no phosphorylation of the substrate was observed. Immunoprecipitation with anti-phosphotyrosine antibody revealed that most of the radioactivity was associated in the tyrosine residue(s) (data not shown). These results confirm that p47phox is phosphorylated in vitro by Src kinase.

Inhibition of Src Prevents Hyperoxia-Induced Translocation of p47phox to Cell Periphery—By having established a role for Src in hyperoxia-induced generation of ROS, we next investigated whether hyperoxia promoted migration of p47phox to the cell periphery and possible involvement of Src in p47phox translocation. To determine whether exposure to hyperoxia-mediated translocation of p47phox to the cell periphery, HPAECs were transfected with GFP vector or GFP-p47phox plasmids for 48 h. Cells were exposed to either normoxia or hyperoxia for 1 h and were examined by immunofluorescence microscopy. As shown in Fig. 8A, most of the GFP-p47phox was localized in the cytoplasm and perinuclear region in normoxic cells, and hyperoxia enhanced the distribution of GFP-p47phox from the cytoplasm toward the cell periphery that appeared punctated and

**Fig. 4. Effect of overexpression of wild type (WT) Src or dominant-negative (−) Src on hyperoxia-induced ROS production in HPAECs.** A, cells grown to ~60% confluence in 35-mm dishes were transfected with either empty vector or WT Src or dominant-negative (−) Src cDNAs with FuGENE 6 as described under “Experimental Procedures.” After 48 h of transfection, cells were exposed to normoxia (N) or hyperoxia (HO) for 1 h; cell lysates were prepared in lysis buffer, subjected to 10% SDS-PAGE, and Western-blotted (IB) with anti-Src antibody. B, after 48 h of transfection, cells were exposed to either normoxia or hyperoxia (1 h), and total ROS released into the medium was measured by Amplex Red fluorescence. Values are means ± S.D. of three independent experiments. *, significantly different from normoxic cells (p < 0.01); **, significantly different from control cells exposed to hyperoxia (p < 0.01); ***, significantly different from control cells exposed to hyperoxia (p < 0.05).
Wester blotting for immunodetectable p47
agarose-conjugated anti-phosphotyrosine antibody for 18 h under nondenaturing conditions, and the immunoprecipitates were analyzed by SDS-PAGE and Western blotting (IB) with mouse monoclonal anti-phosphotyrosine antibody (4G10, 1:1000 dilution). Changes in tyrosine phosphorylation of p47phox because of hyperoxia were calculated from three independent experiments and expressed as percent of control. B, HPAECs (~60% confluence in 35-mm dishes) were infected with adenoviral vector control or vector containing cDNA for wild type p47phox (10 pfu/cell) for 24 h. Cell lysates were analyzed by Western blotting for overexpression of p47phox with polyclonal goat anti-p47phox antibody. C, vector control or adenoviral infected (10 pfu/cell, 24 h) HPAECs in 35-mm dishes were pretreated with medium alone or medium containing PP2 (1 µM) for 30 min prior to exposure to either normoxia or hyperoxia for 2 h. Cell lysates (500 µg of protein) were subjected to immunoprecipitation with agarose-conjugated anti-phosphotyrosine antibody for 18 h under nondenaturing conditions, and the immunoprecipitates were analyzed by Western blotting for immunodetectable p47phox using monoclonal anti-p47phox antibody. Shown is a representative blot from three separate experiments. D, HPAECs (~90% confluence in 35-mm dishes) were labeled with [32P]orthophosphate (50 μCi/ml) in phosphate-free Dulbecco’s modified Eagle’s medium for 2 h. The radioactive medium was aspirated, and cells were pretreated with complete EGM-2 alone or EGM-2 containing PP2 (1 µM) for 30 min prior to exposure to either normoxia or hyperoxia for 3 h. Total cell lysates (500 µg of protein) were subjected to immunoprecipitation with goat polyclonal anti-p47phox antibody for 18 h, and immunoprecipitates were separated by SDS-PAGE, transferred to PVDF membranes, and analyzed by autoradiography. After complete decay of the radioactivity, the same membranes were probed for total p47phox by Western blotting. Values are means ± S.D. of three independent experiments, and fold increases in the incorporation of [32P]orthophosphate into p47phox were normalized to total p47phox in the immunoprecipitates. The effect of PP2 on hyperoxia-induced [32P]phosphorylation of p47phox was calculated by image analysis and normalized to total p47phox. *, significantly different from cells exposed to normoxia (p < 0.05); **, significantly different from cells exposed to hyperoxia (p < 0.01).

Hyperoxia-Induced Tyrosine Phosphorylation of p47phox is Attenuated by PP2 in HPAECs. A, HPAECs grown to ~95% confluence in 6-mm dishes were exposed to normoxia (N) or hyperoxia (HO) for 2 h. Cell lysates (500 µg of protein) were subjected to immunoprecipitation (IP) with polyclonal goat anti-p47phox antibody for 18 h under nondenaturing conditions, and the immunoprecipitates were analyzed by SDS-PAGE and Western blotting (IB) with mouse monoclonal anti-phosphotyrosine antibody (4G10, 1:1000 dilution). Changes in tyrosine phosphorylation of p47phox because of hyperoxia were calculated from three independent experiments and expressed as percent of control. B, HPAECs (~60% confluence in 35-mm dishes) were infected with adenoviral vector control or vector containing cDNA for wild type p47phox (10 pfu/cell) for 24 h. Cell lysates were analyzed by Western blotting for overexpression of p47phox with polyclonal goat anti-p47phox antibody. C, vector control or adenoviral infected (10 pfu/cell, 24 h) HPAECs in 35-mm dishes were pretreated with medium alone or medium containing PP2 (1 µM) for 30 min prior to exposure to either normoxia or hyperoxia for 2 h. Cell lysates (500 µg of protein) were subjected to immunoprecipitation with agarose-conjugated anti-phosphotyrosine antibody for 18 h under nondenaturing conditions, and the immunoprecipitates were analyzed by Western blotting for immunodetectable p47phox using monoclonal anti-p47phox antibody. Shown is a representative blot from three separate experiments. D, HPAECs (~90% confluence in 35-mm dishes) were labeled with [32P]orthophosphate (50 μCi/ml) in phosphate-free Dulbecco’s modified Eagle’s medium for 2 h. The radioactive medium was aspirated, and cells were pretreated with complete EGM-2 alone or EGM-2 containing PP2 (1 µM) for 30 min prior to exposure to either normoxia or hyperoxia for 3 h. Total cell lysates (500 µg of protein) were subjected to immunoprecipitation with goat polyclonal anti-p47phox antibody for 18 h, and immunoprecipitates were separated by SDS-PAGE, transferred to PVDF membranes, and analyzed by autoradiography. After complete decay of the radioactivity, the same membranes were probed for total p47phox by Western blotting. Values are means ± S.D. of three independent experiments, and fold increases in the incorporation of [32P]orthophosphate into p47phox were normalized to total p47phox in the immunoprecipitates. The effect of PP2 on hyperoxia-induced [32P]phosphorylation of p47phox was calculated by image analysis and normalized to total p47phox. *, significantly different from cells exposed to normoxia (p < 0.05); **, significantly different from cells exposed to hyperoxia (p < 0.01).
hyperoxia (2 h) revealed translocation of both p47phox and Src to the cell periphery. Additionally, merging of the immunofluorescence stains of p47phox (green) and Src (red) showed colocalization (yellow) at the cell periphery (Fig. 9B). Furthermore, pretreatment of HPAECs with PP2 for 1 h blocked the enhanced association of p47phox with Src as evidenced by immunoprecipitation and Western blotting (Fig. 9C). A similar association between Src and p47phox was observed in HPAECs infected with the adenoviral construct of p47phox (10 multiplicity of infection) for 24 h (data not shown). We also determined by immunostaining whether the translocated Src to the cell periphery after exposure to hyperoxia is phosphorylated. As shown in Fig. 10, hyperoxia enhanced staining of phospho-Src at the cell periphery, compared with normoxia, and pretreatment with PP2, but not PP3, prevented a hyperoxia-induced increase in immunostaining of phospho-Src and p47phox. These results show that hyperoxia stimulates movement of phospho-Src and p47phox to the cell periphery and enhanced interaction and association between Src and p47phox.

Inhibition of Src Prevents Hyperoxia-induced Association of p47phox with gp91phox—As assembly between p47phox and membrane-associated gp91phox and p22phox is critical in NADPH oxidase activation and ROS production, we evaluated the effects of inhibition of protein-tyrosine phosphatase and Src kinase on hyperoxia-mediated association between p47phox and gp91phox. HPAECs (~60% confluence) were infected with vector or with p47phox WT and were pretreated with vanadate (10 μM) or PP2 (1 μM) for 30 min prior to exposure to either normoxia or hyperoxia for 3 h. Co-immunoprecipitation studies revealed that hyperoxia enhanced the association between p47phox and gp91phox compared with normoxia (Fig. 11). Furthermore, pretreatment of cells with vanadate enhanced the association between p47phox and gp91phox under normoxic and hyperoxic conditions (Fig. 11). Moreover, PP2 (1 μM) blocked the hyperoxia-mediated association between p47phox and gp91phox as well as the effect of vanadate alone or vanadate plus hyperoxia on the association between the two components (Fig. 11). These results are consistent with the role of Src in tyrosine phosphorylation and targeting of p47phox to the cell periphery and assembly with gp91phox required in NADPH oxidase activation and ROS generation.

**DISCUSSION**

Activated NADPH oxidase of phagocytes and nonphagocytic cells is a multiprotein complex consisting of at least three cytosolic and two membrane-associated components plus two small molecular weight G proteins. One important step in the activation of NADPH oxidase is the assembly of p47phox, p67phox, and Rac1 or Rac2 with gp91phox and p22phox (1, 34–38). The mechanism(s) of activation of leukocyte and nonphagocytic NADPH oxidase is complex and unclear. In resting cells, the p47phox, p67phox, and Rac-1 or Rac2 are distributed in the cytosol, and activation of cells by bacterial products or agonists results in the phosphorylation of some of the cytosolic components prior to assembly and migration to the membrane for assembly with the cytochrome b558 (39). In phagocytes, one of the cytosolic protein components phosphorylated is p47phox that is crucial for the assembly and activation of the NADPH oxidase (36, 40–42). A number of earlier studies in leukocytes have shown that p47phox is phosphorylated on several serine residues located between amino acids 303 and 379. Upon stimulation with angiotensin II, p47phox is phosphorylated at serine and tyrosine residues in vascular smooth muscle cells (18).

We demonstrated a role for ERK and p38 MAPK in hyperoxia-induced activation of NADPH oxidase and production of ROS in HPAECs (10). In the present study, we show that hyperoxia-induced activation of NADPH oxidase involves Src and Src-dependent tyrosine phosphorylation of p47phox. In addition, we provide evidence for in vitro phosphorylation of p47phox by Src and interaction between Src and p47phox in the regulation of hyperoxia-induced O2·− generation.

Activation of NADPH oxidase is a major pathway of hyperoxia-induced ROS production in lung endothelial cells (10, 43). This is supported by the findings that exposure of HPAECs to hyperoxia (1–12 h) stimulates O2·− production that was blocked by diphenyleneiodonium, an inhibitor of flavoproteins or by antisense to p22phox (10). Additionally, siRNA for p47phox completely abolished hyperoxia-mediated ROS formation (Fig. 12), confirming a major role for NADPH oxidase. Although signaling pathways whereby hyperoxia regulates endothelial NADPH oxidase have not been completely defined, involvement of MAPKs in NADPH oxidase activation and ROS production has been reported (10, 44).

Previous studies in human vascular and rat thoracic aortic VSMCs demonstrated that angiotensin II-dependent stimulation of NADPH oxidase is regulated in part by c-Src (18). Our data clearly implicate a role for c-Src, but not Yes, Lyn, or Hck, in hyperoxia-induced p47phox phosphorylation and ROS generation. PP2, but not the inactive analog PP3, and the dominant-negative Src attenuated hyperoxia-mediated ROS production, p47phox tyrosine phosphorylation, and translocation of p47phox to the cell periphery. This is consistent with an earlier report on the role of a-Src in angiotensin II-dependent O2·− generation and phosphorylation/translocation...
of p47<sub>phox</sub> in human VSMCs (18, 45). However, in rat thoracic VSMCs, angiotensin II stimulation of NADPH oxidase and ROS production seem to be biphasic. The first rapid phase of ROS production requires PKC activation, whereas the second sustained phase involves phosphatidylinositol 3-kinase, epidermal growth factor-receptor kinase, and Src and Rac signaling pathways (46). Although dominant-negative Rac1 completely abolished hyperoxia-induced O<sub>2</sub><sup>·</sup> production, it is unclear if phosphatidylinositol 3-kinase 3-kine and transactivation of growth factor receptors contribute to hyperoxia-induced NADPH oxidase activation and O<sub>2</sub><sup>·</sup> formation in HPAECs.

The mechanism(s) by which Src regulates NADPH oxidase remains poorly defined. Our data show that Src activation by hyperoxia is upstream of p47<sub>phox</sub> phosphorylation and oxidase activation. We also demonstrate for the first time that p47<sub>phox</sub> co-immunoprecipitates with Src under conditions of hyperoxia (1–3 h) and in vivo and in vitro tyrosine phosphorylation of p47<sub>phox</sub> by Src (Figs. 5, 7, and 10). Experiments with the Src kinase inhibitor, PP2, suggest that hyperoxia-induced phosphorylation of Src is not only critical to tyrosine phosphorylation of p47<sub>phox</sub> but also for the interaction between Src and p47<sub>phox</sub> as evidenced by co-immunoprecipitation studies (Figs. 9 and 10). Although our results indicate a direct role for Src-dependent tyrosine phosphorylation of p47<sub>phox</sub> in lung EC NADPH oxidase activation and ROS generation, the roles of other potential intermediate serine/threonine kinases in the phosphorylation of p47<sub>phox</sub> and oxidase activation are not known. Most interestingly, phosphoamino acids analysis of acid digests of 32P-labeled p47<sub>phox</sub> immunoprecipitates shows the majority of the radioactivity present in the phosphotyrosine residue and practically none in phosphoserine or phosphothreonine residues (Fig. 6). These results suggest that Src-dependent tyrosine phosphorylation of p47<sub>phox</sub> represents a novel post-transcriptional modification in hyperoxia-induced activation of NADPH oxidase and ROS production in human ECs. As reported earlier, angiotensin II stimulated Src-dependent serine phosphorylation of p47<sub>phox</sub> in VSMCs, suggesting involvement of other intermediate serine kinases such as phosphatidylinositol 3-kinase/AKT, PKC, p21-activated kinase, and Raf-1 in the activation of NADPH oxidase (47). Although serine phosphorylation of p47<sub>phox</sub> by PKC and other kinases is well established, an involvement of the Src

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2 V. Natarajan, unpublished results.
family of nonreceptor kinases in tyrosine phosphorylation of p47\textsuperscript{phox} has not been reported before in either phagocytic or nonphagocytic cells. The important findings of this study are the association between Src and p47\textsuperscript{phox} and the role of Src in tyrosine phosphorylation of p47\textsuperscript{phox} in HPAECs. Among the various protein kinases, PKC\textalpha, -\beta, -\delta, and -\zeta have been shown to phosphorylate p47\textsuperscript{phox} and regulate NADPH oxidase activity in phagocytic cells (13–16). Very little is known regarding the role of PKC isoforms regulating p47\textsuperscript{phox} phosphorylation and NADPH oxidase activation in nonphagocytic cells. Stimulation

![Fig. 9](http://www.jbc.org/)

**Fig. 9. Inhibition of Src kinase prevents hyperoxia-induced association of Src with p47\textsuperscript{phox}.** A, HPAECs grown to ~90% confluence in 60-mm dishes were exposed to either normoxia (N) or hyperoxia (HO) for 2 h, and cell lysates (500 \mu g of protein) were subjected to immunoprecipitation (IP) with IgG or monoclonal anti-Src antibodies conjugated to agarose under nondenaturing conditions as described under “Experimental Procedures.” Equal volume of the immunoprecipitates were subjected to 10% SDS-PAGE and probed with rabbit polyclonal anti-Src (1:2000 dilution), goat polyclonal p47\textsuperscript{phox} (1:1000 dilution), or rabbit polyclonal anti-p67\textsuperscript{phox} (1:500 dilution) antibodies. Shown are representative blots from three independent experiments, and relative pixel intensities for each of the blots were determined by image analysis and normalized to total Src. IB, immunoblot. * Significantly different from normoxia (p < 0.05). B, HPAECs grown to ~90% confluence on glass coverslips were exposed to either normoxia or hyperoxia for 2 h. Cells were washed, fixed, permeabilized, probed with anti-p47\textsuperscript{phox} antibody or with anti-Src antibodies, stained with secondary antibodies, and examined by immunofluorescence microscopy using a 60\x oil objective. C, HPAECs grown to ~90% confluence in 60-mm dishes were pretreated with PP2 (1 \mu M) for 30 min prior to exposure to either normoxia or hyperoxia for 2 h. Total cell lysates (500 \mu g of protein) were subjected to immunoprecipitation with mouse monoclonal anti-Src antibody (1:100 dilution) under non-denaturing conditions. Equal volumes of the immunoprecipitates were separated by SDS-PAGE (10% gels) and probed with goat polyclonal p47\textsuperscript{phox} antibody (1:1000 dilution). The effect of PP2 on the association between Src and p47\textsuperscript{phox} was calculated from image analysis of the Western blots and normalized to total Src in the immunoprecipitates. Shown is a representative Western blot of three independent experiments.
of human lung ECs with TNF-α resulted in the phosphorylation of p47phox with TNF-α-treated cells (13). In addition to serving as a target for PKC, p47phox acts as a regulator of PKC. In neutrophils, a direct interaction between isotypes I and II and p47phox has been described, and cells lacking p47phox showed differences in the recruitment and processing of PKC isotypes to particulate fractions and in the phosphorylation of the cytoskeletal protein, coronin (48). We have investigated the effect of PKC inhibitors, such as bisindolylmaleimide and GF109203X, on hyperoxia-induced ROS production. Although the PKC inhibitors attenuated hyperoxia-induced ROS production by ~40% (data not shown), [32P]orthophosphate labeling of HPAECs revealed that most of the incorporated radioactivity was associated with the phosphotyrosine residue of the p47phox immunoprecipitates and very negligible incorporation into phosphoserine plus phos-
phothreonine residues (Fig. 6). This suggests that hyperoxia-induced activation of PKC may be involved in serine/threonine phosphorylation of other subcomponents of the NADPH oxidase required for assembly and activation. Further studies are necessary to understand the mechanisms of PKC-dependent activation of NADPH oxidase subcomponents (such as p67phox and NOX family) and ROS production in response to hyperoxia. It is possible that ERK and p38 MAPK-regulated ROS production in hyperoxia (10) may involve phosphorylation of other NADPH oxidase subcomponents and not p47phox.

In this study, we show that Src phosphorylates p47phox and induces translocation of p47phox to the cell periphery and ROS production via NADPH oxidase activation. We also found that Src is constitutively associated with p47phox and p67phox as evidenced by co-immunoprecipitation and Western blot analysis (Fig. 9). More importantly, hyperoxia (1–3 h) significantly enhanced the association of Src with p47phox but not with p67phox.

The present results do not rule out the possible association of Src and p67phox with other proteins such as the cytoskeletal proteins that may function as the protein platform for the assembly of NADPH oxidase components. In addition to p47phox, hyperoxia increased tyrosine phosphorylation of cortactin, an actin-binding protein and a substrate for Src kinase (49), that was blocked by PP2, and co-immunoprecipitation of cortactin revealed strong association between cortactin, p47phox, and Src in hyperoxia. At present, the Src-mediated tyrosine phosphorylation sites on p47phox have not been mapped either in vivo or in vitro studies. Also it is presently unknown whether tyrosine phosphorylation by Src or other tyrosine kinase(s) causes any consequential changes of p47phox similar to serine phosphorylation-induced conformational changes in the activation of phagocytic NADPH oxidase (50–52).

In summary, the present study implicates c-Src as a key nonreceptor tyrosine kinase regulating hyperoxia-mediated ROS generation through the activation of NADPH oxidase in human lung ECs. The activation of endothelial NADPH oxidase was dependent on Src-induced tyrosine phosphorylation of p47phox. Furthermore, inhibition of Src by PP2 or dominant-negative Src prevented hyperoxia-induced phosphorylation of p47phox and decreased association between Src and p47phox and ROS production. Recently, the families of gp91phox-like proteins (termed as NOX proteins) as well as two proteins with homology to p47phox and p67phox (termed as p41phox and p51phox) have been identified in nonphagocytes that may modulate NADPH oxidase activity (53). Future studies will address the role of tyrosine phosphorylation of p47phox and its homolog (p41phox) by Src family nonreceptor kinase(s), mapping of phosphorylation sites, and the physiological significance of tyrosine phosphorylation in the activation of endothelial NADPH oxidase.

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Src-mediated Tyrosine Phosphorylation of p47phox in Hyperoxia-induced Activation of NADPH Oxidase and Generation of Reactive Oxygen Species in Lung Endothelial Cells

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