Hyperoxia-induced NAD(P)H oxidase activation and regulation by MAP kinases in human lung endothelial cells

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PATIENTS with acute respiratory distress syndrome (ARDS) or chronic obstructive pulmonary diseases (COPD) and subjects suffering from severe hypoxia due to drowning, life-threatening soft-tissue infections, and exposure to smoke and toxic gases are often rescued by oxygen therapy (13). Although oxygen therapy is given to the critically ill patients, prolonged exposure to high concentrations of oxygen results in ventilator-induced lung injury (24). Thus exposure to supraphysiological concentrations of oxygen (hyperoxia) results in sustained elevation of inspired oxygen, extensive damage to the alveolar-capillary barrier, increased permeability, and decreased pulmonary function (11). Hyperoxia-induced pathological changes in the lung tissue are often accompanied by injury and death of endothelial and epithelial cells (14). Apoptosis plays an important role in animal models of hyperoxic lung injury as well as in ARDS (28). Molecular mechanisms of hyperoxia-induced lung injury and cell death are complex and regulated by generation of overwhelming levels of reactive oxygen species (ROS), cytokine-mediated inflammation, loss of antioxidant defense mechanisms and modulation of signal transduction pathways that regulate the expression of stress responsive and apoptotic regulatory genes (4, 35).

Hyperoxia-induced formation of superoxide (O2-) hydrogen peroxide (H2O2), hydroxide (HO-) and ONOO- and their potential role in pulmonary oxygen toxicity, as well as the efficacy of superoxide dismutase (SOD) for pulmonary defense against oxygen toxicity, have been emphasized (52). It was shown that exposure to hyperoxia induced both NADPH- and NADH-dependent generation of O2- in lung and liver nuclei (58). Hyperoxia was shown to be involved in neutrophil retention in the lung (38). Allopurinol, an inhibitor of xanthine oxidase, was shown to decrease the hyperoxia-induced neutrophilic alveolar response but not the alveolar injury, ruling out the role of xanthine oxidase in hyperoxia-induced lung injury (8). Prolonged exposure to hyperoxia stimulated cyanide-insensitive release of H2O2 by alveolar macrophages, indicating a role for NADPH oxidase (30). Hyperoxia induced formation of H2O2 in the brain and was implicated as a mediator of oxygen toxicity to the central nervous system (44). The involvement of xanthine oxidase in hyperoxia-induced lung injury is unclear, as no increase in conversion of xanthine dehydrogenase to xanthine oxidase in the lung during hyperoxia has been observed (19). Administration of liposome-entrapped catalase and SOD pro-

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tected rats against hyperoxic oxygen toxicity, indicating that O$_2^-$ and H$_2$O$_2$ are prime species in mediating oxygen toxicity (21). The vascular bed surrounding the lung is extremely vulnerable to oxygen toxicity during hyperoxic treatment (15). Endothelial cells (ECs) of the pulmonary vasculature are one of the prime targets of hyperoxic insult resulting in extensive vascular leakiness, further augmenting oxidative injury of the lung tissue (14, 15). Cultured bovine ECs, upon exposure to hyperoxia, were shown to release higher levels of H$_2$O$_2$ (29). In bovine aortic ECs, xanthine oxidase did not appear to contribute significantly to hyperoxia-induced generation of ROS (43). The hyperoxia-induced release of H$_2$O$_2$ by vascular ECs was sensitive to changes in oxygen concentrations over a narrow range (31).

Aerobes generate ROS by nonenzymatic and enzymatic mechanisms (50). Molecular O$_2$ is activated by enzymatic electron transfer in subcellular organelles such as mitochondria, endoplasmic reticulum, nuclear membranes, peroxisomes, plasma membranes, and cytosol (25). Mammalian cells possess several potential sources of generation of ROS that include but are not limited to the mitochondrial electron transport system, arachidonic acid metabolism by cyclooxygenase/lipoxygenase, cytochrome P-450, xanthine oxidase, NAD(P)H oxidase, nitric oxide synthase, peroxidase, and other heme proteins (3). However, the professional phagocytes of the immune system (neutrophils, eosinophils, monocytes, and macrophages) primarily generate O$_2^-$ mediated by a tightly regulated NADPH oxidase system for phagocytosis and destruction of invading pathogens (34, 47). Although the phagocytic NADPH oxidase has been characterized more extensively than the vascular isoform, recent studies suggest that a number of tissues contain NAD(P)H oxidase or other oxidases of gp91 phox homologs designated as NOX-1, NOX-3, and NOX-4, which are involved in generation of O$_2^-$/ROS (17, 48, 50, 57). The generation of O$_2^-$ is slow and low in nonphagocytic cells but fast and vigorous in phagocytes that are responding to microorganisms or cytokine challenge (17, 48, 50, 57). The large quantities of ROS generated by phagocytes serve in the defense against invading microbes, whereas the low levels of ROS (O$_2^-$) produced by nonphagocytic cells have been attributed to their cellular signal-mediating functions as signaling molecules (3, 22, 33, 55). Among the nonphagocytic cells examined so far, endothelial NAD(P)H oxidase has been investigated more extensively compared with its counterpart in the vascular smooth muscle cells (3). The activity and expression of NAD(P)H oxidase-like enzyme have been observed in ECs from bovine and porcine pulmonary arteries, rat coronary microvascular tissue, and human umbilical vein, and also in these cells the presence of one or more of the phagocytic NAD(P)H oxidase subcomponents has been demonstrated (6, 23, 26). Recent studies suggest that vascular NAD(P)H oxidase may be responsible for excessive generation of O$_2^-$ in atherosclerosis, ischemic lung, pulmonary hypertension, and diabetes (1, 16, 20, 22, 36). Activation of the vascular NAD(P)H oxidase is mediated by hormones, cytokines, mechanical stress, and ischemia-reperfusion injury (1, 16, 20).

Despite the fact that a majority of the earlier studies reported the formation/release of H$_2$O$_2$ independent from the xanthine oxidase-mediated mechanism, other potential contributor(s) responsible for hyperoxia-induced formation/release of O$_2^-$ and ROS in ECs warrant investigation. Therefore, our current studies were conducted to establish the nature, mechanism, and regulation of hyperoxia-induced generation of ROS in human pulmonary artery endothelial cells (HPAECs). For the first time, our results show 1) the activation of NAD(P)H oxidase and minimal role of mitochondrial electron transport and xanthine oxidase in hyperoxia-induced generation of ROS, 2) the presence of phagocytic NAD(P)H oxidase subcomponents in human lung ECs, and 3) a role of p38 MAPK and ERK in the regulation of hyperoxia-induced activation of NAD(P)H oxidase in HPAECs.

**MATERIALS AND METHODS**

**Materials.** HPAECs (passage 4) were obtained from Clonetics (San Diego, CA). MCDB medium and phosphate-buff ered saline (PBS) were obtained from Biofluids (Rockville, MD). Nonessential amino acids, trypsin, FBS, penicillin-streptomycin, Tris-HCl, EGTA, MgCl$_2$, glycerophosphate, Triton X-100, sodium orthovanadate, aprotinin, leupeptin, pepstatin, Twin 20, ferricytochrome C, human erythrocyte SOD, bovine liver catalase, H$_2$O$_2$ (30%), diphenyleneiodo- dine (DPI), lucigenin, and DMSO were all obtained from Sigma (St. Louis, MO). Amplex Red Hydrogen Peroxide Assay kit, hydroethidine, and 6-carboxy-2, 7'-dichlorodihydrofluorescein diacetate dihydroxyethyl ester (DCFDA) were obtained from Molecular Probes (Eugene, OR). 5,5'-Dimethyl-1-pyrroline-N-oxide (DMPO) spin trap was obtained from Dojindo Laboratories (Gaitersburg, MD). [γ-32P]ATP (specific activity 5 Ci/mmol in 10 mM Tris buffer) and 2-deoxy-d-[3H]glucose were obtained from New England Nuclear (Wilmington, DE). SB-202190, SB-203580, and PD-98059 were obtained from Calbiochem (San Diego, CA). Endothelial cell growth factor (ECGF) and antibodies against recombinant subcomponents of phagocytic NADPH oxidase (phox 22, phox 47, phox 67 and gp 91) were obtained from Upstate Biotechnology (Lake Placid, NY). Phospho-specific p38 MAPK and ERK1/ERK2 antibodies were purchased from Cell Signaling (Beverly, MA). Polyclonal antibodies to ERK1, ERK2, p38 MAPK, and c-Jun NH$_2$-terminal kinase (JNK) were procured from Santa Cruz Biotechnology (Santa Cruz, CA). The enhanced chemiluminescence kit for the detection of proteins by Western blots was obtained from Amersham (Arlington Heights, IL).

**Endothelial cell culture.** HPAECs, at passages between 5 and 9 were grown to ~80% confluence in MCDB medium supplemented with 10% FBS, 100 U/ml penicillin and streptomycin, 5 µg/ml ECGF, 1% nonessential amino acids, and 1 µg/ml hydrocortisone at 37°C in a 5% CO$_2$-95% air atmosphere as described earlier (40). Cells were grown in 35- or 100-mm dishes or T-75 flasks and were used in all of the experiments.

**Assay of endothelial injury and cytotoxicity.** The morphology of ECs and detachment of cells from the monolayers were assayed by phase-contrast microscopy. The uptake of trypan blue and 2-deoxy-d-[^3]H]glucose release by the cells were measured as indexes of hyperoxia-induced cytotoxicity (39).
**HYPEROXIA-INDUCED ACTIVATION OF NAD(P)H OXIDASE**

Exposure of cells to hyperoxia. HPAECs in complete MCDB medium were placed in a dry and airtight modular incubator chamber (Billups-Rothenberg, Del Mar, CA), flushed continuously with 95% O₂-5% CO₂ for 30 min until the oxygen level inside the chamber reached ~95%, and placed in a cell culture incubator at 37°C for the desired lengths of time of incubation. The concentration of O₂ inside the chamber was continuously monitored with a digital oxygen monitor. The buffering capacity of cell culture medium did not alter throughout the study period under hyperoxic exposure and maintained neutral pH (~7.4).

**Determination of O₂• release by cytochrome c reduction and hydroethidine fluorescence.** O₂• release by HPAECs was measured by SOD-inhibitable cytochrome c reduction assay (18). HPAECs (~80% confluent in 60-mm dishes) were exposed to either normoxia (95% air-5% CO₂) or hyperoxia (95% O₂-5% CO₂) for indicated time of in the presence of ferricytochrome C (33 μM) in 1.0 ml of phenol red-free medium (GIBCO-BRL medium 199) without or with SOD (100 μg) and catalase (100 μg) or 10 μM hydroethidine in phenol red-free medium (GIBCO-BRL medium 199). Wherever necessary, we included different medium containing catalase, SOD, and catalase (100 μg) and 10 μM hydroethidine at required concentrations, in the incubation medium while exposing the cells to normoxia and hyperoxia. At the end of incubation, the medium was collected and centrifuged at 4,000 g for 5 min, and absorbance of the medium was measured at 550 nm against appropriate blanks for cytochrome c reduction. The amount of O₂• released by the cells into the medium was calculated using the micromolar absorption coefficient of cytochrome c (19.1) and expressed as nanomoles of O₂• released/milligram of protein. For measurement of O₂• by hydroethidine fluorescence, medium was collected separately, cells were washed once with 1.0 ml of ice-cold medium 199 and scraped, and cell lysates were prepared by sonicating on ice with a probe sonicator at a setting of 5 for 15 s in 1.0 ml of medium 199 to prepare cell lysates. Fluorescence of oxidized hydroethidine in the medium and cell lysates, an index of formation of O₂•, was determined on an Amino Bowman series 2 spectrofluorometer with excitation and emission set at 488 and 610 nm, respectively, using appropriate blanks and expressed as a percentage of normoxic controls.

**Determination of H₂O₂ by Amplex red assay.** H₂O₂ formation in the medium was determined by fluorescence method using Amplex Red Hydrogen Peroxide Assay Kit (Molecular Probes). HPAECs (~80% confluent in 60-mm dishes) were exposed to normoxia (95% air-5% CO₂) and hyperoxia (95% O₂-5% CO₂) for indicated time of in 1.0 ml of phenol red-free medium (GIBCO-BRL medium 199) in the absence and presence of pharmacological inhibitors for specified lengths of time. At the end of incubation, the medium was collected and centrifuged at 4,000 g for 5 min, and fluorescence of the medium was measured on an Amino Bowman series 2 spectrofluorometer with excitation and emission set at 560 and 590 nm, respectively, using appropriate blanks and expressed as percentage of normoxic controls.

**ROS measurement by DCFDA fluorescence.** Formation of ROS in HPAECs was determined by DCFDA fluorescence method (54). HPAECs (~80% confluent in 35-mm dishes) were loaded with 10 μM DCFDA for 30 min in complete MCDB medium at 37°C in a 95% air-5% CO₂ environment. At the end of incubation, the medium containing DCFDA was aspirated, cells were washed once with complete MCDB medium, 1.0 ml of complete MCDB medium was added, and, if necessary, cells were preincubated with test compounds for indicated time followed by exposure to normoxia (95% air-5% CO₂) and hyperoxia (95% O₂-5% CO₂) for desired lengths of time. At the end of exposure to normoxia and hyperoxia, the dishes containing cells were placed on ice, cells were scraped, and the medium containing cells was transferred to 1.5-ml microfuge tubes and centrifuged at 8,000 g for 10 min at 4°C. The medium was aspirated, and the cell pellet was washed twice with ice-cold PBS and sonicated on ice with a probe sonicator at a setting of 5 for 15 s in 500 μl of ice-cold PBS to prepare cell lysates. Fluorescence of oxidized DCFDA in cell lysates, an index of formation of ROS, was measured on an Amino Bowman series 2 spectrofluorometer with excitation and emission set at 490 and 530 nm, respectively, using appropriate blanks. The extent of ROS formation was expressed as a percentage of normoxic control.

**ROS detection in cells by fluorescence microscopy.** Hyperoxia-induced ROS formation in cells was detected by fluorescence microscopy. HPAECs (~80% confluent) in 35-mm dishes were loaded with DCFDA (10 μM) for 30 min in complete MCDB medium at 37°C in a 95% air-5% CO₂ environment. At the end of incubation, the medium containing DCFDA was aspirated, cells were washed once with complete MCDB medium, 1.0 ml of complete MCDB medium (1:4 dilution in blocking buffer) for 1 h at room temperature with test compounds for the desired amount of time followed by exposure to normoxia (95% air-5% CO₂) and hyperoxia (95% O₂-5% CO₂) for required lengths of time. At the end of incubation, cells were washed two times with PBS and examined under a Nikon Eclipse TE 300 microscope with excitation and emission set at 490 and 530 nm, respectively. Fluorescence of oxidized DCFDA in cells was captured with Sony digital DKC 5000 camera.

**Electron paramagnetic resonance spectroscopy and spin trapping.** HPAECs (~80% confluent) in 35-mm dishes were exposed, in the presence of 50 mM DMPO in complete MCDB medium, to normoxia (95% air-5% CO₂) and hyperoxia (95% O₂-5% CO₂) at 37°C for required lengths of time. Wherever necessary, pharmacological agents such as DPI (100 μM) or SOD (100 μg) were included in the medium throughout the experimental exposure. Electron paramagnetic resonance (EPR) spectra were recorded in flat cells at room temperature with a Bruker ESP 300 spectrometer operating at X-band using the following parameters: microwave power, 20 mW; modulation amplitude, 0.5; modulation frequency, 100 kHz; receiver gain, 5 × 105; and the samples were loaded into the TM-110 cavity (60).

**Localization of NADPH oxidase subcomponents by immunofluorescence microscopy.** Immunohistochemical localization of NADPH oxidase subcomponents (p47 phox and gp 91) in HPAECs was done using antibodies against p47 phox and gp 91 subunits for immunohistochemical staining, followed by fluorescence microscopic examination. HPAECs were grown on coverslips to ~80% confluence in an atmosphere of 95% air-5% CO₂ and washed with 2 ml of prewarmed PBS (37°C). The coverslips were immediately treated with fixation-permeabilization solution (0.5% Triton X-100 and 3% paraformaldehyde) for 2 min immediately followed by treatment with 3% paraformaldehyde for 20 min at room temperature. The coverslips were washed three times with PBS for 5 min and incubated with blocking buffer [1% BSA in Tris-buffered saline containing 1% Tween (TBS-T)] for 30 min at room temperature. The coverslips were incubated with primary (p47 phox and gp 91) antibodies (1:400 dilution in blocking buffer) for 1 h at room temperature. The coverslips were gently washed three times with TBS-T for 5 min and incubated with Alexa Fluor 488 anti-goat antibody (1:200 dilution) for 1 h at room temperature followed by washing with TBS-T. Mounting medium (Kirkgaard & Perry Laboratories, Gaithersburg, MD) was applied.
and coverslips were sealed. The cells were examined for fluorescence on Nikon inverted fluorescence microscope ECLIPSE TE300.

**Determination of NADPH oxidase activity by chemiluminescence assay.** NAD(P)H oxidase activity in intact cells was assayed by lucigenin chemiluminescence assay (18). HPAECs (~80% confluent) in 35-mm dishes, after exposure to normoxia (95% air-5% CO\(_2\)) and hyperoxia (95% O\(_2\)-5% CO\(_2\)) for required lengths of time, were gently scraped and centrifuged at 400 g for 10 min at 4°C. The cell pellet was resuspended in a known volume (250 \(\mu\)l) of ice-cold phenol red-free medium (medium 199) containing either NADPH (1 \(\mu\)M) or lucigenin (20 \(\mu\)M), 50 \(\mu\)l of cell suspension (0.1 \(\times\) 10\(^6\) cells) were added to initiate the reaction followed by immediate measurement of chemiluminescence in a Packard scintillation counter in out-of-coincidence mode. Wherever necessary, pharmacological agents such as DPI (100 \(\mu\)M) or SOD (100 \(\mu\)g) were also included in the incubation mixture. Appropriate blanks and controls were established. Chemiluminescence was recorded. Neither NADPH nor NADH enhanced the background chemiluminescence of lucigenin alone (30–40 counts per min). Chemiluminescence was measured continuously for 12 min, and the activity of NAD(P)H oxidase was expressed as counts per million per cells.

**Preparation of cell lysates and Western blotting.** Preparation of cell lysates and Western blotting of proteins were performed as described earlier (41). After the HPAECs were exposed to normoxia (95% air-5% CO\(_2\)) and hyperoxia (95% O\(_2\)-5% CO\(_2\)) in complete MCDB medium for required lengths of time, cells were rinsed twice with ice-cold PBS, scraped in 150 mM NaCl, 2 mM EGTA, 5 mM glycerophosphate, 1 mM NADPH (1 \(\mu\)M) phenol red-free medium (medium 199) containing either NADPH (1 \(\mu\)M-100 \(\mu\)M) or lucigenin (20 \(\mu\)M), 50 \(\mu\)l of cell suspension (0.1 \(\times\) 10\(^6\) cells) were added to initiate the reaction followed by immediate measurement of chemiluminescence in a Packard scintillation counter in out-of-coincidence mode. Wherever necessary, pharmacological agents such as DPI (100 \(\mu\)M) or SOD (100 \(\mu\)g) were also included in the incubation mixture. Appropriate blanks and controls were established. Chemiluminescence was recorded. Neither NADPH nor NADH enhanced the background chemiluminescence of lucigenin alone (30–40 counts per min). Chemiluminescence was measured continuously for 12 min, and the activity of NAD(P)H oxidase was expressed as counts per million per cells.

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**Infection of HPAECs with p38 MAPK dominant-negative adenoviral constructs.** HPAECs were cultured in 35-mm dishes until they reached ~80% confluence. Each dish was infected with either vector or p38 dominant-negative p38 MAPK adenovirus constructs [50 plaque-forming units (PFU)/cell; ~3 × 10\(^6\) cells/dish]. After 24 and 48 h of infection, the medium was replaced with fresh complete MCDB medium, and the cells were exposed to hyperoxia and analyzed for expression of p38 MAPK by Western blotting and generation of ROS as described earlier. HPAECs infected with p38 MAPK dominant-negative or p38 wild-type or vector controls were exposed to normoxia and hyperoxia (3 h) followed by determination of intracellular ROS by the DCFDA oxidation method.

**Statistical analysis.** Statistical analysis was carried out by ANOVA using SigmaStat (Jandel). The level of statistical significance was taken as \(P < 0.05\).

**RESULTS**

Hyperoxia induces generation of \(\text{O}_2^-/\text{ROS}\) in HPAECs. Hyperoxia-induced \(\text{O}_2^-\) generation (intracellular formation and extracellular release) in intact HPAECs was measured by SOD-inhibitable cytochrome c reduction, hydroethidine fluorescence, and EPR spin trapping assays. As shown in Fig. 1A, cells released significantly higher amounts of \(\text{O}_2^-\) at 1 h (~2.5-fold) and, upon increasing the duration of hyper-
oxic exposure to 3 or 12 h, did not further enhance the release of $O_2^-$ (SOD-inhibitable cytochrome c reduction). In cells exposed to hyperoxia (12 h), the release of $O_2^-$ was completely inhibited by the flavo-enzyme inhibitor DPI (100 μM) (Fig. 1B). Alternatively, HPAECs exposed to hyperoxia (3 h), compared with the cells exposed to normoxia (3 h), in the presence of hydroethidine, which specifically reacts with $O_2^-$, showed significant enhancement of intracellular fluorescence of oxidized hydroethidine, which was significantly inhibited by SOD (100 μg) and DPI (100 μM) (Fig. 2). EPR spectroscopic studies revealed a marked increase in the signal of hydroxyl (HO•) radical in HPAECs exposed to hyperoxia (3 h) compared with cells exposed to normoxia (3 h) (Fig. 3, A and B) as measured by the formation of DMPO-OH spin adduct. The amplitude of the hydroxyl radical signal in cells exposed to hyperoxia was markedly reduced by SOD (100 μg) and DPI (100 μM), confirming $O_2^-$ as the precursor of observed HO• species and involvement of a flavin-dependent enzyme such as NAD(P)/H oxidase during hyperoxia-induced formation of HO• in HPAECs. Hyperoxia-induced intracellular generation of ROS was also studied by DCFDA oxidation. Fluorescence microscopic examination of HPAECs preloaded with DCFDA and exposed to hyperoxia (3 h) revealed enhanced intracellular fluorescence due to formation of oxidized DCFDA compared with their counterparts exposed to normoxia (3 h) (Fig. 4A). Spectrofluorometric assay of lysates of HPAECs preloaded with DCFDA and exposed to hyperoxia (3 h) exhibited significantly increased oxidation of intracellular DCFDA (twofold) compared with the cells exposed to normoxia (3 h) (Fig. 4B). DPI (100 μM) significantly inhibited hyperoxia-induced generation of intracellular ROS in HPAECs (Fig. 4C). These results show that hyperoxia-enhanced generation of $O_2^-$/ROS was DPI sensitive in HPAECs.

Hyperoxia induces formation of $H_2O_2$ in HPAECs. HPAECs exposed to hyperoxia (3 h) compared with the cells exposed to normoxia (3 h) showed significant accumulation of extracellular $H_2O_2$ (150% of normoxic control) in the medium as measured by Amplex red fluorescence, which was completely attenuated by treatment with DPI (100 μM) (Fig. 5). These results indicate that hyperoxia induced the formation of $H_2O_2$ in the medium from dismutation of $O_2^-$ released by the cells and the participation of a flavin-dependent enzyme such as NAD(P)/H oxidase in hyperoxia-induced formation of extracellular $H_2O_2$ due to its sensitivity to DPI.
DPI, but not rotenone or oxypurinol, inhibits hyperoxia-induced formation of ROS in HPAECs. Experiments were conducted utilizing the DCFDA fluorescence method to identify the source of generation of ROS in HPAECs exposed to hyperoxia. Metabolic blockers such as DPI, rotenone, and oxypurinol were used as inhibitors of flavin-dependent enzyme such as NAD(P)H oxidase, mitochondrial electron transport chain, and xanthine oxidase, respectively. Hyperoxia (12 h)-induced formation of intracellular ROS in HPAECs was enhanced (170% of normoxic control) and was significantly inhibited by DPI (100 μM) (125% of normoxic control), but not by rotenone (100 μM) or oxypurinol (100 μM) (Fig. 6). These results further demonstrate a role of NAD(P)H oxidase, but not of either mitochondrial electron transport chain or xanthine oxidase, in hyperoxia-induced formation of intracellular ROS in HPAECs.

**Fig. 4.** Hyperoxia induced formation of ROS in HPAECs as measured by 6-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (DCFDA) fluorescence. HPAECs (~80% confluent, 3 × 10⁵ cells/35-mm dish) were preloaded with DCFDA (10 μM) in complete MCDB medium under a 95% air-5% CO₂ atmosphere for 30 min and exposed to normoxia (95% air-5% CO₂) and hyperoxia (95% O₂-5% CO₂) at 37°C for desired lengths of time (3 h in A and B, 12 h in C). DPI-inhibitable formation of ROS in cells (C) was determined by including DPI (100 μM) throughout the incubation in the medium. At the end of exposure, formation of reactive oxygen species (ROS) in cells was examined under fluorescence microscope (A) and determined by fluorimetric measurement of extent of oxidation of DCFDA (B and C) as described in MATERIALS AND METHODS. Results are expressed as means ± SD from 3 individual experiments in triplicate. *Significantly different at P < 0.05 compared with normoxic controls. **Significantly different at P < 0.05 compared with hyperoxia.

**Fig. 5.** Hyperoxia induced H₂O₂ formation by HPAECs as measured by Amplex red fluorescence. HPAECs (~80% confluent, 1.0 × 10⁶ cells/60-mm dishes) were exposed to normoxia (95% air-5% CO₂) and hyperoxia (95% O₂-5% CO₂) at 37°C for 3 h in 1.0 ml of phenol red-free medium (GIBCO-BRL medium 199) in the absence or presence of 100 μM DPI. At the end of exposure, formation of H₂O₂ in the medium was measured fluorimetrically using the Amplex Red Hydrogen Peroxide Assay kit (Molecular Probes) as outlined in MATERIALS AND METHODS. Results are expressed as means ± SD from 3 individual experiments in triplicate. *Significantly different at P < 0.05 compared with normoxic controls. **Significantly different at P < 0.05 compared with hyperoxic treatment.

**Fig. 6.** DPI but not rotenone and oxypurinol inhibited hyperoxia-induced formation of ROS by HPAECs. HPAECs (~80% confluent, 3 × 10⁵ cells/35-mm dish) were preloaded with DCFDA (10 μM) in complete MCDB medium under a 95% air-5% CO₂ atmosphere for 30 min and exposed to normoxia (95% air-5% CO₂) and hyperoxia (95% O₂-5% CO₂) at 37°C for 12 h in the absence or presence of DPI (100 μM), oxypurinol (100 μM), and rotenone (100 μM). At the end of exposure, formation of ROS in cells was determined by fluorimetric measurement of extent of oxidation of DCFDA as described in MATERIALS AND METHODS. Results are expressed as means ± SD from 3 individual experiments in triplicate. *Significantly different at P < 0.05 compared with normoxic controls. **Significantly different at P < 0.05 compared with hyperoxic treatment.
ence of either NADPH or NADH did not cause detectable chemiluminescence (data not shown). In intact HPAECs, exogenous NADPH caused a significant and dramatic increase in lucigenin chemiluminescence at 1-μM concentration. When we further increased the concentration of NADPH to 50 μM, the chemiluminescence almost doubled and showed no further change at 100 μM NADPH (Fig. 7A). Both DPI (100 μM) and SOD (100 μg) nearly abolished the NADPH-dependent lucigenin chemiluminescence in intact HPAECs (Fig. 7A). NADH-dependent lucigenin chemiluminescence in intact HPAECs was not only significant and linear with tested concentrations of NADH (1, 50, and 100 μM) (Fig. 7B) but was also lower by several orders of magnitude compared with the NADPH-dependent lucigenin chemiluminescence (Fig. 7, A and B). Although the NADH-dependent lucigenin chemiluminescence in intact HPAECs was almost completely inhibited by SOD (100 μg), it was marginally blocked by DPI (100 μM) (Fig. 7B) in contrast to almost entire inhibition of NADPH-dependent lucigenin chemiluminescence by DPI (Fig. 7, A and B). NADH- and NADPH-dependent lucigenin chemiluminescence was significantly enhanced in intact HPAECs exposed to hyperoxia (12 h) compared with that in normoxia (12 h), where the latter was greater than the former by severalfold (Fig. 7C). These results show that hyperoxia significantly enhanced both the NADPH- and NADH-dependent O2•− production, indicating the activation of NAD(P)H oxidase. Furthermore, both DPI and SOD attenuated the NADPH-dependent generation of O2•− by HPAECs.

HPAECs contain phagocytic NADPH oxidase subcomponents. The presence of phagocytic NADPH subcomponents in HPAECs was examined by immunofluorescence microscopy and Western blotting. HPAECs exhibited intense and moderate fluorescence due to reactivities to human neutrophil p47 phox and gp 91 antibodies, indicating the presence of p47 phox and gp 91 in HPAECs (Fig. 8A). SDS-PAGE and Western blotting analysis of lysates of HPAECs revealed the presence of gp 91, p67 phox, and p22 phox subcomponents, similar to the NADPH subcomponents of human neutrophils (Fig. 8B). The only phagocytic subcomponent of NADPH oxidase not detectable by SDS-PAGE and Western blotting analysis in HPAECs was p40 phox (data not shown).

p22 phox antisense blocks hyperoxia-induced formation of ROS in HPAECs. We measured the role of NAD(P)H oxidase in hyperoxia-induced formation of intracellular ROS by DCFDA fluorescence in HPAECs by exposing cells to hyperoxia after overnight transfection with p22 phox antisense plasmid. The intracellular generation of ROS in HPAECs transfected with vector control was significantly higher in hyperoxia (3 h) (158% of normoxic vector control), whereas in the cells transfected with p22 phox antisense, the generation of ROS was significantly lower after both normoxia (3 h) and hyperoxia (3 h) (48% of normoxic vector control and 60% of hyperoxic vector control) (Fig. 9A).

Figure 9B shows diminished expression of p22 phox subcomponent of NAD(P)H oxidase in cells transfected with p22 phox antisense. The intracellular ROS by DCFDA fluorescence due to lucigenin chemiluminescence in a Packard scintillation counter in out-of-coincidence mode. Wherever necessary, pharmacological agents such as DPI (100 μM) or SOD (100 μg) were also included in the incubation medium. The extent of formation of O2•− was expressed as counts per min (cpm). Results are expressed as means ± SD from 3 individual experiments in triplicate. *Significantly different at P < 0.05 compared with normoxic controls. **Significantly different at P < 0.01 compared with hyperoxic treatment.
with p22 phox antisense for 12 h as revealed by Western blotting analysis. These results suggest a role for p22 phox in activation of NAD(P)H oxidase and formation of intracellular ROS in hyperoxia.

Hyperoxia activates MAPKs in HPAECs. The activation of MAPKs in HPAECs upon exposure to hyperoxia (3 h) was studied by SDS-PAGE and Western blotting analysis. As shown in Fig. 10, the extent of the phosphorylation of ERK and p38 MAPK in cells exposed to hyperoxia (3 h) was markedly higher than that in the cells exposed to normoxia (3 h). No such increase in the phosphorylation of JNK was noticed in the cells exposed to hyperoxia (Fig. 10). These results demonstrate that hyperoxia activates both ERK and p38 MAPK in HPAECs.

MAPK inhibitors attenuate hyperoxia-induced ROS formation in HPAECs. We studied the role of MAPKs in hyperoxia-induced formation of intracellular ROS by exposing HPAECs to hyperoxia (3 h) in the absence or presence of various MAPK-specific inhibitors and measured the extent of phosphorylation of ERK, p38 MAPK, and JNK as described in Materials and Methods. Numerical values at the bottom indicate the ratios of intensity of phosphorylated vs. nonphosphorylated bands of MAPKs. Results shown are representative blots of at least 3 independent experiments.

Fig. 8. HPAECs contain NAD(P)H oxidase subcomponents similar to phagocytic NAD(P)H oxidase subcomponents. A: immunohistochemical localization of NAD(P)H oxidase subcomponents (p47 phox and gp 91) in lysates of HPAECs was done using antibodies against neutrophil p47 phox and gp 91 subunits for immunohistochemical staining followed by fluorescence microscopic examination as described in Materials and Methods. B: lysates of HPAECs were prepared, and equal amounts of proteins were subjected to SDS-PAGE on 12% gels and analyzed for different subcomponents of NAD(P)H oxidase by Western blotting with anti-gp 91, anti-p67, anti-p47, and anti-p22 antibodies raised against neutrophil NAD(P)H subcomponents. Cytosol and membranes of neutrophil in equal amounts of protein were simultaneously subjected to SDS-PAGE and Western blotting as positive controls for various NAD(P)H oxidase subcomponents.
CO2) in complete MCDB medium at 37° for 1 h, preloaded with DCFDA (10 μM) for 30 min under a 95% air-5% CO2 atmosphere, and exposed to normoxia (95% air-5% CO2) and hyperoxia (95% O2-5% CO2) in complete MCDB medium at 37°C for 3 h. At the end of exposure, formation of ROS in cells was determined by fluorimetric measurement of extent of oxidation of DCFDA as described in MATERIALS AND METHODS. Results are expressed as means ± SD from 3 individual experiments in triplicate. *Significantly different at P < 0.05 compared with hyperoxic treatment.

Fig. 11. MAPK inhibitors attenuated hyperoxia-induced ROS formation in HPAECs as measured by DCFDA fluorescence. HPAECs (~80% confluent, 3 × 10^5 cells/35-mm dish) were pretreated with p38 MAPK inhibitors SB-202190 (20 μM) and SB-203580 (20 μM) (A) and MEK inhibitor PD-98059 (20 μM) (B) for 1 h, preloaded with DCFDA (10 μM) for 30 min under a 95% air-5% CO2 atmosphere, and exposed to normoxia (95% air-5% CO2) and hyperoxia (95% O2-5% CO2) in complete MCDB medium at 37°C for 3 h. The MEK1/2 inhibitor PD-98059 (20 μM), although less effective than the p38 MAPK-specific inhibitors, also significantly attenuated the hyperoxia-induced intracellular generation of ROS in HPAECs (190% of the normoxic control, Fig. 11B). These results show a role for both ERK and p38 MAPK in hyperoxia-induced ROS generation in HPAECs.

Infection of HPAECs with p38 MAPK dominant-negative adenoviral constructs attenuates hyperoxia-induced generation of ROS. To further establish the role of p38 MAPK in hyperoxia-induced generation of ROS, we infected HPAECs infected with vector control and dominant-negative p38 MAPK adenoviral constructs to normoxia (3 h) and hyperoxia (3 h), and the extent of intracellular oxidation of DCFDA was determined. As shown in Fig. 12A, hyperoxia-induced generation of ROS was significantly attenuated in cells infected with dominant-negative p38 MAPK adenoviral constructs (125% of normoxic vector controls) compared with vector control infected cells (275% of normoxic vector controls). Correspondingly, the expression of p38 MAPK protein in cells infected with dominant-negative con-structs was markedly enhanced, as revealed by Western blotting of cell lysates (Fig. 12B). These results further support the role of p38 MAPK in hyperoxia-induced generation of ROS in HPAECs.

DISCUSSION

In the present study, we employed various established techniques (5) to determine the generation/release of O2·− and ROS by the HPAECs under normoxic and hyperoxic exposure. HPAECs exposed to hyperoxia for 1, 3, and 12 h released significantly greater amounts of O2·− as measured by an increase in either SOD-inhibitable cytochrome c reduction or the fluorescence of hydroethidine in the medium (Figs. 1, A and B, and 2). Our results from the Amplex red fluorescence assay also showed that hyperoxia (3 h) induced significant formation of extracellular H2O2 compared with normoxia (3 h) in HPAECs (Fig. 5). EPR spectroscopic examination of HPAECs exposed to hyperoxia (3 h) compared with those exposed to normoxia (3 h) revealed the intracellular formation of SOD-inhibitable

Fig. 12. Effect of p38 MAPK dominant-negative adenoviral construct on hyperoxia-induced generation of ROS in HPAECs. HPAECs (~80% confluent, 3 × 10^5 cells/35-mm dish) were infected with vector control and p38 MAPK dominant-negative (dom.-ve) adenoviral constructs (50 plaque-forming units/dish) for 24 and 48 h followed by preloading with DCFDA (10 μM) for 30 min under a 95% air-5% CO2 atmosphere and exposure to normoxia (95% air-5% CO2) and hyperoxia (95% O2-5% CO2) in complete MCDB medium at 37°C for 3 h as described in MATERIALS AND METHODS. At the end of incubation, intracellular generation of ROS was determined by measuring the fluorescence of oxidized DCFDA as described in MATERIALS AND METHODS (A). Results shown in A are averages of 3 independent experiments. *Significantly different at P < 0.05 compared with normoxic controls. **Significantly different at P < 0.05 compared with hyperoxic treatment.

The expression of p38 MAPK protein in cells infected with dominant-negative con-
DMPO-OH, suggesting the formation of HO· from O$_2^-$ and H$_2$O$_2$ (Fig. 3, A and B). We also demonstrated that hyperoxia (3 h) induced significant generation of intracellular ROS as measured by DCFDA fluorescence in HPAECs compared with normoxia (3 h) (Fig. 4, A–C). DCFDA diffuses passively into the cells and undergoes hydrolysis mediated by the intracellular esterases to a nonfluorescent derivative, and, upon oxidation by ROS (O$_2^-$ and HO·), it is converted into a fluorophore that serves as an indicator of formation of ROS (18, 54). Measurement of extracellular O$_2^-$ by the SOD-inhibitable cytochrome $c$ reduction spectrophotometric assay is problematic due to dismutation of O$_2^-$ into H$_2$O$_2$, which subsequently oxidizes O$_2^-$-reduced cytochrome $c$ back to the oxidized form leading to erroneous determination of O$_2^-$ (18). To overcome this problem, addition of catalase to the incubation medium is necessary (18). The hydroethidine fluorescence assay for determination of O$_2^-$ and fluorimetric determination of H$_2$O$_2$ can be used as a complementary method to the SOD-inhibitable cytochrome $c$ reduction assay for determination of O$_2^-$: O$_2^-$ generated intracellularly is converted to various species of ROS, which can be determined by EPR spin-trapping method and spectrofluorometric assay of DCFDA fluorescence. In the current investigation, all of the employed methods to determine the ROS substantiated the hyperoxia-induced formation of ROS in HPAECs. DCFDA spectrofluorometric assay is a well-established technique and convenient for studies with intact cells. We and others have employed this technique, which yields reproducible results comparable to the studies that have used other methods for the determination of ROS (1, 18). It is for those reasons that in the current investigation, we widely employed the DCFDA spectrofluorometric assay for the determination of intracellular generation of ROS in HPAECs under normoxic and hyperoxic exposure.

Several studies have revealed the activities of both NADH and NADPH oxidase(s) in vascular ECs (37, 49). Our results also demonstrate that HPAECs contain an NADH- and NADPH-dependent oxidase system that activates molecular oxygen to O$_2^-$, which we observed HO· EPR signal, which reflects the formation of HO· as a predominant radical formed in HPAECs under hyperoxia. EPR DMPO spin-trapping analysis also revealed the formation of O$_2^-$ as a minor species of ROS in HPAECs during hyperoxia. Because HPAECs in the current study were exposed to hyperoxia in complete medium, trace amounts of Fe present in the medium could have contributed to the formation of HO· from H$_2$O$_2$ generated from the dismutation of O$_2^-$: SOD used in our studies, at a concentration of 100 μg, dismutated O$_2^-$, resulting in formation of H$_2$O$_2$, and might have complexed with Fe, rendering it inaccessible for the homolysis of H$_2$O$_2$ to form HO·. This could have been the reason for the diminished intensity of HO· EPR signal in HPAECs treated with SOD during hyperoxia. It was also evident from our current study that hyperoxia-induced generation of intracellular ROS generation was only significantly inhibited by DPI but not by oxypurinol or rotenone (Fig. 6). These results suggest that NAD(P)H oxidase is involved in the hyperoxia-induced generation of both extracellular and intracellular ROS (O$_2^-$, H$_2$O$_2$, HO·) in HPAECs.

On the other hand, studies conducted by others suggest the role of mitochondrial electron transport chain in the generation of ROS in ECs during exposure to hyperoxia (46, 59). One such study by Sanders et al. (46) suggests that the mitochondrial electron transport chain is responsible for the generation of ROS and other reactive intermediates in sheep pulmonary microvascular ECs on exposure to hyperoxia. In contrast to the study by Sanders et al. (46) in which sheep pulmonary microvascular ECs were used, in our current studies, HPAECs were used to study the mechanisms of generation of ROS under hyperoxia. We believe that ECs from different sources not only from different species but also from different vessels, such as microvessels and macrovessels, appear to possess different mechanisms of generation of ROS under hyperoxia. In addition, in the study conducted by Sanders et al. (46) the duration of exposure of sheep pulmonary microvascular ECs to hyperoxia was shorter (30 min), whereas in our studies HPAECs were exposed to hyperoxia (3 h).

Fig. 13. Proposed mechanism of activation and regulation of NAD(P)H oxidase in lung endothelial cells by hyperoxia. RhoGDI; Rho guanine nucleotide dissociation-inhibitor alpha.
NADPH-dependent generation of \( \text{O}_2^- \) and the role of NADPH oxidase was suggested (30).

Several nonphagocytic cells such as the vascular smooth muscle and ECs have been shown to contain many of the subcomponents of the phagocytic NADPH oxidase complex (3, 6, 29, 26). Although the exact role in EC physiology and function and the mechanism of activation of vascular EC NAD(P)H oxidase are yet to be understood thoroughly, it is clear from our results that HPAECS possess most of the subcomponents of human neutrophil NADPH oxidase. The only phagocytic subcomponent of NADPH oxidase not detectable by SDS-PAGE and Western blotting analysis in HPAECS was p40 phox. Our results revealed that transfection with p22 phox antisense attenuated hyperoxia-induced generation of ROS in many cultured cell systems (19, 43). Also, it was shown that prolonged exposure to hyperoxia stimulated cyanide-insensitive release of \( \text{H}_2\text{O}_2 \) by alveolar macrophages, and the role of NADPH oxidase was suggested (30).

Similarly, hyperoxia induced both NADH- and NADPH-dependent generation of \( \text{O}_2^- \) in lung and liver nuclei (58). These studies and our current results strongly support the notion that an oxygen sensor like NAD(P)H oxidase (27) may be an active contributor of ROS during hyperoxia in the vascular endothelium.

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