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Original Research Communication

Hyperoxia Alters Phorbol Ester-Induced Phospholipase D Activation in Bovine Lung Microvascular Endothelial Cells

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ABSTRACT

We investigated the effect of hyperoxia on phospholipase D (PLD) activation in bovine lung microvascular endothelial cells (BLMVECs). Generation of intracellular reactive oxygen species in BLMVECs exposed to hyperoxia for 2 or 24 h was three-fold higher compared with normoxic cells as measured by dichlorodihydrofluorescein di(acetoxymethyl ester) fluorescence. Exposure of BLMVECs to hyperoxia for 2 or 24 h attenuated 12-*O*-tetradecanoylphorbol 13-acetate (TPA)-mediated PLD activation compared with normoxic cells, however, hyperoxia did not alter basal PLD activity. Antioxidants, such as propyl gallate and pyrrolidine dithiocarbamate, reversed the effect of hyperoxia on TPA-induced PLD activity. Furthermore, the TPA-induced PLD activation was inhibited not only by the protein kinase C inhibitor, Go6976, but also by the tyrosine kinase inhibitor, genistein, and by the Src kinase specific inhibitor, PP-2, suggesting the involvement of protein kinase C and also tyrosine kinases in TPA-induced PLD activition. Western blot analysis of cell lysates from the hyperoxic (2 or 24 h) BLMVECs stimulated with TPA with anti-phosphotyrosine antibody showed an attenuation in overall tyrosine phosphorylation of proteins. In conclusion, we have demonstrated that hyperoxia enhanced the generation of reactive oxygen species in lung microvascular endothelial cells and attenuated TPA-induced protein tyrosine phosphorylation and PLD activation. As protein tyrosine phosphorylation and PLD play important roles in inflammatory responses, this could provide a mechanism for the regulation of endothelial barrier function during hyperoxic lung injury. *Antioxid. Redox Signal.* 5, 217–228.

INTRODUCTION

OXYGEN THERAPY is required in patients with acute respiratory distress syndrome precipitated by sepsis, primary bacterial or viral pneumonia, fat embolism, near drowning, massive blood transfusion, and inhalation of smoke or other toxic gases. Patients with chronic obstructive pulmonary diseases due to emphysema, asthma, and bronchitis also require oxygen intervention as a result of respiratory failure. Similarly, ventilator-induced lung injury is known to occur as a result of prolonged exposure to high oxygen concentrations. Thus, sustained elevation in inspired oxygen ($FiO_2 > 60\%$) results in inflammatory changes, alveolar infiltration, and eventually pulmonary fibrosis.

One of the earliest and most extensive targets of hyperoxic injury is the pulmonary capillary endothelium. Endothelial cells (ECs) exposed to hyperoxia show an increase in release of lactate dehydrogenase, impaired cell growth, and increased permeability (6). Morphological changes associated with hyperoxic cells include enlargement and early vacuolization, gross distortions, regular stellate appearance after 24 h, and detachment from the culture dish by 72 h (46), strongly suggesting cytoskeletal alterations. Expression of intracellular adhesion molecule increases in response to hyperoxia sug-

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gesting the possibility of increased adherence of neutrophils to pulmonary endothelium (2, 21, 57), whereas expression of vascular endothelial growth factor is decreased in rat lung during hyperoxia, indicating that angiogenesis may be impaired (44).

The metabolic status of the cell during hyperoxia is also altered. The tricarboxylic acid cycle is impaired (51) along with a concomitant elevation of glycolytic activity in the lungs of rats exposed to hyperoxia (1). Transcription of the antioxidant enzyme, manganese superoxide dismutase, is induced severalfold during hyperoxia, suggesting up-regulation of antioxidant defense mechanism(s) (10). Hyperoxia also increases the expression of a variety of stress-responsive genes, including heme oxygenase-1, c-fos, c-jun, and CAAT-enhancer binding (C/EBP) (9, 47). Macrophage cell line (RAW 264.7) exposed to hyperoxia undergoes apoptosis through activation of extracellularly regulated kinase (ERK) (22, 45). Whereas the activity of phospholipase A_2 decreased upon hyperoxic exposure of lung (15), levels of arachidonic acid metabolites such as leukotriene B_4 and prostaglandins increased in bovine pulmonary artery ECs (BPAECs) after 48 h of hyperoxia (18). Reactive oxygen species (ROS) have been implicated in hyperoxia-induced biochemical changes (52). Previous studies have demonstrated that exogenously administered ROS, such as hydrogen peroxide (H_2O_2), fatty acid hydroperoxide, 4-hydroxynonenal, and oxidized low-density lipoprotein, modulated signal transduction pathways of phospholipases A_2, C, and D in ECs (31, 33). Phospholipase D (PLD) plays an integral role in many cellular events, such as membrane trafficking, proliferation, senesence, mitogenesis, oncogenesis, and inflammation (24). PLD catalyzes the hydrolysis of phosphatidylcholine to generate phosphatidic acid (PA) and choline. PA is a lipid second messenger that mediates the functional role of PLD (13). PA can be converted to other bioactive lipids, such as diacylglycerol (DAG) and lysophosphatidic acid (LPA), by lipid phosphate phosphatase and phospholipase A_1/A_2 (3, 55, 56), respectively. DAG activates protein kinase C (PKC), whereas LPA is known to act through the endothelial differentiation gene receptors regulating several functions, such as cellular migration, proliferation, and permeability (28). PLD is stimulated by various hormones, neurotransmitters, growth factors, cytokines, and other agonists that activate cell-surface receptors (11). Activation of PLD requires its interaction with small G proteins, such as ADP-ribosylation factor (Arf) and members of the Rho family of GTPases (RhoA, Rac1, and cdc42). Regulation of oxidant-induced PLD activation by PKC (27), Src (19, 42), and p38 mitogen-activated protein kinase is cell type-specific (36).

The aim of the present study was to investigate the effect of intracellularly generated ROS on PLD activation in bovine lung microvascular ECs (BLMVECs). We used hyperoxia as a model system for generation of intracellular ROS, and our results show for the first time that the intracellular ROS generated during hyperoxia attenuated phorbol ester-induced PLD activation in BLMVECs. Furthermore, coincubation of the cells with antioxidants such as propyl gallate (PG) and pyrrolidine dithiocarbamate (PDTC) blocked the effect of hyperoxia on phorbol ester-induced PLD activation. We also show that the phorbol ester-induced PLD activation in BLMVECs is tyrosine kinase-dependent. In BLMVECs, phorbol ester increased tyrosine phosphorylation of proteins migrating at ~60 kDa and ~125 kDa, and hyperoxia attenuated 12-*O*-tetradecanoylphorbol 13-acetate (TPA)-induced total tyrosine phosphorylation, as well as distribution within the cells.

MATERIALS AND METHODS

Materials

Minimum essential medium (MEM), sodium orthovanadate, nonessential amino acids, trypsin-EDTA, penicillin–streptomycin, fetal bovine serum, PG, PDTC, and TPA were obtained form Sigma (St. Louis, MO, U.S.A.). Go6976, genistein, Brij 35 detergent (polyoxyethylene glycol dodecyl ether), and PP-2 were obtained from Calbiochem (San Diego, CA, U.S.A.). BLMVECs (passage 4) were purchased from Cell Systems (University of Washington, Seattle, WA, U.S.A.). EC growth supplement and affinity-purified monoclonal anti-phosphotyrosine antibody (4G10) were obtained from Upstate Biotechnology (Lake Placid, NY, U.S.A.). An enhanced chemiluminescence kit was from Amersham (Arlington Heights, IL, U.S.A.). [^{32}P]Orthophosphate (carrier free), [choline-*methyl*-^{3}H]dipalmitoylphosphatidylcholine (DPPC) and [γ-^{32}P]ATP in 10 m*M* Tricine buffer (specific activity 6,000 Ci/mmol) were purchased from DuPont NEN (Boston, MA, U.S.A.).

EC culture

BLMVECs cultured in MEM were maintained at 37°C in a humidified atmosphere of 5% CO_2/95% air and grown to contact-inhibited monolayers with a typical cobblestone morphology (30). Cells from each primary flask were detached with 0.05% trypsin, resuspended in fresh medium, and cultured in complete medium, to 90% confluency before exposure to normoxic or hyperoxic (95% O_2/5% CO_2) conditions. Cells from passages 4–9 were used in all the experiments.

Retroviral infected stable human dermal microvascular endothelial cell (HDMVEC) lines of PKCα and PKCε

Infectious amphotropic retroviruses were produced from MSCV2.1 PKCα, PKCε, and control MSCV2.1 backbone as previously described (43). In brief, the murine ecotropic packaging line GP+ E-86 (E-86) (25) was transfected with MSCV2.1 constructs, and transient supernatants collected 48 h later containing infectious virions were then used to infect the amphotropic packaging line GP+ EnvAm12 (Am-12) (25). Individual colonies of Am-12 were selected with G418 (0.75 mg of dry powder/ml) containing medium. Viral titers from selected clones were evaluated by infection of NIH-3Y3 fibroblasts using dilutions of virus-containing supernatants from multiple clones. G418-selected Am-12 clones were further analyzed by Southern blot analysis to confirm the integrity of the provirus. Viral supernatants from three clones, Am-12 MSCV2.1 PKCα, PKC$\varepsilon\varepsilon$, and Am-12 MSCV2.1, producing 10^6 colony-forming units/ml were used to infect HDMVECs, and clones of transduced HDMVECs, resistant to 250 μg/ml G418 were further characterized by Southern blot analysis.

Measurement of intracellular ROS production through the oxidation of dichlorodihydrofluorescein di(acetoxymethyl ester) (DCFDA)

Intracellularly generated ROS were measured by fluorescence microscopy based on the oxidation of DCFDA. Cells grown on glass cover slips were placed in a Modular Incubator Chamber (Billups-Rothenberg, Del Mar, CA, U.S.A.), which was flushed for 15 min with 95% O_2/5% CO_2 and maintained for 2 h or 24 h at 37°C. The cells were washed with buffer A (20 m*M* HEPES, 1.5 m*M* $CaCl_2$, 4.9 m*M* KCl, 137 m*M* NaCl, 1.2 m*M* NaH_2PO_4, 1.2 m*M* $MgSO_4$, 15 m*M* D-glucose, pH 7.4) and incubated with 10 μ*M* DCFDA (Molecular Probes) in buffer A for 30 min. The nonpolar dye diffuses passively into cells and is hydrolyzed by intracellular esterases to a nonfluorescent derivative, dichlorofluorescein (DCF). The cover slips were then gently washed for 30 min with indicator-free buffer A at room temperature to allow deesterification of the eye. DCF fluorescence was recorded at a field of two or three connected monolayer cells on a cover slip in a perfusion chamber mounted on the stage of a modified Nikon Diaphot inverted epifluorescence microscope. DCF fluorescence was excited at 480 ± 20 nm using a xenon short arc lamp (UXL-75X c̄ Ushio Inc.) and band pass interference filters (Omega Optical) with selected wavelength bands of emitted fluorescence at 535 ± 25 nm. Emitted DCF fluorescence was collected and measured using a spectrofluorometer (PTI DeltaScan). As DCF fluorescence intensity from BLMVECs increased linearly during the measurement period (~10 s), the slope of DCF fluorescence intensity increase was used to quantify the intracellularly generated ROS.

Determination of PLD activation in intact ECs

BLMVECs in 35-mm dishes (5×10^5 cells/dish) were incubated with [^{32}P]orthophosphate (5 μCi/ml) for 18–24 h in phosphate-free Dulbecco's modified Eagle's medium (DMEM) containing 2% fetal bovine serum. Cells exposed to normoxia were incubated at 37°C in 95% air/5% CO_2, whereas cells exposed to hyperoxia were flushed for 15 min with 95% O_2/5% CO_2 in a Modular Incubator Chamber, after which the cells were incubated at 37°C for the indicated time periods. The radioactive medium was aspirated, cells were incubated in serum-free MEM or MEM containing agonist at concentrations indicated in the presence of 0.05% butanol. Incubations were terminated by addition of 1 ml of methanol/HCl (100:1, vol/vol), lipids were extracted in chloroform/methanol/water (1:1:0.9, by volume), and phosphatidylbutanol (PBt) (formed an index of PLD activation) was analyzed by thin-layer chromatography (30). Radioactivity associated with [^{32}P]PBt was quantified by liquid scintillation counting. All experiments were done in triplicate, values were normalized to 1×10^6 dpm in total lipid extract, and [^{32}P]PBt formed was expressed as disintegrations per minute per dish or percentage of control.

Measurement of PKC activity

BLMVECs grown in 100-mm dishes were incubated in normoxia (95% air/ 5% CO_2) or hyperoxia (95% O_2/5% CO_2) for 24 h. Medium was aspirated, and cells were exposed to serum-free MEM or MEM containing 25 n*M* TPA under normoxic or hyperoxic conditions for 30 min. Cells were scraped into PKC assay buffer [50 m*M* Tris (pH 7.5), 5 m*M* EDTA, 10 m*M* EGTA, 10 m*M* benzamidine, 0.3% (wt/vol) β-mercaptoethanol, 10 n*M* okadaic acid, and protease inhibitors] and sonicated with a probe sonicator (3 × 15 s). Cytosolic and particulate fractions were isolated by centrifugation at 105,000 *g* for 90 min, and PKC activity was measured in total cell lysates and cytosolic and particulate fractions using the Biotrak PKC enzyme assay kit from Amersham Pharmacia Biotech (Buckinghamshire, U.K.).

Lipid peroxidation assay

Lipid peroxidation was determined by the thiobarbituric acid reaction assay according to published procedures (41). The extent of lipid peroxidation was calculated from a standard curve established using 1,1,3,3-tetraethoxypropane. Lipid peroxidation was expressed as nanomoles of thiobarbituric acid reactive substances (TBARS) formed per milligram of protein.

In vitro *PLD assay*

Activities of PLD1 and PLD2 were determined in lysates of cells exposed to normoxia and hyperoxia for 24 h by the [^{3}H]choline release assay (8, 42). After exposure to normoxia and hyperoxia, cells were washed with ice-cold phosphate-buffered saline, scraped, centrifuged for 5 min at 1,000 *g*, and resuspended in 50 m*M* Na-HEPES buffer (pH 7.4) containing 1 μ*M* dithiothreitol (DTT), 10 μg/ml protease inhibitors, 1 μg/ml aprotinin, and 1 μg/ml leupeptin, and cell lysates were prepared by sonication of cells on ice at a setting of 5 with a probe sonicator. Liposomal substrate [dioleoylphosphatidylethanolamine (DOPE)/phosphatidylinositol 4,5-bisphosphate (PIP_2)/DPPC in a molar ratio of 16:1.4:1] was prepared by sonicating the lipid mixture with a probe sonicator at a setting of 6 for 5 × 30 s at room temperature in vesicle buffer containing 50 m*M* Na-HEPES (pH 7.5), 3 m*M* EGTA, 80 m*M* KCl, 1 m*M* DTT, and 3 m*M* $MgCl_2$. [Choline-*methyl*-^{3}H]DPPC was added to the liposomal substrate to give 50,000–70,000 dpm/assay. Equal volumes of cell lysates (10 μl) were added to the reaction mixture containing the liposomal substrate in a final volume of 100 μl in 50 m*M* Na-HEPES (pH 7.5), 3 m*M* EGTA, 80 m*M* KCl, 1 m*M* DTT, 3 m*M* $MgCl_2$, and 2 m*M* $CaCl_2$ and incubated at 37°C for 30 min. To differentiate between PLD1 and PLD2 activities, the incubation mixtures contained Arf-guanosine 5′-*O*-(3-thiotriphosphate) (1 μg) and Rho-guanosine 5′-*O*-(3-thiotriphosphate) (1 μg), wherever required (8). The reactions were terminated by the addition of 200 μl of 10% TCA and 100 μl of 1% bovine serum albumin. The mixture was gently vortexed and centrifuged in a microfuge at 10,000 *g* for 5 min. Radioactivity in the supernatant was measured by liquid scintillation spectroscopy. PLD activity was expressed as picomoles of the substrate hydrolyzed per milligram of protein in 30 min.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting

Proteins of BLMVECs were separated on 12% SDS-PAGE gels, transferred to Immobilon-P membranes by electroblotting, incubated with anti-phosphotyrosine antibodies

(1:3,000 dilution) or anti-Src antibodies (1:500 dilution), and visualized using a chemiluminescent kit (ECL, Amersham Pharmacia Biotech) (35, 42).

Statistical analysis

All experiments were done in triplicate and data expressed as means ± SD. Statistical comparisons were made by Student's *t*-test and by analysis of variance. Statistical significance was accepted at $p < 0.05$.

RESULTS

Hyperoxia increases generation of intracellular ROS

High oxygen tension is known to increase free radical production in mammalian cells (18, 48). Incubation of BLMVECs in 95% O_2/5% CO_2, compared with 95% air/5% CO_2, increased lipid peroxidation measured as TBARS and rate of intracellular ROS generation as measured by DCFDA oxidation after 2 and 24 h of exposure (Fig. 1). Quantitative analysis of fluorescence intensity revealed that ROS production in hyperoxic cells was about threefold higher compared with normoxic cells at both time points of exposure (Table 1). A similar increase in fluorescence intensity of DCFDA in the hyperoxic cells (2 or 24 h) compared with control cells was also observed under fluorescence microscopy (data not shown). Exposure of BLMVECs to hyperoxia did not cause cytotoxicity as measured by 2-[^{3}H]deoxyglucose release (data not shown).

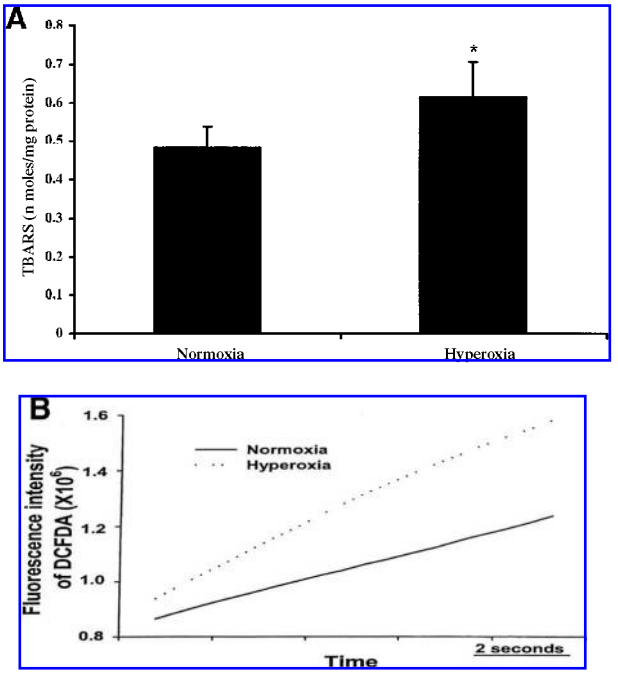


FIG. 1. Hyperoxia increases TBARS and intracellular generation of ROS. BLMVECs were grown on cover slips and incubated in normoxia or hyperoxia for 2 h (**A**) or 24 h (**B**). TBARS were assayed (A) in control and hyperoxic cells as described in Materials and Methods. For ROS measurements, cells were incubated with 10 μ*M* DCFDA in MEM for 30 min. The cover slips were washed to remove the dye not taken up by the cells, and the fluorescence was recorded by a Nikon Diaphot inverted epifluorescence microscope. Autofluorescence from unloaded cells was subtracted automatically from sample fluorescence recordings. Slopes for intracellular oxidation of the dye due to ROS generation over a 10-s time period from normoxic and hyperoxic cells were compared (B). *Significantly different ($p < 0.05$) from normoxic cells.

TABLE 1. HYPEROXIA INCREASES DCFDA OXIDATION

	Fluorescence intensity (% control)	
	2 h	*24 h*
Normoxia	100 ± 7	100 ± 12
Hyperoxia	330 ± 40	256 ± 26

BLMVECs grown on glass cover slips were exposed to normoxia or hyperoxia for 2 or 24 h as indicated in Fig. 1. Cells were incubated with 10 μ*M* DCFDA for 30 min, and fluorescence was recorded by a Nikon Diaphot inverted epifluorescence microscope. Intracellular oxidation of DCFDA due to ROS generation over a 10-sec time period from normoxic and hyperoxic cells is compared. Values are means ± SD of three independent determinations.

Hyperoxia attenuates agonist-induced PLD activation

As hyperoxia increased ROS generation in ECs, its effect on agonist-induced PLD activation was studied. BLMVECs were exposed to hyperoxia for 2 or 24 h and stimulated with 25 n*M* TPA for 30 min. Hyperoxia attenuated TPA-induced PLD activation as quantified by [^{32}P]PBt formation, an index of PLD activation in intact cells (Fig. 2). Furthermore, the TPA-induced [^{32}P]PBt formation in hyperoxic cells was sig-

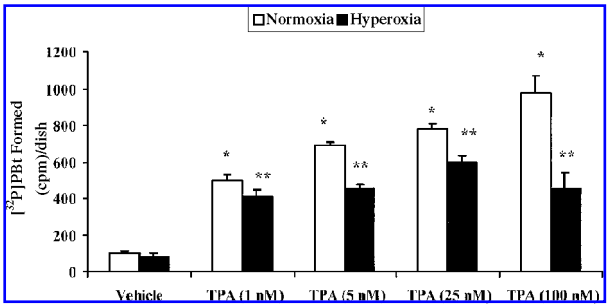


FIG. 2. Dose dependence of TPA-induced PLD activation in BLMVECs. Cells (35-mm dishes) were labeled with [^{32}P]orthophosphate (5 μCi/dish) in phosphate-free DMEM for 24 h. Medium was aspirated, cells were incubated in normoxia or hyperoxia for 2 h in the presence of vehicle or different concentrations of TPA, and incubations were continued for an additional 30 min. Lipids were extracted under acidic conditions, and [^{32}P]PBt was quantified as described in Materials and Methods. Values represent means ± SD of triplicate samples from three experiments. *Significantly different ($p < 0.05$) from normoxic control values. **Significantly different ($p < 0.05$) from TPA-treated normoxic cells.

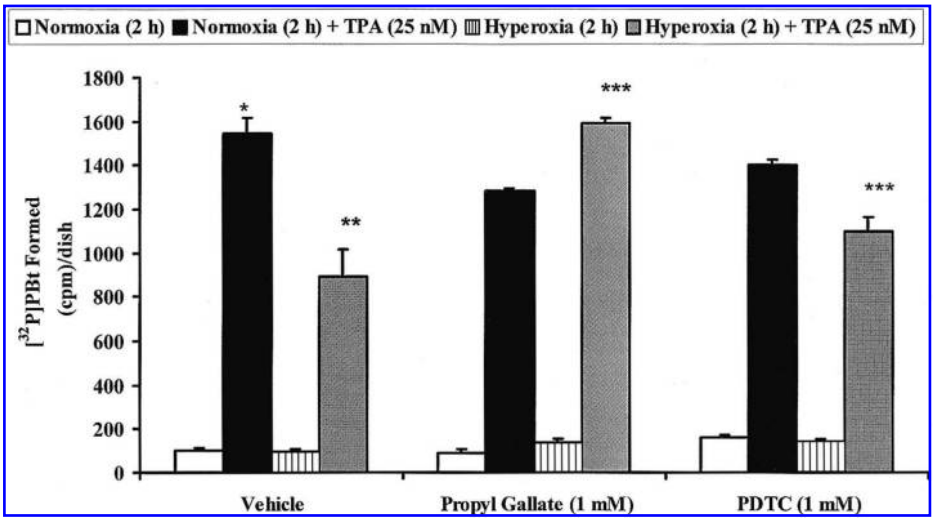


FIG. 3. Antioxidants reverse the attenuation of TPA-induced PLD activation in hyperoxic BLMVECs. Cells (35-mm dishes) were labeled with [^{32}P]orthophosphate (5 μCi/dish) in phosphate-free DMEM for 24 h. Medium was aspirated, and cells were incubated in normoxia or hyperoxia for 2 h in the presence of vehicle, PG (1 m*M*), or PDTC (1 m*M*). Medium was removed, and the cells were stimulated with TPA (25 n*M*) for 30 min. Lipids were extracted under acidic conditions, and [^{32}P]PBt was quantified as described in Materials and Methods. Values represent means ± SD of triplicate samples from three experiments. *Significantly different ($p < 0.05$) from normoxic cells. **Significantly different ($p < 0.05$) from normoxia + TPA-treated cells. ***Significantly different ($p < 0.05$) from hyperoxia + TPA-treated cells.

nificantly lower at all concentrations tested compared with normoxic cells (Fig. 2). These data suggest that hyperoxia partially reduced TPA-mediated PLD activation.

Antioxidants reverse the effect of hyperoxia on TPA-induced PLD activation

To support further the role of intracellularly generated ROS during hyperoxia in modulating TPA-induced PLD activation, the effects of PG and PDTC were investigated. BLMVECs labeled with [^{32}P]orthophosphate for 18 h were treated with PG (1 m*M*) or PDTC (1 m*M*) and simultaneously exposed to hyperoxia for 2 h before challenging with vehicle or TPA (25 n*M*) for 30 min. As shown in Fig 3, both PG and PDTC reversed the effect of hyperoxia on TPA-induced PLD activation. Interestingly, PG, but not PDTC attenuated TPA-induced PLD activation under normoxic conditions. These results suggest that ROS generated by hyperoxia modulate TPA-induced PLD activation.

Effect of Go6976 and PKC overexpression on TPA-induced [^{32}P]PBt formation

As TPA directly activates classical and atypical PKC isotypes (49), we examined the effect of PKC inhibitors on TPA-induced PLD activation. Pretreatment of BLMVECs with Go6976 (5 μ*M*) for 30 min blocked TPA-mediated accumulation of [^{32}P]PBt (Fig 4). Western blotting of BLMVEC lysates with different PKC isotype antibodies revealed that PKCα and PKCε were the two major isoenzymes present in BLMVECs (data not shown). To investigate the role PKCα and PKCε on PLD activation, ECs stably overexpressing either PKCα or PKCε isotypes were challenged with TPA. As shown in Fig. 5A, overexpression of PKCα increased TPA-induced [^{32}P]PBt formation by twofold under normoxic conditions, whereas exposure of control and PKCα overexpressing cells to hyperoxia attenuated TPA-induced [^{32}P]PBt accumulation. However, exposure of PKCε overexpressing cells to hyperoxia had no effect on TPA-induced [^{32}P]PBt formation (Fig. 5B). These results indicate that PKCα may play a role in TPA-induced PLD activation.

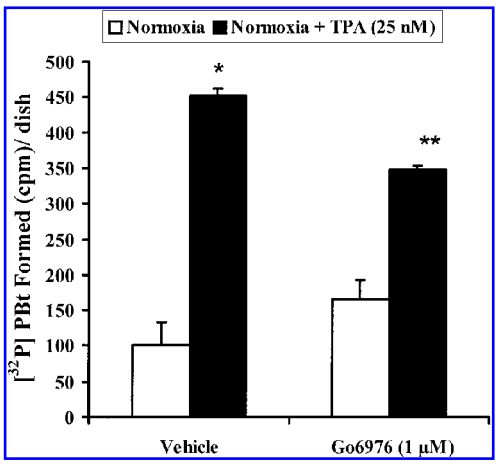
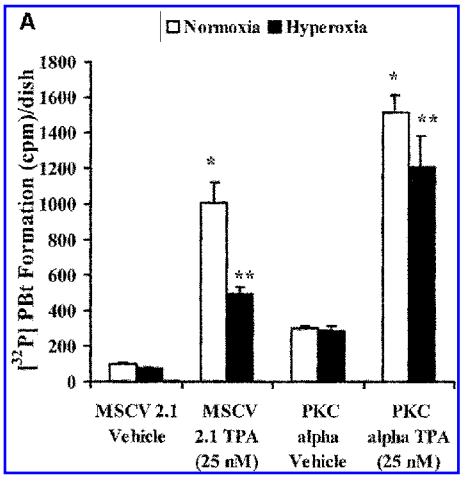


FIG. 4. Go6976 inhibits TPA-induced PLD activation in BLMVECS. Cells (35-mm dishes) were labeled with [^{32}P]orthophosphate (5 μCi/dish) in phosphate-free DMEM for 24 h. Medium was aspirated, cells were incubated with Go6976 (1 μ*M*) for 2 h in MEM, and then cells were stimulated with TPA (25 n*M*) for 30 min. Lipids were extracted under acidic conditions, and [^{32}P]PBt was quantified as described in Materials and Methods. Values represent means ± SD of three independent experiments in triplicate. *Significantly different ($p < 0.05$) from normoxic cells. **Significantly different ($p < 0.005$) from normoxia + TPA treatments.



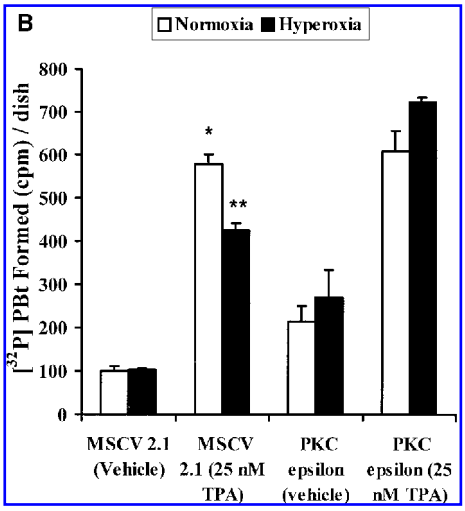


FIG. 5. Phospholipase D activity in HDMVECs PKCα (A) or PKCε (B). Vector control or PKCα (A) or PKCε overexpressing cells (35-mm dishes) were labeled with [^{32}P]orthophosphate (5 μCi/dish) in phosphate-free DMEM for 24 h in normoxia or hyperoxia. Cells were incubated in vehicle or TPA (25 n*M*) for 30 min prior to acidic extraction of lipids. [^{32}P]PBt was quantified as described in Materials and Methods. Values represent means ± SD of triplicate samples from three experiments. *Significantly different ($p < 0.05$) from normoxic control. **Significantly different ($p < 0.05$) from normoxia + TPA treatment.

Effect of hyperoxia on PKC activity

As our results show that TPA-induced PLD activation in hyperoxic cells was significantly lower as compared with that in normoxic cells, we examined the effect of hyperoxia on PKC activity in cell lysates and subcellular fractions. The total PKC activity was 1.5-fold higher in particulate fractions of cells exposed to hyperoxia compared with particulate fractions from normoxic cells, consistent with earlier reports that ROS such as hydrogen peroxide increased PKC activity (data not shown) (27, 54). Also, [^{3}H]phorbol dibutyrate ([^{3}H]-PDBu) binding studies with normoxic and hyperoxic cells showed no significant difference between the two treatments (data not shown),

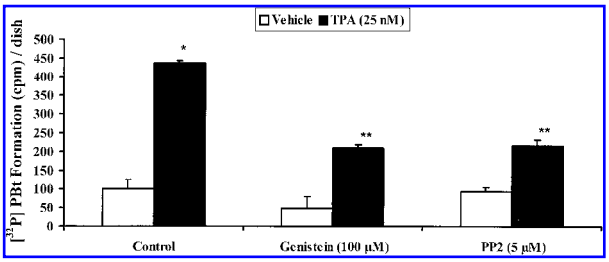


FIG. 6. Tyrosine kinase inhibitors block TPA-induced PLD activation in BLMVECS. Cells (35-mm dishes) were labeled with [^{32}P]orthophosphate (5 μCi/dish) in phosphate-free DMEM for 24 h. Medium was aspirated, cells were incubated with vehicle, genistein (100 μ*M*), or PP-2 (5 μ*M*) for 2 h in MEM, and cells were stimulated with TPA (25 n*M*) for 30 min. Lipids were extracted under acidic conditions, and [^{32}P]PBt was quantified as described in Materials and Methods. Values represent means ± SD of triplicate samples from three experiments. *Significantly different ($p < 0.05$) from control cells. **Significantly different ($p < 0.005$) from TPA-treated cells in the absence of genistein or PP-2.

suggesting that exposure of BLMVECs to hyperoxia for 24 h increased total PKC activity without altering binding capacity. These results also suggest that the TPA-induced effect on PLD in hyperoxia was likely due to modulation of PKC-dependent target protein(s) downstream from PKC.

Tyrosine kinase inhibitors attenuate TPA-induced PLD activation

We next examined the effects of genistein, an inhibitor of protein tyrosine kinase, and PP-2, a specific Src kinase inhibitor, on PLD activation. Genistein (100 μ*M*) significantly inhibited TPA-induced [^{32}P]PBt accumulation without altering the basal PLD activity (Fig. 6). The effect of genistein on

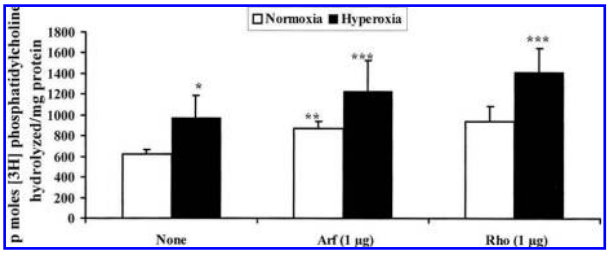


FIG. 7. Hyperoxia enhances PLD1 and PLD2 activities in cell lysates. BLMVECs (35-mm dishes) were incubated in normoxia or hyperoxia for 24 h in MEM. Lysates from normoxia- and hyperoxia-exposed cells were prepared as described in Materials and Methods. Cell lysates were mixed with DOPE, DPPC plus PIP_2 liposomes, and PLD activity was determined *in vitro* in the presence of Arf (1 μg) and Rho (1 μg), as described in Materials and Methods. PLD activity is expressed as pmol [^{3}H] phosphatidylcholine hydrolyzed/30 min/mg of protein. Values are means ± SD ($n = 3$). *Significantly different from normoxia control ($p < 0.05$). **Significantly different from normoxia control ($p < 0.05$). ***Significantly different from hyperoxia control ($p < 0.05$).

TABLE 2. EFFECT OF HYPEROXIA ON TPA-MEDIATED PROTEIN TYROSINE PHOSPHORYLATION

	Protein tyrosine phosphorylation (% control)			
Treatment	*2 h*		*24 h*	
	Vehicle	*TPA (25 nM)*	*Vehicle*	*TPA (25 nM)*
Normoxia	100	244	100	987
Hyperoxia	114	95	225	470

BLMVECs (5×10^5 cells/dish) were exposed to normoxia or hyperoxia for 2 h or 24 h. Cells were challenged with medium or medium containing TPA (25 n*M*) for 30 min, and total cell lysates were subjected to SDS-PAGE and Western blotting with anti-phosphotyrosine antibody. Tyrosine-phosphorylated proteins were detected as indicated in Fig. 8 and quantified by densitometric scanning and image analysis. The densities of all the tyrosine phosphorylated bands were summed up and expressed as total protein tyrosine-phosphorylation. Values are averages from three independent experiments.

TPA-induced [^{32}P]PBt formation was dose dependent with ~50% inhibition at 100 μ*M* concentration of genistein (data not shown). To clarify further the role of tyrosine kinase(s) on TPA-induced PLD activation, we examined the effect of PP-2 on [^{32}P]PBt formation. Pretreatment of BLMVECs with PP-2 (5 μ*M*) inhibited TPA-induced [^{32}P]PBt formation by ~50% compared with control cells without PP-2 treatment. These data suggest that TPA-induced PLD activation was dependent on PKC-mediated activation of tyrosine kinases, probably Src kinase.

Effect of hyperoxia on PLD1 and PLD2 activities in cell lysates

We next investigated the effect of hyperoxia on PLD1 and PLD2 activities in total cell lysates from normoxic and hyperoxic BLMVECs. Cell lysates from normoxic BLMVECs exhibited PIP_2-dependent release of [^{3}H]choline from phosphatidylcholine that was ~1.5-fold higher in cells exposed to hyperoxia for 24 h (Fig. 7). Addition of Arf or Rho further enhanced the PIP_2 dependent PLD1 activity in control as well as hyperoxic cell lysates (Fig. 7). These results demonstrate that both PLD1 and PLD2 activities were higher in hyperoxic cell lysates compared with normoxia, suggesting modulation of TPA-dependent downstream target proteins in attenuating PLD during hyperoxia.

TPA enhances protein tyrosine phosphorylation in BLMVECs

As presented in Table 2, BLMVECs exposed to hyperoxia (2 and 24 h) exhibited enhanced overall tyrosine phosphorylation of proteins as determined by SDS-PAGE and western blotting analysis utilizing anti-phosphotyrosine antibody. Previous studies from our laboratory have shown that ROS activated

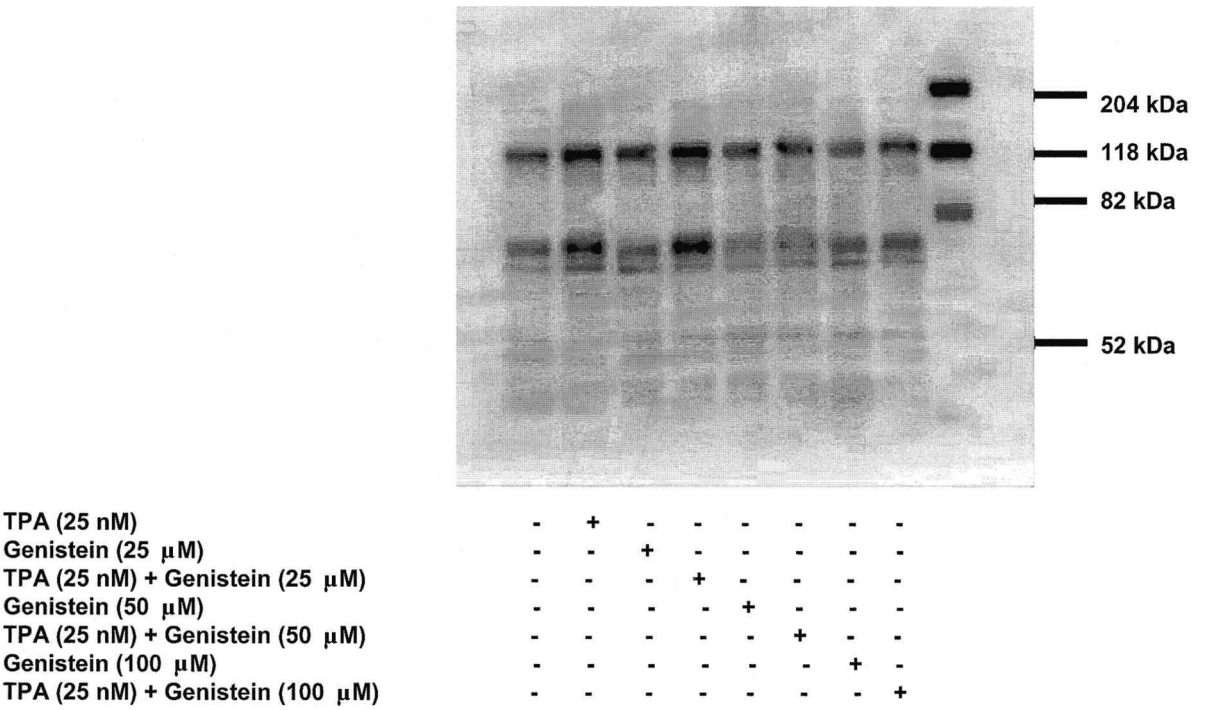


FIG. 8. Tyrosine kinase inhibitors attenuate TPA-induced total tyrosine phosphorylation in BLMVECs. Cells (100 mm) were pretreated with MEM or MEM plus genistein (25, 50, or 100 μ*M*) for 2 h. Medium was aspirated, and cells were incubated in MEM or MEM plus TPA (25 n*M*) for 30 min. Cell lysates (10 μg of protein) in RIPA buffer were subjected to SDS-PAGE, and proteins were transferred to polyvinylidene difluoride membranes and immunoblotted with anti-phosphotyrosine antibody. Tyrosine phosphorylated proteins were detected by enhanced chemiluminescence.

PLD via a tyrosine kinase-dependent pathway in BPAECs (31). However, in BPAECs, TPA (25–100 n*M*) had no effect on tyrosine phosphorylation of proteins as determined by Western blotting with anti-phosphotyrosine antibodies (35). In contrast to BPAECs, the TPA-induced PLD activation in BLMVECs was partially blocked by genistein and PP-2, suggesting a role for TPA-mediated tyrosine kinase stimulation. To characterize further the effect of TPA on protein tyrosine phosphorylation, BLMVECs were treated with MEM or MEM plus genistein (100 μ*M*) for 2 h before challenging with TPA (25 n*M*). As shown in Fig. 8, TPA increased tyrosine phosphorylation of proteins migrating at ~70 kDa and ~125 kDa compared with control cells. Interestingly, a significant basal phosphorylation of ~125-kDa and ~70-kDa proteins was observed in cells incubated overnight in serum-deficient medium (0.5% fetal bovine serum). Pretreatment of BLMVECs with genistein blocked the TPA-induced increases in tyrosine phosphorylation (Fig. 8). These results show that in BLMVECs, TPA-induced protein tyrosine phosphorylation was attenuated by genistein, a tyrosine kinase inhibitor.

DISCUSSION

In this study, we have investigated the role of intracellularly generated ROS by hyperoxia in agonist-induced signaling pathways in microvascular ECs. BLMVECs exposed to hyperoxia (2–24 h) increased lipid peroxidation and ROS production with no detectable cytotoxicity. However, TPA-induced protein tyrosine phosphorylation and PLD activation were attenuated by hyperoxia compared with normoxia. In our experiments, it was important to test the possibility that ROS generated intracellularly were contributing to the changes in TPA-induced protein tyrosine phosphorylation and PLD activation. PG has been shown as an efficient antioxidant against superoxide anion and hydroxyl radical activity with low and little cytotoxic properties (5, 20). PDTC is also used as an antioxidant with a broad specificity in radical quenching (16). Pretreatment of cells with antioxidants such as PG or PDTC reversed the effects of hyperoxia on TPA-mediated responses. Treatment of cells with a cell-permeable superoxide dismutase mimetic, MnTyp, did not alter the effects of hyperoxia on TPA-mediated responses (data not shown). Interestingly, earlier studies with BPAECs showed that TPA did not stimulate protein tyrosine phosphorylation and TPA-mediated PLD activation was tyrosine kinase-independent (30, 35). However, protein tyrosine phosphorylation in response to TPA was enhanced in lung microvascular ECs and TPA-induced PLD activation was sensitive to tyrosine kinase inhibitors, such as genistein and PP-2.

Hyperoxia exposure is different from exogenous addition of hydrogen peroxide to the ECs. We have measured ~2–3 μ*M* hydrogen peroxide produced by human pulmonary artery ECs upon exposure to hyperoxia for 3 h (Natarajan *et al.,* unpublished observations). In our earlier studies, we used 100 μ*M* hydrogen peroxide, which stimulated PLD activity in BPAECs, whereas the current study utilized BLMVECs. Therefore, the microvascular ECs that were used in the current study apparently behave differently from the ECs obtained from the macrovessels. In addition, low amounts of hydrogen peroxide produced during hyperoxia will not induce the same magnitude of activation of PLD as induced by exogenous addition of 100 μ*M* hydrogen peroxide. Also, in our earlier studies, we challenged ECs with 100 μ*M* of hydrogen peroxide for 30 min to 1 h as opposed to exposing the BLMVECs in the current study for 2–24 h. It is also becoming clearer that hyperoxia-induced signaling events are different from those induced by exogenous addition of hydrogen peroxide. It has been established by other investigators that hyperoxia up-regulates several genes, including genes for antioxidants and antioxidant enzymes, which may thus participate in offering an adaptive response to oxidative stress in cells during prolonged hyperoxia. We have noticed that the PLD response to oxidants in cells depends upon (a) type of cell system studied, (b) type of oxidant utilized, (c) the process of generation of oxidant, *i.e.,* endogenous versus exogenous, and (d) the adaptive response of the ECs to oxidative stress. Therefore, it is not reasonable to anticipate inhibition of PLD stimulation by TPA in BLMVECs upon exposure to hyperoxia as opposed to activation of PLD by 100 μ*M* hydrogen peroxide in BPAECs. It is conceivable to argue that BLMVECs have a different strategy to handle ROS generated during prolonged hyperoxia exposure wherein tight control of PLD (attenuation) may play an adaptive role, and we are currently investigating the physiological significance and mechanism of attenuation and activation of PLD in different EC lines exposed to different oxidants, hypoxia, and hyperoxia.

Exogenously added ROS activated PLD in ECs and fibroblasts (27, 30, 31, 33). In BPAECs, the ROS-induced PLD activation was not blocked by inhibitors of PKC, such as bisindolylmaleimide or calphostin C, but was modulated by tyrosine kinase inhibitors, genistein and PP-2 (42). Interestingly, in ECs derived from macrovessels such as pulmonary artery, TPA did not enhance protein tyrosine phosphorylation (32). However, in the present study, in the lung microvascular ECs, TPA increased protein tyrosine phosphorylation, which was blocked by genistein, suggesting a PKC-dependent activation of tyrosine kinases. A similar effect of genistein on TPA-induced PLD activation and protein tyrosine phosphorylation was observed in osteoblasts (23). Phorbol ester-enhanced tyrosine phosphorylation of cellular proteins has been previously reported and was associated with the intermediate activation of PKC. For example, enriched fractions of integral membrane proteins from Jurkat cells showed an enhanced tyrosine phosphorylation in the presence of TPA and PDBu (4). In human neuroblastoma cells, the docking protein p130Cas was tyrosine-phosphorylated during TPA-induced differentiation, which was blocked by the PKC inhibitor GF109203X (14). Our results show that the basal tyrosine phosphorylation was enhanced in hyperoxia compared with that in normoxic cells; however, the TPA-induced tyrosine phosphorylation was attenuated by hyperoxia.

The mechanism(s) of TPA-induced PLD activation modulated by hyperoxia is unclear. ROS generated in excess during hyperoxia may modulate PKC activity, thereby altering the TPA-mediated PLD stimulation. However, in BLMVECs, hyperoxia did not inhibit PKC activity as determined by *in vitro* assay, suggesting involvement of signaling pathways down-

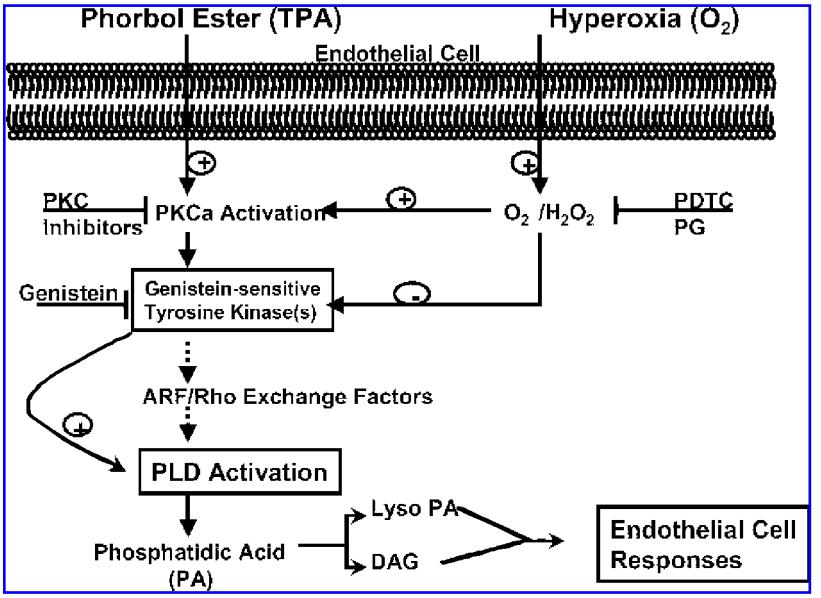


FIG. 9. Proposed mechanism of hyperoxia-induced alteration of TPA-induced activation of PLD in lung microvascular ECs.

stream from PKC. The possibility of changes in PLD activity in response to hyperoxia was also considered; however, hyperoxia did not modulate the activities of PLD1 or PLD2 as measured in cell lysates in the presence of PIP_2 and/or Arf and Rho. These results suggest that attenuation of TPA-induced PLD activation by hyperoxia was not directly due to modulation of PKC or PLD activity. We previously demonstrated that diperoxovanadate, an oxidant and inhibitor of protein tyrosine phosphatases, enhanced Src activity and tyrosine phosphorylation of Src (53). Src kinase activity is regulated by numerous phosphorylation sites. Phosphorylation of Y-416 (autophosphorylation site) increases its activity, whereas phosphorylation of Y-527 represses its activity. In addition, Thr^{34}, Thr^{46}, and Ser^{72} are phosphorylated by cyclin-dependent kinase 2 and cyclin B, and Ser^{12} and Ser^{48} are phosphorylated during PKC activation (55). However, TPA-induced PLD activation in BLMVECs was insensitive to PP-2 (data not shown), whereas genistein blocked [^{32}P]PBt formation mediated by TPA (Fig. 6). Recent study suggests that guanine nucleotide-exchange factor(s) for Arf is regulated by unidentified tyrosine kinase(s) in HL-60 cells (17). It is possible that TPA-dependent activation of PKC modulates guanine nucleotide-exchange factor(s) via genistein-sensitive tyrosine kinase, regulating PLD stimulation (Fig. 9). Our current study shows that the TPA-mediated protein tyrosine phosphorylation and PLD activation are attenuated by hyperoxia in BLMVECs. As hyperoxia increases lipid peroxidation and ROS production in BLMVECs (Figs. 1 and 2), it is possible that the intracellularly generated ROS modulate the genistein-sensitive tyrosine kinase and/or the guanine nucleotide-exchange factors. Further investigations in regard to identifying the particular genistein-sensitive tyrosine kinase(s) that modulates guanine nucleotide-exchange factors and regulation of TPA-mediated PLD activation under hyperoxia are necessary.

These are only a few reports on the effect of hyperoxia on PKC or tyrosine kinases. In rat type 2 epithelial cells, hyperoxia attenuated epidermal growth factor receptor phosphorylation (37). Our data show that TPA-induced tyrosine phosphorylation was reduced after 2 or 24 h of hyperoxia. Furthermore, immunofluorescence analysis of total tyrosine-phosphorylated proteins showed differences in the localization of the phosphorylated proteins. In normoxic cells, TPA caused translocation of the tyrosine-phosphorylated proteins to the plasma membrane; however, such a translocation was not seen in hyperoxic cells challenged with TPA (data not shown). Similar changes in the distribution of epidermal growth factor and transforming growth factor-α receptors were observed in the retina of rats exposed to hyperoxia (47). Hyperoxia also activated mitogen-activated protein kinases (45), p53 (38), p21 ras (48), GADD (40), and bcl (39). Activation of these kinases during hyperoxia has been implicated in the onset of apoptosis in epithelial cells and macrophages.

The functional significance of decreased TPA-induced PLD activation in hyperoxia is unclear. Agonist-induced activation of PLD results in transient accumulation of PA, a known second messenger in mammalian cells (13). PA is known to activate NADPH oxidase (26), bind to Raf, activate ERK (12, 50), stimulate phosphatidylinositol 4-kinase (29), increase intracellular Ca^{2+} (13), and cause actin-strain fibers (28). Additionally, PA can be metabolized to LPA by phospholipase A_1/A_2 (3) or DAG by lipid phosphate phosphatase (7). Both LPA and DAG modulate a number of cell signaling enzymes and regulate cellular functions. Although hyperoxia activated endothelial NADPH oxidase (Natarajan *et al.,* unpublished observations), the role of PA generated by PLD signaling by hyperoxia in EC barrier function is under investigation.

In conclusion, we have demonstrated that hyperoxia enhanced the generation of ROS in lung microvascular ECs and attenuated TPA-induced protein tyrosine phosphorylation and PLD activation. As PLD plays an important role in inflammatory responses, this could provide a mechanism for the regulation of endothelial barrier function during hyperoxic lung injury.

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ABBREVIATIONS

Arf, ADP-ribosylation factor; BLMVEC, bovine lung microvascular endothelial cell; BPAEC, bovine pulmonary artery endothelial cell; DAG, diacylglycerol; DCF, dichlorofluorescein; DCFDA, dichlorodihydrofluorescein di(acetoxymethyl ester); DMEM, Dulbecco's modified Eagle's medium; DOPE, dioleoylphosphatidylethanolamine; DPPC, dipalmitoylphosphatidylcholine; DTT, dithiothreitol; EC, endothelial cell; ERK, extracellular signal-regulated kinase; HDMVEC, human dermal microvascular endothelial cell; LPA, lysophosphatidic acid; PA, phosphatidic acid; PBt, phosphatidylbutanol; PDBu, phorbol dibutyrate; PDTC, pyrrolidine dithiocarbamate; PG, propyl gallate; PIP_2, phosphatidylinositol 4,5-bisphosphate; PKC, protein kinase C; PLD, phospholipase D; ROS, reactive oxygen species; SDS, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TBARs, thiobarbituric acid reactive substances; TPA, 12-*O*-tetradecanoylphorbol 13-acetate.

REFERENCES

1. Allen CB, Guo XL, and White CW. Changes in pulmonary expression of hexokinase and glucose transporter mRNAs in rats adapted to hyperoxia. *Am J Physiol* 274: L320–L329, 1998.
2. Aoki T, Suzuki Y, Suzuki K, Miyata A, Oyamada Y, Takasugi T, Mori M, Fujita H, and Yamaguchi K. Modulation of ICAM-1 expression by extracellular glutathione in hyperoxia-exposed human pulmonary artery endothelial cells. *Am J Respir Cell Mol Biol* 15: 319–327, 1996.
3. Billah MM, Lapetina EG, and Cuatrecasas P. Phospholipase A2 activity specific for phosphatidic acid. A possible mechanism for the production of arachidonic acid in platelets. *J Biol Chem* 256: 5399–5403, 1981.
4. Borowski P, Roloff S, Medem S, Kuhl R, and Laufs R. Protein kinase C-alpha produces reciprocal effects on the phorbol ester stimulated tyrosine phosphorylation of a 50 kDa kinase in Jurkat cells. *Biol Chem* 380: 403–412, 1999.
5. Bors W, Michel C, and Stettmaier K. Electron paramagnetic studies of radical species of proanthocyanidins and gallate esters. *Arch Biochem Biophys* 374: 347–355, 2000.
6. Bowman CM, Butler EN, and Repine JE. Hyperoxia damages cultured endothelial cells causing increased neutrophil adherence. *Am Rev Respir Dis* 128: 469–472, 1983.
7. Brindley DN and Waggoner DW. Phosphatidate phosphohydrolase and signal transduction. *Chem Phys Lipids* 80: 45–57, 1996.
8. Brown HA, Gutowski S, Moomaw CR, Slaughter C, and Sternweis PC. ADP-ribosylation factor, a small GTP-dependent regulatory protein, stimulates phospholipase D activity. *Cell* 75: 1137–1144, 1993.
9. Choi AM, Sylvester S, Otterbein L, and Holbrook NJ. Molecular responses to hyperoxia in vivo: relationship to increased tolerance in aged rats. *Am J Respir Cell Mol Biol* 13: 74–82, 1995.
10. Clerch LB, Massaro D, and Berkovich A. Molecular mechanisms of antioxidant enzyme expression in lung during exposure to and recovery from hyperoxia. *Am J Physiol* 274: L313–L319, 1998.
11. Daniel LW, Sciorra VA, and Ghosh S. Phospholipase D, tumor promoters, proliferation and prostaglandins. *Biochim Biophys Acta* 1439: 265–276, 1999.
12. Dorman CM and Johnson SE. Activated Raf inhibits avian myogenesis through a MAPK-dependent mechanism. *Oncogene* 18: 5167–5176, 1999.
13. English D, Cui Y, and Siddiqui RA. Messenger functions of phosphatidic acid. *Chem Phys Lipids* 80: 117–132, 1996.
14. Fagerstrom S, Pahlman S, and Nanberg E. Protein kinase C-dependent tyrosine phosphorylation of p130cas in differentiating neuroblastoma cells. *J Biol Chem* 273: 2336–2343, 1998.
15. Giulivi C, Lavagno CC, Lucesoli F, Bermudez MJ, and Boveris A. Lung damage in paraquat poisoning and hyperbaric oxygen exposure: superoxide-mediated inhibition of phospholipase A2. *Free Radic Biol Med* 18: 203–213, 1995.
16. Haddad JJ and Land SC. Redox signaling-mediated regulation of lipopolysaccharide-induced proinflammatory cytokine biosynthesis in alveolar epithelial cells. *Antiox Redox Signal* 4: 179–193, 2002.
17. Houle MG and Bourgoin S. Regulation of phospholipase D by phosphorylation-dependent mechanisms. *Biochim Biophys Acta* 1439: 135–149, 1999.
18. Jackson RM, Chandler DB, and Fulmer JD. Production of arachidonic acid metabolites by endothelial cells in hyperoxia. *J Appl Physiol* 61: 584–591, 1986.
19. Jiang H, Luo JQ, Urano T, Frankel P, Lu Z, Foster DA, and Feig LA. Involvement of Ral GTPase in v-Src-induced phospholipase D activation. *Nature* 378: 409–412, 1995.
20. Kahl R and Hilderbrandt AG. Methodology for studying antioxidant activity and mechanisms of action of antioxidants. *Food Chem Toxicol* 24: 1007–1014, 1986.
21. Kang BH, Crapo JD, Wegner CD, Letts LG, and Chang LY. Intercellular adhesion molecule-1 expression on the alveolar epithelium and its modification by hyperoxia. *Am J Respir Cell Mol Biol* 9: 350–355, 1993.
22. Kazzaz JA, Xu J, Palaia TA, Mantell L, Fein AM, and Horowitz S. Cellular oxygen toxicity. Oxidant injury without apoptosis. *J Biol Chem* 271: 15182–15186, 1996.
23. Kozawa O, Suzuki A, and Oiso Y. Tyrosine kinase regulates phospholipase D activation at a point downstream from protein kinase C in osteoblast-like cells. *J Cell Biochem* 57: 251–255, 1995.
24. Liscovitch M, Czarny M, Fiucci G, Lavie Y, and Tang X. Localization and possible functions of phospholipase D isozymes. *Biochim Biophys Acta* 1439: 245–263, 1999.
25. Markowitz D, Goff S, and Bank A. A safe packaging line for gene transfer: separating viral genes on two different plasmids. *J Virol* 62: 1120–1124, 1988.

26. McPhail LC, Waite KA, Regier DS, Nixon JB, Qualliotine-Mann D, Zhang WX, Wallin R, and Sergeant S. A novel protein kinase target for the lipid second messenger phosphatidic acid. *Biochim Biophys Acta* 1439: 277–290, 1999.
27. Min DS, Kim EG, and Exton JH. Involvement of tyrosine phosphorylation and protein kinase C in the activation of phospholipase D by H_2O_2 in Swiss 3T3 fibroblasts. *J Biol Chem* 273: 29986–29994, 1998.
28. Moolenaar WH. Lysophosphatidic acid, a multifunctional phospholipid messenger. *J Biol Chem* 270: 12949–12952, 1995.
29. Moritz A, De Graan PN, Gispen WH, and Wirtz KW. Phosphatidic acid is a specific activator of phosphatidylinositol-4-phosphate kinase. *J Biol Chem* 267: 7207–7210, 1992.
30. Natarajan V and Garcia JG. Agonist-induced activation of phospholipase D in bovine pulmonary artery endothelial cells: regulation by protein kinase C and calcium. *J Lab Clin Med* 121: 337–347, 1993.
31. Natarajan V, Taher MM, Roehm B, Parinandi NL, Schmid HH, Kiss Z, and Garcia JG. Activation of endothelial cell phospholipase D by hydrogen peroxide and fatty acid hydroperoxide. *J Biol Chem* 268: 930–937, 1993.
32. Natarajan V, Vepa S, Verma RS, and Scribner WM. Role of protein tyrosine phosphorylation in H_2O_2-induced activation of endothelial cell phospholipase D. *Am J Physiol* 271: L400–L408, 1996.
33. Natarajan V, Scribner WM, and Vepa S. Phosphatase inhibitors potentiate 4-hydroxynonenal-induced phospholipase D activation in vascular endothelial cells. *Am J Respir Cell Mol Biol* 17: 251–259, 1997.
34. Natarajan V, Scribner WM, Al-Hassani M, and Vepa S. Reactive oxygen species signaling through regulation of protein tyrosine phosphorylation in endothelial cells. *Environ Health Perspect* 106 Suppl 5: 1205–1212, 1998.
35. Natarajan V, Vepa S, Shamlal R, Al Hassani M, Ramasarma T, Ravishankar HN, and Scribner WM. Tyrosine kinases and calcium dependent activation of endothelial cell phospholipase D by diperoxovanadate. *Mol Cell Biochem* 183: 113–124, 1998.
36. Natarajan V, Scribner WM, Morris AJ, Roy S, Vepa S, Yang J, Wadgaonkar R, Reddy SPM, Garcia JGN, and Parinandi NL. Role of p38 mitogen activated protein kinase in diperoxovanadate-induced phospholipase D activation in endothelial cells. *Am J Physiol* 281: L435–L449, 2001.
37. Nici L, Medina M, and Frackelton AR. The epidermal growth factor receptor network in type 2 pneumocytes exposed to hyperoxia in vitro. *Am J Physiol* 270: L242–L250, 1996.
38. O'Reilly MA, Staversky RJ, Stripp BR, and Finkelstein JN. Exposure to hyperoxia induces p53 expression in mouse lung epithelium. *Am J Respir Cell Mol Biol* 18: 43–50, 1998.
39. O'Reilly MA, Staversky RJ, Huyck HL, Watkins RH, LoMonaco MB, D'Angio CT, Baggs RB, Maniscalco WM, and Pryhuber GS. Bcl-2 family gene expression during severe hyperoxia induced lung injury. *Lab Invest* 80: 1845–1854, 2000.
40. O'Reilly MA, Staversky RJ, Watkins RH, Maniscalco WM, and Keng PC. p53-independent induction of GADD45 and GADD153 in mouse lungs exposed to hyperoxia. *Am J Physiol Lung Cell Mol Physiol* 278: L552–L559, 2000.
41. Parinandi NL, Weis BK, Natarajan V, and Schmid HH. Peroxidative modification of phospholipids in myocardial membranes. *Arch Biochem Biophys* 280: 45–52, 1990.
42. Parinandi NL, Roy S, Shi S, Cummings RJ, Morris AJ, Garcia JG, and Natarajan V. Role of Src kinase in diperoxovanadate-mediated activation of phospholipase D in endothelial cells. *Arch Biochem Biophys* 396: 231–243, 2001.
43. Pei Y, Barber LA, Murphy RC, Johnson CA, Kelley SW, Dy LC, Fertel RH, Nguyen TM, Williams DA, and Travers JB. Activation of the epidermal platelet-activating factor receptor results in cytokine and cyclooxygenase-2 biosynthesis. *J Immunol* 161: 1954–1961, 1998.
44. Perkett AE, and Klekamp JG. Vascular endothelial growth factor expression is decreased in rat lung following exposure to 24 or 48 hours of hyperoxia: implications for endothelial cell survival. *Chest* 114: 52S–53S, 1998.
45. Petrache I, Choi ME, Otterbein LE, Chin BY, Mantell LL, Horowitz S, and Choi AM. Mitogen-activated protein kinase pathway mediates hyperoxia-induced apoptosis in cultured macrophage cells. *Am J Physiol* 277: L589–L595, 1999.
46. Phillips PG and Tsan MF. Hyperoxia causes increased albumin permeability of cultured endothelial monolayers. *J Appl Physiol* 64: 1196–1202, 1988.
47. Powers MR and Planck SR. Immunolocalization of transforming growth factor-alpha and its receptor in the normal and hyperoxia-exposed neonatal rat retina. *Curr Eye Res* 16: 177–182, 1997.
48. Rancourt RC, Keng PC, Helt CE, and O'Reilly MA. The role of p21(CIP1/WAF1) in growth of epithelial cells exposed to hyperoxia. *Am J Physiol Lung Cell Mol Physiol* 280: L617–L626, 2001.
49. Rasmussen H, Isales CM, Calle R, Throckmorton D, Anderson M, Gasalla-Herraiz J, and McCarthy R. Diacylglycerol production, Ca^{2+} influx, and protein kinase C activation in sustained cellular responses. *Endocr Rev* 16: 649–681, 1995.
50. Rizzo MA, Shome K, Vasudevan C, Stolz DB, Sung TC, Frohman MA, Watkins SC, and Romero G. Phospholipase D and its product, phosphatidic acid, mediate agonist-dependent raf-1 translocation to the plasma membrane and the activation of the mitogen-activated protein kinase pathway. *J Biol Chem* 274: 1131–1139, 1999.
51. Sagone AL Jr. Effect of hyperoxia on the carbohydrate metabolism of human lymphocytes. *Am J Hematol* 18: 269–274, 1985.
52. Sanders SP, Zweier JL, Kuppusamy P, Harrison SJ, Bassett DJ, Gabrielson EW, and Sylvester JT. Hyperoxic sheep pulmonary microvascular endothelial cells generate free radicals via mitochondrial electron transport. *J Clin Invest* 91: 46–52, 1993.
53. Shi S, Garcia JC, Roy S, Parinandi NL, and Natarajan V. Involvement of c-Src in diperoxovanadate-induced endothelial cell barrier dysfunction. *Am J Physiol Lung Cell Mol Physiol* 279: L441–L451, 2000.
54. Taher MM, Garcia JG, and Natarajan V. Hydroperoxide-induced diacylglycerol formation and protein kinase C activation in vascular endothelial cells. *Arch Biochem Biophys* 303: 260–266, 1993.
55. van Hoek ML, Allen CS, and Parsons SJ. Phosphotyrosine phosphatase activity associated with c-Src in large multi-

meric complexes isolated from adrenal medullary chromaffin cells. *Biochem J* 326 (Pt 1): 271–277, 1997.
56. Waggoner DW, Xu J, Singh I, Jasinska R, Zhang QX, and Brindley DN. Structural organization of mammalian lipid phosphate phosphatases: implications for signal transduction. *Biochim Biophys Acta* 1439: 299–316, 1999.
57. Welty SE, Rivera JL, Elliston JF, Smith CV, Zeb T, Ballantyne CM, Montgomery CA, and Hansen TN. Increases in lung tissue expression of intercellular adhesion molecule-1 are associated with hyperoxic lung injury and inflammation in mice. *Am J Respir Cell Mol Biol* 9: 393–400, 1993.

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2. Paramjit S Tappia, Tushi Singal, Melissa R Dent, Girma Asemu, Rabban Mangat, Naranjan S Dhalla. 2006. Phospholipid-mediated signaling in diseased myocardium. *Future Lipidology* **1**:6, 701-717. [CrossRef]
3. Saradhadevi Varadharaj , Tonya Watkins , Arturo J. Cardounel , Joe G.N. Garcia , Jay L. Zweier , Periannan Kuppusamy , Viswanathan Natarajan , Narasimham L. Parinandi . 2005. Vitamin C-Induced Loss of Redox-Dependent Viability in Lung Microvascular Endothelial Cells. *Antioxidants & Redox Signaling* **7**:1-2, 287-300. [Abstract] [Full Text PDF] [Full Text PDF with Links]
4. Luke B. Ravi, Joy G. Mohanty, Francis J. Chrest, Rajadas Jayakumar, Enika Nagababu, Peter V. Usatyuk, Viswanathan Natarajan, Joseph M. Rifkind. 2004. Influence of beta-amyloid fibrils on the interactions between red blood cells and endothelial cells. *Neurological Research* **26**:5, 579-585. [CrossRef]
5. Vijay Kumar Kutala , Narasimham L. Parinandi , Ramasamy P. Pandian , Periannan Kuppusamy . 2004. Simultaneous Measurement of Oxygenation in Intracellular and Extracellular Compartments of Lung Microvascular Endothelial Cells. *Antioxidants & Redox Signaling* **6**:3, 597-603. [Abstract] [Full Text PDF] [Full Text PDF with Links]
6. Peter V. Usatyuk , Suryanarayana Vepa , Tonya Watkins , Donghong He , Narasimham L. Parinandi , Viswanathan Natarajan . 2003. Redox Regulation of Reactive Oxygen Species-Induced p38 MAP Kinase Activation and Barrier Dysfunction in Lung Microvascular Endothelial Cells. *Antioxidants & Redox Signaling* **5**:6, 723-730. [Abstract] [Full Text PDF] [Full Text PDF with Links]