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Phospholipase D Activation in Endothelial Cells Is Redox Sensitive

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ABSTRACT

Reactive oxygen species (ROS) are implicated in the pathophysiology of a number of vascular disorders, including atherosclerosis. Recent studies indicate that ROS modulate signal transduction in mammalian cells. Previously, we have shown that ROS (hydrogen peroxide, fatty acid hydroperoxide, diperoxovanadate, and 4-hydroxynonenal) enhance protein tyrosine phosphorylation and activate phospholipase D (PLD) in bovine pulmonary artery endothelial cells (BPAECs). In the present study, our aim was to investigate the role of exogenous thiol agents on ROS-induced PLD activation in conjunction with the role of cellular thiols—glutathione (GSH) and protein thiols—on PLD activation and protein tyrosine phosphorylation. Pretreatment of BPAECs with *N*-acetyl-L-cysteine (NAC) or 2-mercaptopropionylglycine (MPG) blocked ROS-induced changes in intracellular GSH and PLD activation. Also, pretreatment with NAC attenuated diperoxovanadate-induced protein tyrosine phosphorylation. Pretreatment of BPAECs with diamide or L-buthionine-(*S,R*)-sulfoximine (BSO), agents that lower intracellular GSH and thiols, enhanced PLD activity. Furthermore, NAC blocked diamide- or BSO-mediated changes in GSH levels, PLD activity, and protein tyrosine phosphorylation. NAC also attenuated diamide-induced tyrosine phosphorylation of proteins between 69 and 118 KDa. These results support the hypothesis that modulation of thiol-redox status (cellular nonprotein and protein thiols) may contribute to the regulation of ROS-induced protein tyrosine phosphorylation and PLD activation in vascular endothelium. Antiox. Redox Signal. 1, 193–210, 1999.

INTRODUCTION

PHOSPHOLIPASE D (PLD) catalyzes the hydrolysis of phosphatidylcholine (PC) to yield phosphatidic acid (PA) and choline (Exton, 1994). Phosphatidic acid formed by the PLD pathway is further metabolized to lysoPA or diacylglycerol (DAG) by phospholipase A1/A2 (Billah *et al.*, 1981) or lipid phosphate phosphatase, respectively (Brindley and Waggoner, 1998). Two major forms of PLD, hPLD1 and hPLD2, have been cloned in mammalian cells (Hammond *et al.*, 1995; Colley *et al.*, 1997; Lopez *et al.*, 1998). Even though the two isoforms of PLD catalyze hydrolysis of PC to PA, they exhibit different cofactor and detergent requirements *in vitro* (Liscovitch and Chalifa-Caspi, 1996). Both hPLD1 and hPLD2 require phosphatidylinositol 4,5-bisphosphate (PIP2) as a lipid activator, whereas hPLD2 activity requires a mixture of PC and a detergent, like Triton X-100, for the activity (Exton, 1997b; Morris *et al.*, 1997). Also, hPLD1 activity is enhanced by small G-proteins such as Rho, Arf,

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Rac, and Cdc42 *in vitro* (Brown and Sternweis, 1995; Kwak *et al.*, 1995; Liscovitch and Chalifa-Caspi, 1996; Morris *et al.*, 1997). Furthermore, addition of protein kinase Cα-(PKCα) seems to enhance hPLD1 activity *in vitro* (Kiss, 1996; Singer *et al.*, 1996); however, the exact role of PKCα in PLD1 activation remains unclear.

Activation of PLD by a wide variety of agonists has been regarded as an important mechanism of signal transduction in mammalian cells (Natarajan, 1995; Exton, 1997a; Singer *et al.*, 1997). Reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2), fatty acid hydroperoxide, and 4-hydroxynonenal, stimulated PLD in bovine pulmonary artery endothelial cells (BPAECs) (Natarajan *et al.*, 1993a; Natarajan *et al.*, 1993b). Addition of vanadate to H_2O_2, generating peroxovanadium compounds, synergistically enhanced the protein tyrosine phosphorylation and PLD activation (Bourgoin and Grinstein, 1992; Natarajan *et al.*, 1996a, 1998a). It has been suggested that the ROS-induced PLD stimulation, in fact, was due to inhibition of protein tyrosine phosphatases or activation of tyrosine kinases or both (Posner *et al.*, 1994; Natarajan *et al.*, 1996b). In endothelial cells (ECs), the H_2O_2- or diperoxovanadate (DPV)-induced PLD activation was insensitive to PKC inhibitors or down-regulation of PKC by 12-*O*-tetradecanoyl phorbol 13-acetate (TPA) (Natarajan and Garcia, 1993; Natarajan *et al.*, 1996a). Recently, it was reported that activation of PLD by vanadate plus hydrogen peroxide in Swiss 3T3 cells was dependent on protein tyrosine phosphorylation and protein kinase C (Min *et al.*, 1998). Furthermore, pervanadate enhanced phosphorylation of PLD_1 in HL-60 cells and the PLD_1 immunocomplex was associated with several unidentified tyrosine phosphorylated proteins (Marcil *et al.*, 1997). We also observed that DPV enhanced tyrosine and serine/threonine phosphorylation of PLD_1 in BPAECs (Vepa and Natarajan, unpublished data). In addition to regulation by PKC, tyrosine kinases, and small G proteins (Exton, 1997b), PLD activation by fMLP (N-formyl-Met-Leu-Phe) is also regulated by redox status in HL-60 cells (Nakamura *et al.*, 1998). In the present study, we have investigated the effect of antioxidants and thiol agents on ROS-induced PLD activation and protein tyrosine phosphorylation in vascular ECs because redox-regulation of PLD activation by oxidants may be physiologically important. The results indicate that ROS-mediated PLD activation was attenuated by *N*-acetyl-L-cysteine (NAC) and 2-mercaptopropionylglycine (MPG). Furthermore, NAC blocked ROS-induced alterations in cellular GSH and protein tyrosine phosphorylation, suggesting a role for the redox regulation of signal transduction in vascular ECs.

MATERIALS AND METHODS

Materials

Bovine pulmonary artery endothelial cells (passage 16, CCL-209) were purchased from American Tissue Culture Collection (Rockville, MD). Minimal essential medium (MEM), fetal bovine serum (FBS), trypsin, nonessential amino acids, TPA, sodium orthovanadate, dimethylthiourea (DMTU), MPG, NAC, Triton X-100, bovine serum albumin (BSA), and H_2O_2 were obtained from Sigma Chemical Co. (St. Louis, MO). [^{32}P]Orthophosphate (carrier-free) was from New England Nuclear (Wilmington, DE). Antiphosphotyrosine antibodies (4G10) and endothelial growth factor were purchased from Upstate Biotechnology Inc. (Lake Place, NY). Enhanced chemiluminescence (ECL) kit was obtained from Amersham Life Science (England). Phosphatidylbutanol (PBt) was purchased from Avanti Polar Lipids (Alabaster, AL). Precoated Silica Gel H thin-layer chromatography plates were from Analtech (Newark, DE). Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) precast 8% and 10% gels were obtained from Novex (San Diego, CA). DPV, prepared by mixing equimolar solutions of H_2O_2 and sodium orthovanadate, pH 7.4, and crystallized by ethanol precipitation, was kindly provided by Prof. T. Ramasarma (Indian Institute of Science, Bangalore, India). The crystallized DPV was characterized by nuclear magnetic resonance (NMR) (Shankar and Ramasarma, 1993).

Cell culture

BPAECs were cultured in MEM supplemented with 10% FBS, antibiotics, and growth factors, as discussed previously (Garcia *et al.*, 1992). When confluence was reached, cells

were trypsinized and subcultured in T-75 cm^2 flasks or 35 × 10-mm tissue culture dishes. Confluent cells showed cobblestone morphology under light microscope and stained positive for Factor VIII. All experiments were conducted between 18 and 20 passages.

Assay for PLD activation

BPAECs in 35-mm dishes (5×10^5 cells/dish) were incubated with [^{32}P]orthophosphate (5 μCi/ml) in phosphate-free Dulbecco's minimal Eagle's medium (DMEM) containing 2% FBS for 18–24 hr at 37°C in 95% air and 5% CO_2 incubator (Garcia *et al.*, 1992; Natarajan and Garcia, 1993). The radioactive medium was aspirated, cells were incubated in serum-free MEM or MEM containing oxidants or other agents at concentrations as 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 and PBt formed as a result of PLD activation was analyzed by thin-layer chromatography (TLC) (Natarajan and Garcia, 1993). Radioactivity associated with [^{32}P]PBt was quantified by liquid scintillation counting. All values were normalized to one million total dpm in total lipid extract and [^{32}P]PBt formed was expressed as dpm/dish or % control. Controls and treatments were in triplicates.

Assay of cellular thiols

Determination of cellular thiols (GSH and protein thiols) was based on earlier published procedures of (Harian *et al.*, 1984; Parinandi *et al.*, 1990). BPAECs were grown to confluency (cobble stone morphology) in 60 × 15-mm dishes. Cells were incubated with oxidants, thiol agents (NAC or MPG), antioxidants (dimethylthiourea), and thiol-depleting agents (diamide or BSO) in serum-free MEM wherever necessary as per experimental design for required time periods. At the end of the incubation period, dishes with cells were transformed on to ice, thoroughly washed with ice-cold phosphate-buffered saline (PBS) (containing 0.02% EDTA), and cells pretreated with thiol agents were washed three times with ice-cold PBS (containing 0.02% EDTA) to ensure that all the thiol agent added was removed before intracellular thiol assay was performed. To ensure further the complete removal of thiol agent from the surroundings inside dishes with adhering cells, the PBS wash was checked for the presence or absence of thiols by the 5,5'-dithio-*bis*(2-nitrobenzoic acid (DTNB) test. Extreme caution was exercised to remove completely the exogenously added thiols from the dishes prior to the thiol assay. If the DTNB test was positive for the presence of thiols, cells were washed again with ice-cold PBS (containing 0.02% EDTA) until the DTNB test was negative. Cells were scraped with a Teflon cell scraper after washing, and cells from each dish were taken in a known volume of ice-cold PBS (containing 0.02% EDTA) in microfuge tube. The cells in microfuge tubes, on ice, were sonicated three times for 30 sec with a probe sonicator at a setting of 7. Between each sonication step, there was a 30-sec pause. The final cell sonicate was made to 500 μl and stored on ice for immediate thiol analysis.

For total GSH determination, 1.0 ml of sulfosalicylic acid (2.5% in PBS with 0.02% EDTA) was added to 400 μl of cell sonicate in a glass test tube and vortexed for 1 min at maximum setting followed by addition of 10 μl of ethonolic solution of 0.01% BHT (butylated hydroxytoluene). The mixture was centrifuged at 5,000 rpm for 15 min in a tabletop centrifuge at room temperature. To 1.0 ml of the supernatant, 2.0 ml of Na_2HPO_4 (0.3 *M*, pH 7.4) was added and mixed thoroughly on a vortex mixer. To this mixture, 100 μl of DTNB (0.4% in 1% sodium citrate solution) was added and mixed well. Absorbance at 412 nm against an appropriate blank was measured quickly on Beckman DU-650 spectrophotometer. For protein thiol determination, the pellet obtained in GSH assay after sulfosalicylic acid (2.5% in PBS with 0.02% EDTA) precipitation was mixed with 100 μl of 10% SDS solution, mixed well on a vortex, made up to 400 μl with PBS (with 0.02% EDTA), and then 10 μl of ethanolic solution of 0.01% BHT was added. The subsequent assay method for protein thiols was as described for GSH determination. Protein content in the cell sonicate was determined by bicinchoninic acid protein assay (Pierce, Rockford, IL). Nonpro-

tein thiols (GSH) and protein thiols were calculated from appropriate standard curves prepared with GSH as a standard. Thiols were expressed as μg/mg protein as averages of triplicate samples with ± SD. All data were subjected to one-way analysis of variance (ANOVA) ($p < 0.05$).

SDS-PAGE and western blotting

Serum-starved BPAECs (18–24 hr) were treated with MEM or MEM containing NAC, MPG, DMTU, H_2O_2, vanadate, or DPV for various time periods. Cells were rinsed in ice-cold PBS containing 1 m*M* vanadate and were lysed in a buffer containing 20 m*M* Tris, pH 7.4, 137 m*M* NaCl, 1 m*M* EDTA, 1% NP-40, 0.5% Triton X-100, 2 μg/ml leupeptin, 1 μg/ml aprotinin, 1 mm phenyl methyl sulfonyl fluoride (PMSF) and 1 m*M* vanadate. Cell lysates were sonicated and centrifuged at 14,000 × rpm for 15 min at 4°C. An aliquot of the supernatant was used for protein estimation by BCA assay (Pierce). For Western blot analysis, 40 μl of 6× Laemmli SDS-PAGE buffer was added to 160 μl of cell lysate and samples were boiled for 5 min and stored at −20°C. Cell lysates adjusted to equal protein concentration were subjected to SDS-PAGE in 8% or 14% gels and were electrotransferred onto polyvinylidene difluoride (PVDF) membranes (Vepa *et al.*, 1977). Western blotting was performed on the cell lysates using 4G10 monoclonal antiphosphotyrosine antibody (1:1,000 dilution), and phosphotyrosine-containing proteins were immunodetected by enhanced chemiluminescence.

RESULTS

Antioxidants attenuate ROS-induced PLD activation in BPAECs

Exposure of ^{32}P-labeled BPAECs to H_2O_2 or DPV resulted in an accumulation of [^{32}P]PBt, an index of PLD activation (Morris *et al.*, 1977) (Fig. 1). The [^{32}P]PBt formation induced by H_2O_2 (1 m*M*) or DPV (5 μ*M*) was three-fold and eight-fold higher, respectively, as compared with the cells treated with medium

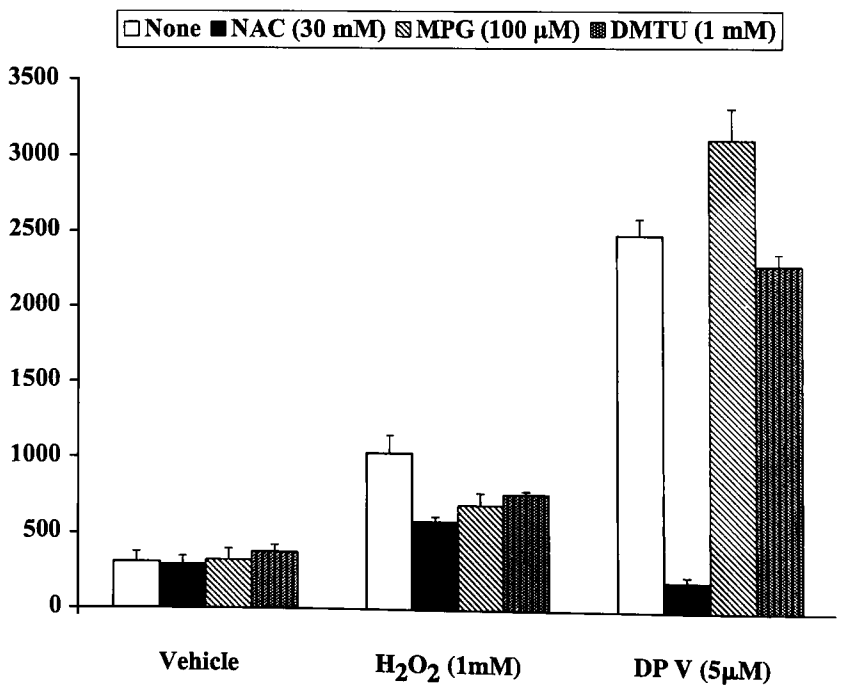

FIG. 1. Effect of thiol agents on ROS-induced PLD activation. BPAECs in 35-mm dishes were labeled with [^{32}P]orthophosphate (5 μCi/dish) in DMEM phosphate-free medium for 18 hr. Cells were washed and pretreated with NAC, MPG, or DMTU for 60 min. The medium was aspirated, and cells were washed with MEM and challenged with H_2O_2 (1 m*M*) or DPV (5 μ*M*) for 30 min in the presence of 0.05% butanol. Lipids were extracted under acidic conditions and [^{32}P]PBt was separated by TLC and quantified as described under Materials and Methods. Values are mean ± SD ($n = 3$).

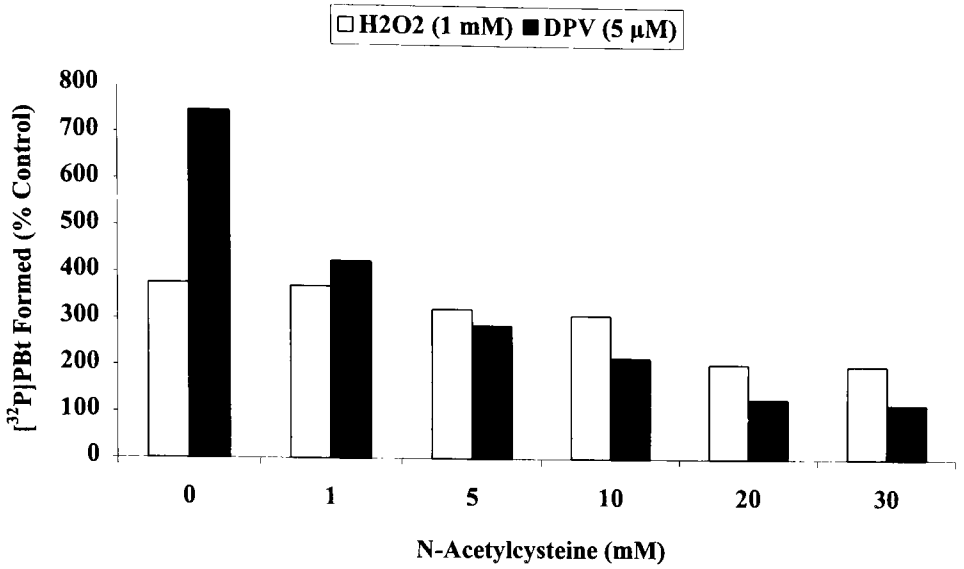

FIG. 2. Dose-dependent attenuation of ROS-induced PLD activation by NAC. BPAECs in 35-mm dishes were labeled with [^{32}P]orthophosphate (5 μCi/dish) in DMEM phosphate-free medium for 18 hr. Cells were washed and pretreated with varying concentrations of NAC for 60 min. The medium was aspirated, and cells were washed with MEM and challenged with H_2O_2 (1 m*M*) or DPV (5 μ*M*) for 30 min in the presence of 0.05% butanol. Lipids were extracted under acidic conditions and [^{32}P]PBt was separated by TLC and quantified as described under Materials and Methods. Data are expressed as % control. Radioactivity after treatment with vehicle alone = 279 ± 42 dpm/dish.

alone. The higher stimulation of PLD seen with DPV as compared to H_2O_2 is attributed to the ability of DPV to be a potent activator of tyrosine kinases and inhibitor of protein tyrosine phosphatases as compared to H_2O_2 (Heffetz *et al.*, 1992; Posner *et al.*, 1994). Because membrane lipids, proteins, and cellular thiols (including GSH) are likely targets for ROS, the role of antioxidants on H_2O_2- and DPV-induced PLD activation was investigated. BPAECs were pre-

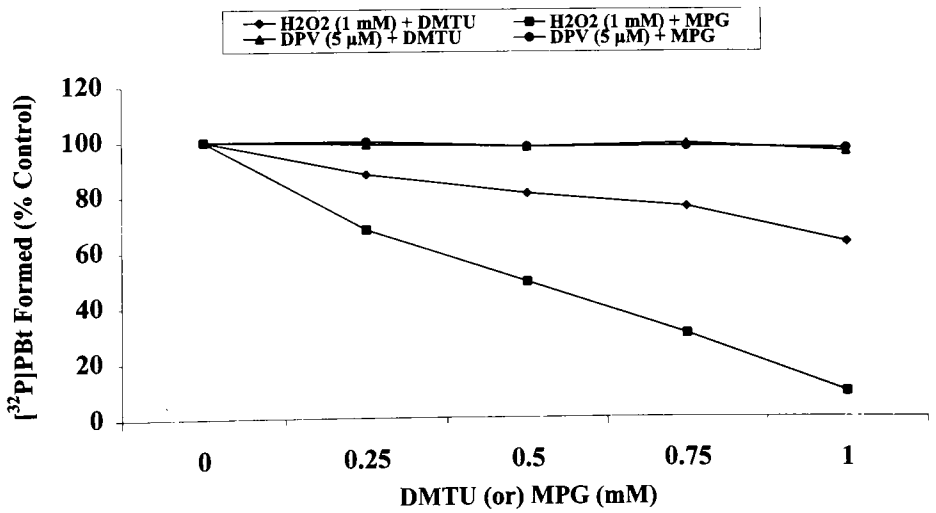

FIG. 3. Effect of DMTU and MPG on ROS-induced PLD activation. BPAECs in 35-mm dishes were labeled with [^{32}P]orthophosphate (5 μCi/dish) in DMEM phosphate-free medium for 18 hr. Cells were washed and pretreated with varying concentrations of MPG or DMTU for 60 min. The medium was aspirated, and cells were washed with MEM and challenged with H_2O_2 (1 m*M*) or DPV (5 μ*M*) for 30 min in the presence of 0.05% butanol. Lipids were extracted under acidic conditions and [^{32}P]PBt was separated by TLC and quantified as described under Materials and Methods. Data are expressed as % control and radioactivity in PBt after treatment with vehicle alone = 374 ± 106 dpm/dish.

treated with NAC or MPG or DMTU, because these thiol agents act not only as antioxidants but also alter the intracellular GSH and protein thiol levels (Moldeus and Cotgreave, 1994). As shown in Fig. 1, NAC inhibited the DPV- or H_2O_2-induced PLD activation. Interestingly, we found that after pretreatment with NAC, the cells have to be extensively rinsed with ice-cold PBS or medium before challenging with DPV or H_2O_2 because NAC reacted with the peroxides and inactivated them. However, antioxidants such as vitamin E or L-ascorbic acid 6-palmitate showed no effect on H_2O_2- and DPV-induced PLD activation (data not shown).

The inhibition of H_2O_2- or DPV-induced [^{32}P]PBt formation by NAC was dose-dependent, with 50% inhibition occurring at 5 m*M* and 1 m*M* NAC for H_2O_2 and DPV, respectively (Fig. 2). Although MPG and DMTU failed to inhibit DPV-mediated PLD stimulation, these agents attenuated the H_2O_2-induced [^{32}P]PBt accumulation (Fig. 3). As shown in Fig. 4, the inhibition of H_2O_2- or DPV-induced PLD activation by NAC (1 m*M*) was time dependent. These results show that ROS-induced PLD activation is blocked by pretreating the ECs with thiol agents suggesting redox regulation.

Effect of NAC, MPG, and DMTU on ROS-induced protein tyrosine phosphorylation

Protein tyrosine phosphorylation resulting from modulation of tyrosine kinases and protein tyrosine phosphatases has been implicated in ROS-induced PLD activation (Natarajan *et al.*, 1996b; Min *et al.*, 1998). Therefore, to determine whether the inhibitory effect of NAC, MPG, or DMTU on H_2O_2- or DPV-induced PLD activation was mediated by blockage of protein tyrosine phosphorylation, lysates from control and cells treated with NAC (10 m*M*) or MPG (100 μ*M*) or DMTU (1 m*M*) were subjected to SDS-PAGE and were analyzed by

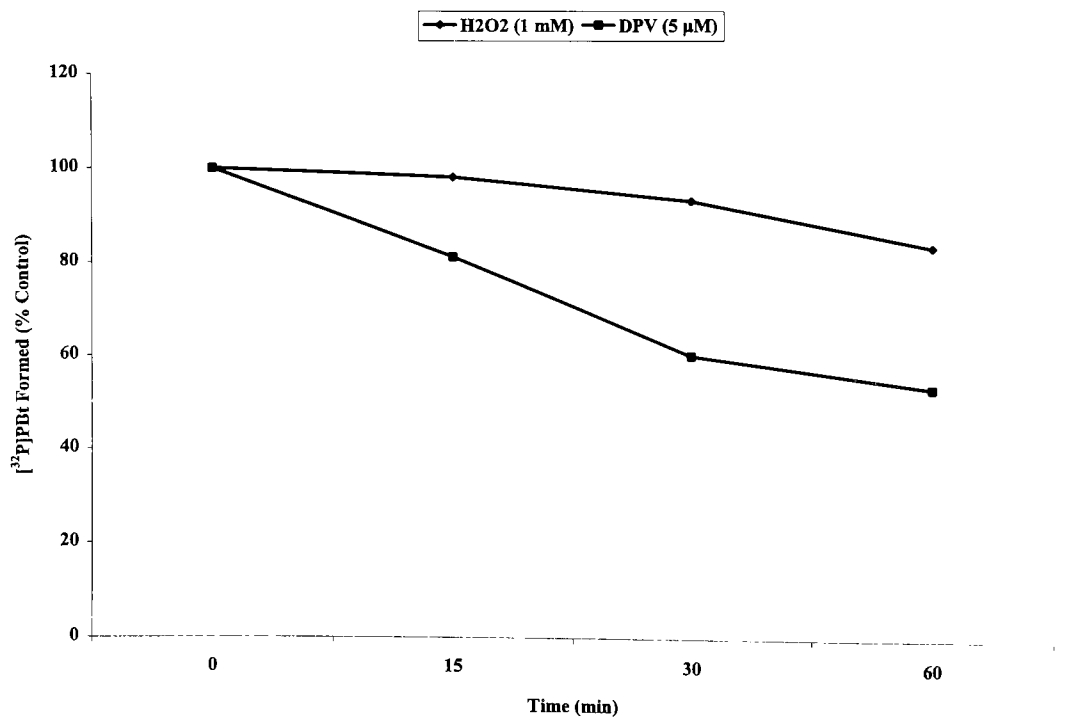

FIG. 4. Time-course of NAC-dependent attenuation of ROS-induced PLD activation. BPAECs in 35-mm dishes were labeled with [^{32}P]orthophosphate (5 μCi/dish) in DMEM phosphate-free medium for 18 hr. Cells were washed and pretreated with 1.0 m*M* NAC for different time intervals. The medium was aspirated, and cells were washed with MEM and challenged with H_2O_2 (1 m*M*) or DPV (5 μ*M*) for 30 min in the presence of 0.05% butanol. Lipids were extracted under acidic conditions and [^{32}P]PBt was separated by TLC and quantified as described under Materials and Methods. Data are expressed as % control.

western blotting with antiphosphotyrosine antibody. As shown in Fig. 5, A and B, exposure of BPAECs to H_2O_2 or DPV caused enhanced tyrosine phosphorylation of several proteins as reported earlier (Natarjan *et al.*, 1996a, 1998a,b). The DPV-mediated protein tyrosine phosphorylation was attenuated by NAC (10 m*M*). As compared to NAC, both MPG and DMTU failed to inhibit DPV-induced PLD activation and protein tyrosine phosphorylation. However, NAC or MPG or DMTU showed no effect on H_2O_2 (1 m*M*)-mediated protein tyrosine phosphorylation. These results indicate that the effect of NAC or MPG or DMTU on H_2O_2- and DPV-induced PLD activation and protein tyrosine phosphorylation are different.

Specificity of NAC on agonist- and ROS-induced PLD activation

Previous studies from our laboratory have shown that a wide variety of agonists such as thrombin, bradykinin, and TPA activated PLD in ECs (Garcia *et al.*, 1992; Natarajan and Garcia, 1993). To investigate the specificity of NAC on agonist- and ROS-induced PLD activation, BPAECs labeled with [^{32}P]orthophosphate were treated with NAC (10 m*M*) for 60 min before challenging with H_2O_2 (1 m*M*) or TPA (100 m*M*) or bradykinin (1 μM). As shown in Table 1, NAC pretreatment had no effect on TPA- or bradykinin-induced [^{32}P]PBt accumulation, however, H_2O_2-mediated [^{32}P]PBt formation was inhibited. These data suggests that the effect of NAC was specific toward ROS-induced PLD activation.

Effect of ROS and thiol agents on intracellular thiols

To assess the role of cellular thiols, including GSH (a major regulator of the redox state of the cells) in ROS-induced PLD activation, BPAECs were challenged with medium alone or medium containing H_2O_2 (1 m*M*) or vanadate (100 μM) or DPV (5 μM) for 30 min, and cellular GSH and protein thiols were measured. Figure 6 shows that treatment of cells with H_2O_2 (1 m*M*) resulted in a drastic and significant decrease in intracellular thiols, including GSH with a maximal decrease (70–80% of control) occurring within 30 min of treatment.

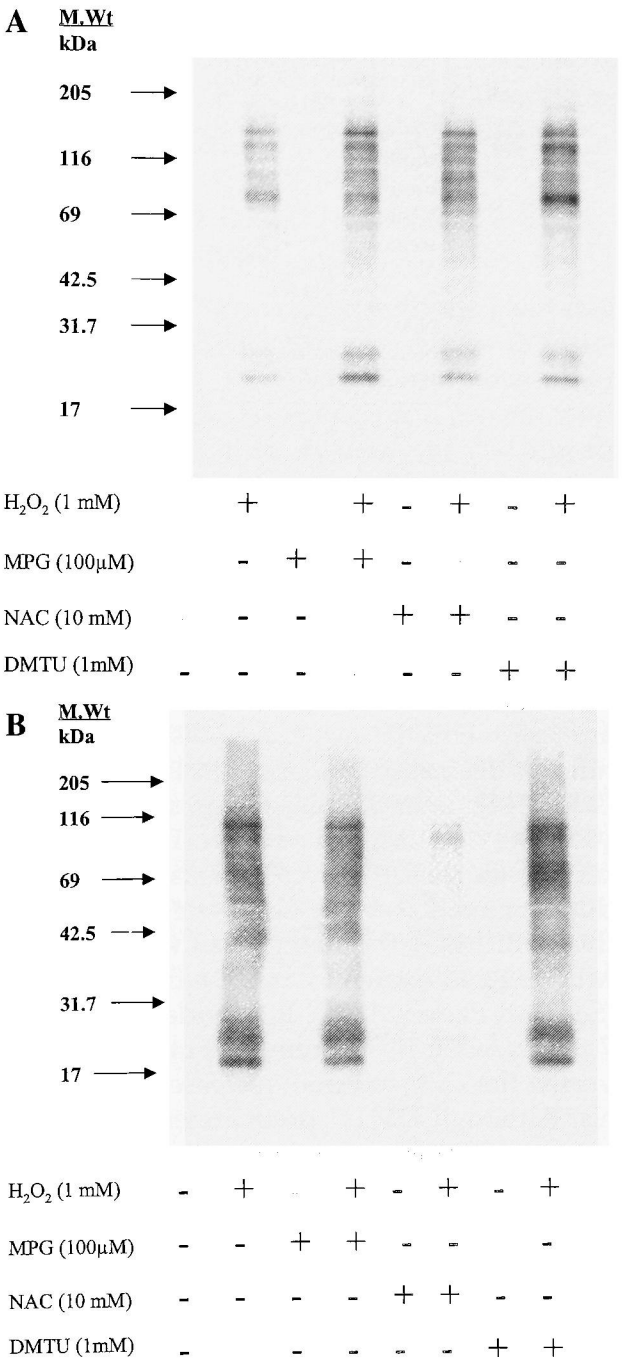

FIG. 5. Effect of NAC, MPG, or DMTU on ROS-induced protein tyrosine phosphorylation. A. BPAECs in 60-mm dishes were pretreated with MPG (100 μM), NAC (30 m*M*), or DMTU (1 m*M*) for 60 min. Cells were washed with MEM (without serum) and were challenged with H_2O_2 (1 m*M*) for 30 min. Cell lysates (10 μg protein) in RIPA buffer were subjected to SDS-PAGE, membrane transfer, and immunoblotting with 4G10 antiphosphotyrosine antibody. Tyrosine phosphorylated proteins were detected using enhanced chemiluminescence. **B.** BPAECs in 60-mm dishes were pretreated with MPG (100 μM), NAC (30 m*M*), or DMTU (1 m*M*) for 60 min. Cells were washed with MEM (without serum) and challenged with DPV (5 μM) for 30 min. Cell lysates (10 μg protein) in RIPA buffer were analyzed by SDS-PAGE as indicated above.

TABLE 1. SPECIFICITY OF N-ACETY-L-CYSTEINE ON ROS-INDUCED PLD ACTIVATION

Pretreatment (60 min)	*Treatment (30 min)*	*[^{32}P] PBt Formed (dpm/dish)*
Vehicle	Vehicle	515 ± 12
Vehicle	H_2O_2 (1 m*M*)	1,239 ± 576
Vehicle	DPV (5 μ*M*)	3,140 ± 198
Vehicle	TPA (100 n*M*)	6,886 ± 755
Vehicle	BRK (1 μ*M*)	1,747 ± 217
NAC (10 m*M*)	Vehicle	336 ± 35
NAC (10 m*M*)	H_2O_2 (1 m*M*)	613 ± 107
NAC (10 m*M*)	DPV (5 μ*M*)	1,276 ± 278
NAC (10 m*M*)	TPA (100 n*M*)	6,116 ± 475
NAC (10 m*M*)	BRK (1 μ*M*)	1,485 ± 161

BPAECs (5×10^5 cells/dish) were labeled with [^{32}P]orthophosphate (5 μCi/dish) in DMEM phosphate-free medium containing 2% FBS for 18 hr. After aspirating the radioactive medium, the cells were pretreated with medium or medium containing NAC (10 m*M*) for 60 min. The cells were washed twice in MEM before challenging with medium alone or medium containing H_2O_2 (1 m*M*) or DPV (5 μ*M*) or TPA (100 n*M*) or bradykinin (BRK) (1 μ*M*) for 30 min in the presence of 0.05% butanol. Lipids were extracted under acidic conditions and the [^{32}P]PBt formed was quantified by TLC as described under Materials and Methods. Each value is an average ± range of two independent experiments in triplicate.

However, DPV (5 μ*M*) caused a small but significant decrease (20% of control; $p < 0.05$) in GSH levels after 30 min of exposure whereas vanadate (100 μM) had no effect. To investigate further the effect of thiol agents on ROS-induced alteration in cellular GSH and protein thiols, BPAECs were pretreated with NAC or MPG, agents known to increase cellular thiols (Sen and Packer, 1996). It is evident from Fig. 7 that NAC or MPG pretreatment partially reversed the DPV-induced decrease in GSH levels. Although DMTU pretreatment showed a marginal effect on intracellular GSH levels in the absence or presence of ROS, NAC pretreatment had a contrasting effect on H_2O_2-mediated depletion of thiol levels.

Although NAC (30 m*M*) increased intracellular GSH levels from 4.5 μg/mg protein to 11.7 μg/mg protein at the end of 30-min incubation period (Fig. 7), in the presence of H_2O_2 (1 m*M*) the intracellular GSH levels remained almost at the same levels even after pretreatment with NAC (30 m*M*) and similar to H_2O_2 (1 m*M*) treatment alone without NAC (Fig. 7).

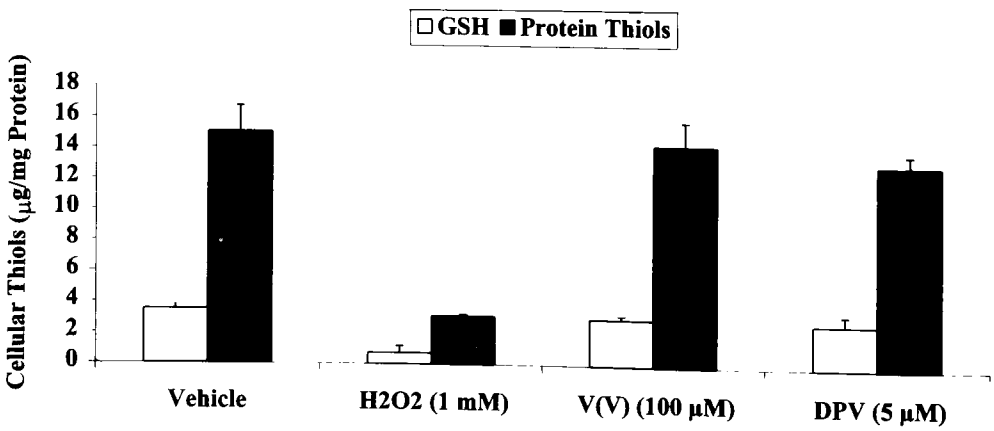

FIG. 6. Effect of ROS on cellular thiols. BPAECs in 60-mm dishes were challenged with MEM or MEM containing H_2O_2 (1 m*M*), vanadate (100 μ*M*), or DPV (5 μ*M*) for 30 min at 37°C in a 95% air/5% CO_2 atmosphere. Cells were thoroughly washed in ice-cold PBS. The cellular GSH and protein thiol levels were determined as described under Materials and Methods. Thiols were expressed as μg/mg protein. Each value is an average of three replicates with ±SD.

However, at lower concentrations of H_2O_2 (0.1 m*M*), pretreatment of BPAECs with NAC resulted in significant restoration of intracellular GSH levels (Fig. 8A). A similar trend in H_2O_2-mediated depletion of protein thiols was observed, and NAC pretreatment failed to restore protein thiols in 1 m*M* H_2O_2 treated cells (Fig. 8B). MPG pretreatment also elevated cellular GSH and protein thiol levels, and the H_2O_2-mediated depletion of GSH levels were not restored by MPG pretreatment (Fig. 7). These results suggest that NAC and MPG were highly effective thiol-redox agents in elevating cellular GSH levels in the absence or presence of ROS. The nature of the ROS employed influenced the restoration of thiol levels, as seen in the comparative study wherein two different oxidants such as H_2O_2 and DPV yielded contrasting results with respect to the thiol status after NAC or MPG pretreatment. This probably depends on the thiol specificity with respect to the oxidizability in presence of ROS, *i.e.*, H_2O_2 at 1 m*M* concentration would have acted as a potent oxidant consuming all the cellular thiols (which include GSH and other thiols) whereas DPV (5 μM) has much less of a thiol-depleting (oxidizing) effect. For comparison, vanadate (100 μM) was used to study its effect on cellular thiols, and observations confirmed that vanadate caused only 15% (from control) reduction in cellular GSH and 5% (from control) reduction in protein thiols (Fig. 6).

Effect of diamide or BSO on intracellular thiols and PLD activation

As the thiol agents NAC and MPG elevated intracellular GSH levels, restored ROS-depleted cellular thiol levels, and attenuated ROS-induced PLD activation and protein tyrosine phosphorylation, we investigated the effect of two well-established thiol-depleting agents, diamide and L-buthionine(*S,R*)-sulfoximine (BSO), on PLD activation in BPAECs. Diamide is a chemical modifier of cellular GSH that oxidizes it to its disulfide, oxidized glutathione (GSSG), and this is not just limited to GSH only but other thiols such as protein thiols are also modified by diamide (Sies, 1985). BSO is a metabolic inhibitor of γ-glutamylcysteine synthetase, which blocks the synthesis of GSH (Meister, 1983; Harian *et al.*, 1984). Treatment of BPAECs with diamide (0.5 m*M*) for 60 min caused a 60% reduction in levels of cellular GSH and nearly a four-fold activation of PLD (Table 2). BSO (1.0 m*M*) pretreatment for

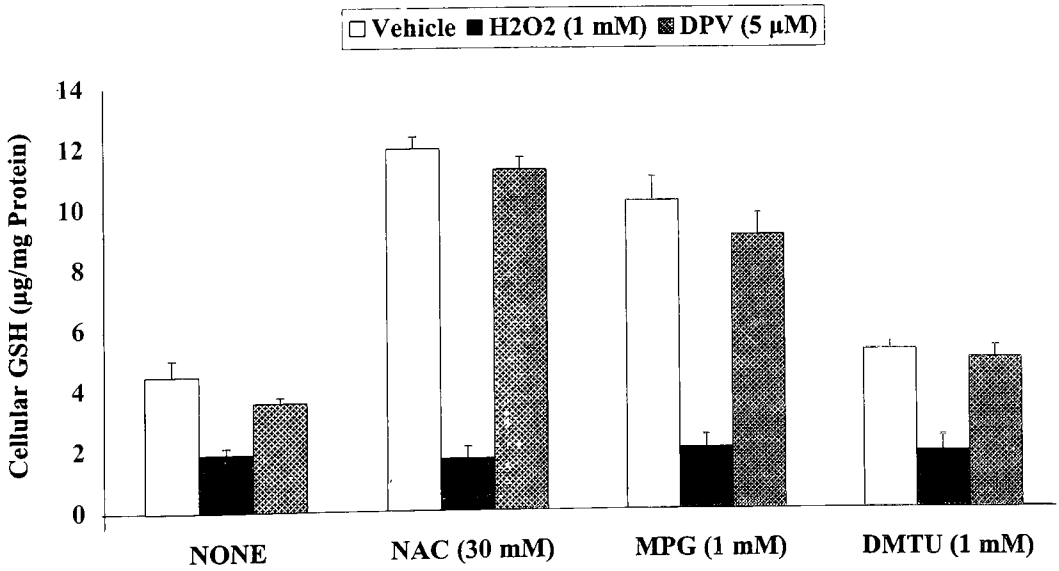

FIG. 7. Effect of NAC, MPG, or DMTU on ROS-mediated changes in cellular thiols. BPAECs in 60-mm dishes were pretreated with MEM or MEM containing NAC (30 m*M*), MPG (100 μM), or DMTU (1 m*M*) for 60 min. Cells were washed extensively with ice-cold PBS and were challenged with MEM or MEM containing H_2O_2 (1 m*M*) or DPV (5 μM) for 30 min at 37°C in 95% air/5% CO_2 atmosphere. Cellular GSH and protein thiols were determined as described under Materials and Methods. Thiols were expressed as μg/mg protein. Each value is an average of three replicates with ±SD.

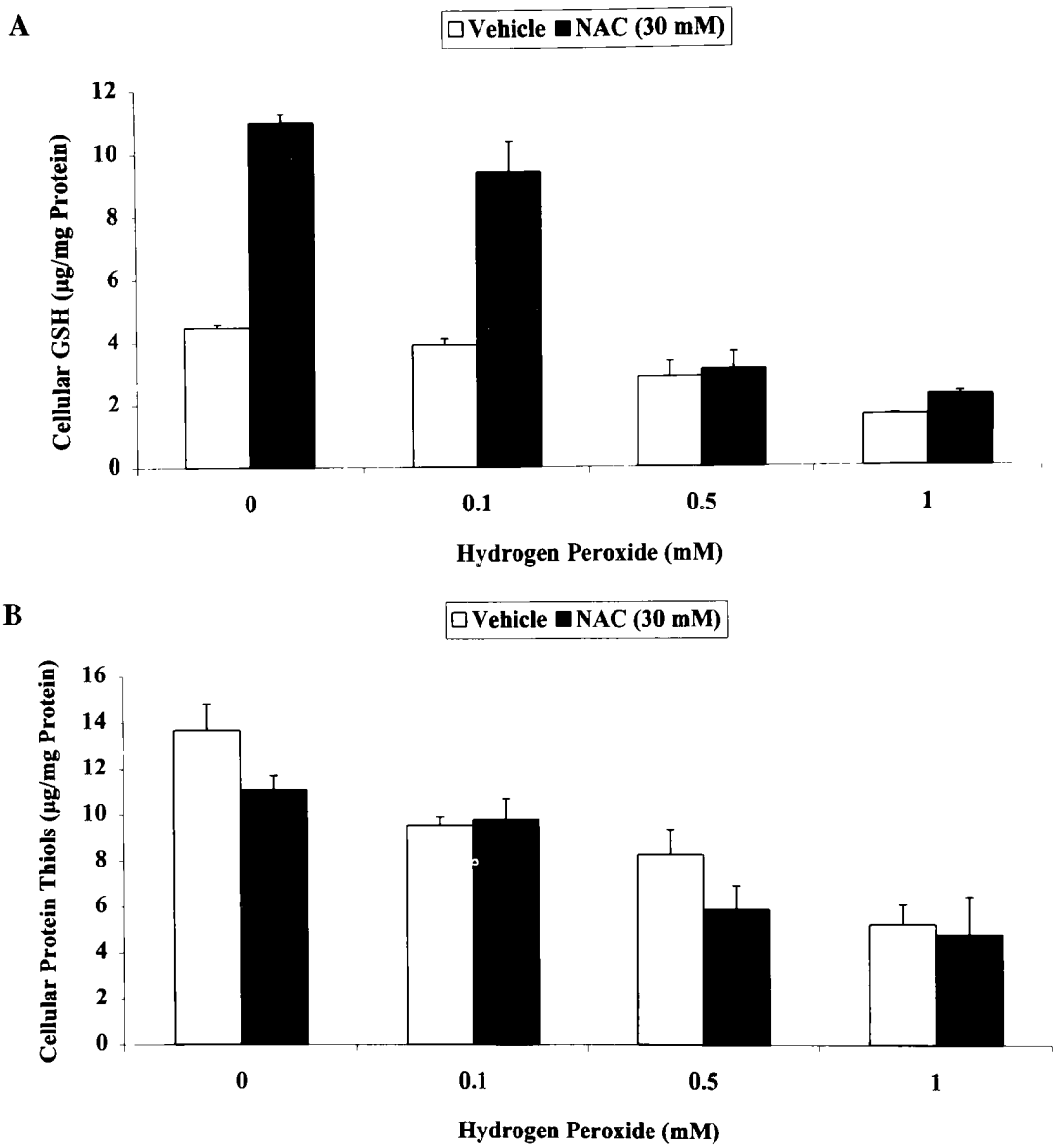

FIG. 8. Dose-dependent depletion of cellular thiols by H_2O_2 and the effect of NAC. BPAECs in 60-mm dishes were pretreated with MEM or MEM containing NAC (30 m*M*) for 60 min. Cells were extensively washed with ice-cold PBS and were challenged with MEM or MEM containing H_2O_2 (1 μM) for 30 min. Cellular GSH **(A)** or cellular protein thiols **(B)** were determined as described under Materials and Methods. Thiols were expressed as μg/mg protein. Each value is an average of three replicates with ±SD.

18 hr caused a 55% decrease in cellular GSH as compared to control BPAECs and induced an accumulation of [^{32}P]PBt (2.5-fold over control) (Table 2). The effect of diamide on intracellular GSH and protein thiols were dose-dependent (Fig. 9). These data further support the hypothesis that alteration in intracellular levels of GSH and protein thiols regulates PLD activation.

Effect of NAC on diamide- or BSO-induced depletion of cellular thiols and PLD activation

To support further the role of cellular thiols in PLD activation, studies on the effect of NAC pretreatment and subsequent exposure of BPAECs to diamide (1 m*M*) revealed that diamide-induced depletion of cellular GSH and

TABLE 2. EFFECT OF DIAMIDE AND BSO ON INTRACELLULAR GSH AND PLD ACTIVATION

Treatment	*GSH (μg/mg protein)*	*[^{32}P] PBt Formed (dpm/dish)*
Vehicle	2.82 ± 0.60	917.00 ± 96.00
Diamide (0.5 m*M*)	0.97 ± 0.35	3,301.00 ± 170.00
BSO (1.0 m*M*, 18 hr)	1.35 ± 0.08	2,284.00 ± 144.00

BPAECs (5×10^5 cells/dish) were labeled with [^{32}P]orthophosphate (5 μCi/dish) in DMEM phosphate-free medium containing 2% FBS for 18 hr. For BSO pretreatment, cells were incubated simultaneously with this agent while labeling with [^{32}P]orthophosphate. Cells were washed in ice-cold PBS and challenged with Diamide (1 m*M*) or BSO (1 m*M*) in the presence of 0.05% butanol for 60 min. For thiols (GSH) determination, BPAECs in 60-mm dishes (after confluence) were treated with BSO (1 m*M*) for 18 hr. [^{32}P]PBt formation and cellular GSH were determined as discussed in the Materials and Methods. Each value is an average ± SD of three replicates.

protein thiols were blocked by NAC in a dose-dependent fashion (Fig. 10A,B). Pretreatment of BPAECs with NAC (1–30 m*M*) also resulted in an attentuation of diamide-induced [^{32}P]PBt accumulation (Fig. 11). Next, we investigated the effect of BSO pretreatment on PLD activation without and with H_2O_2 treatment. As shown in Table 3, treatment of ^{32}P-labeled BPAECs with 100 μ*M* H_2O_2 or 1 m*M* H_2O_2 caused 113% and 193% activation of PLD, respectively as compared to the cells not exposed to H_2O_2. BSO (1 m*M*) pretreatment for 18 hr enhanced [^{32}P]PBt accumulation in BPAECs by 1.8-fold as compared to the control (Table 3). Interestingly, BPAECs pretreated with BSO (1 m*M*) for 18 hr followed by exposure to 100 μ*M* H_2O_2 or 1 m*M* H_2O_2 exhibited a greater accumulation of [^{32}P]PBt (265% and 408%, respectively). This effect of BSO pretreatment was evident only in cells subsequently challenged

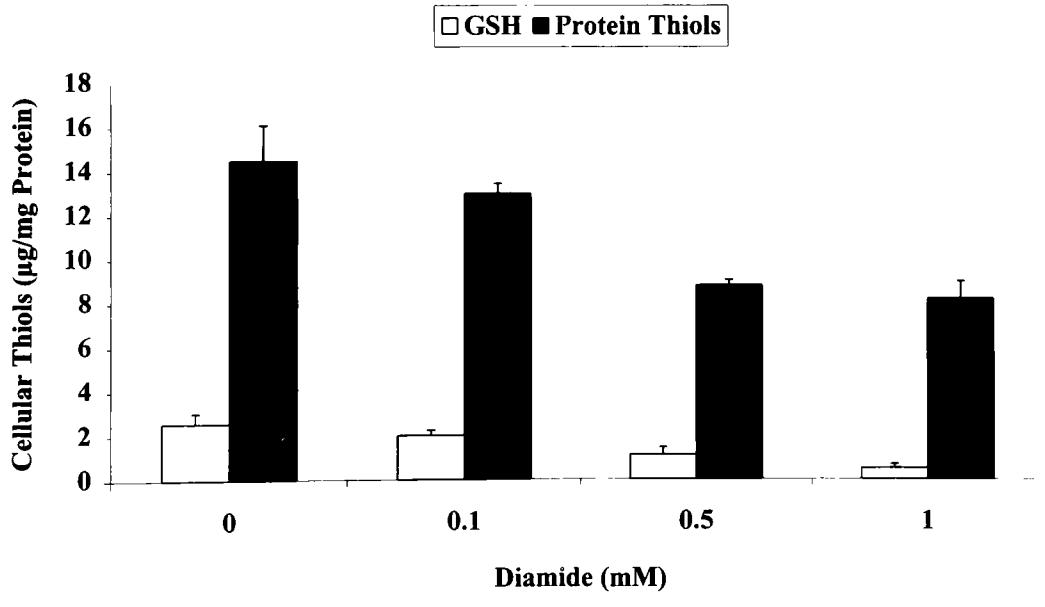

FIG. 9. Effect of diamide on cellular thiols. BPAECs in 60-mm dishes were incubated with varying concentrations of diamonds for 60 min. The cells were washed extensively with ice-cold PBS. Cellular GSH and protein thiols were measured as described under Materials and Methods. Thiols were expressed as μg/mg protein. Each value is an average of three replicates with ±SD.

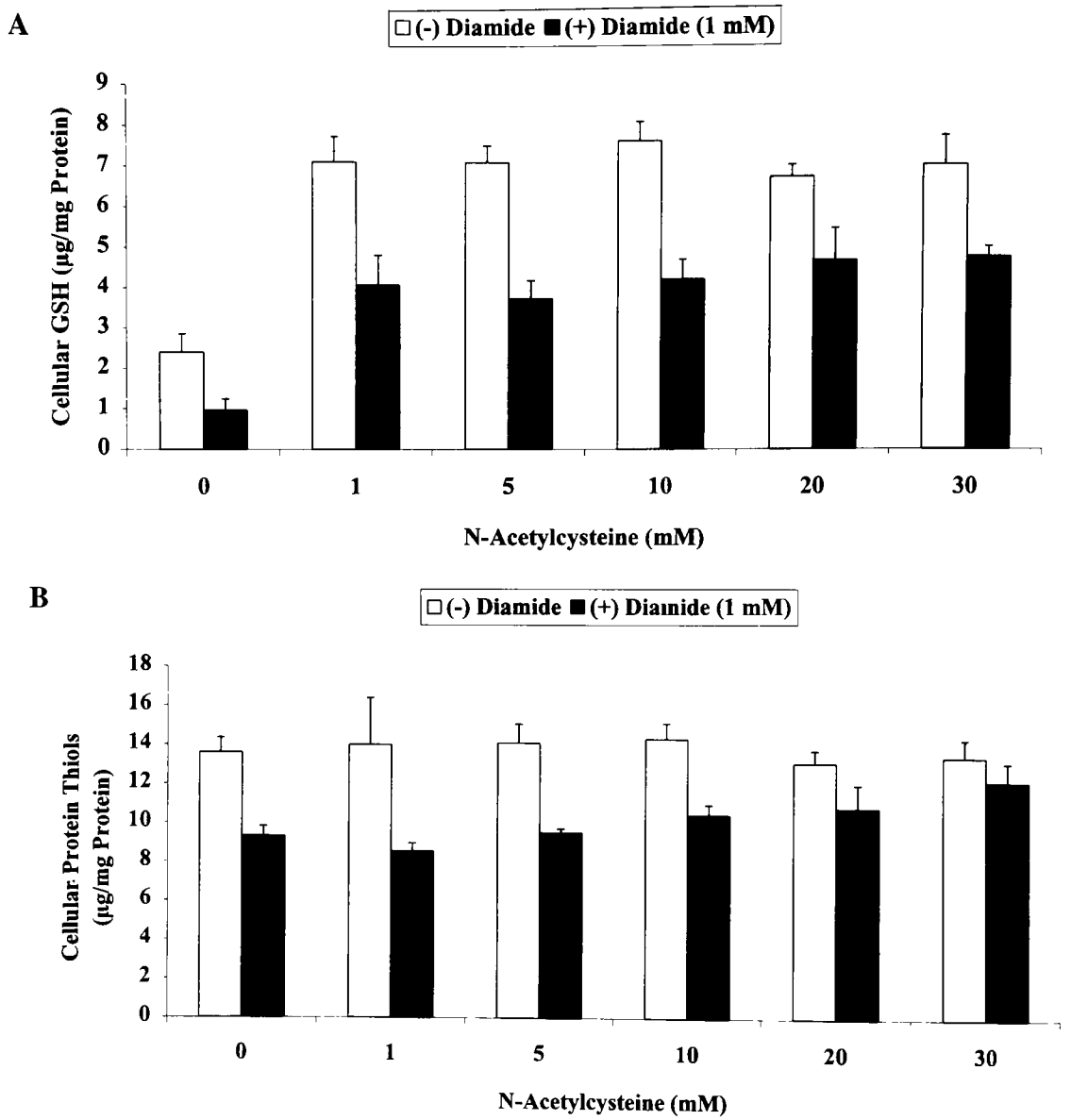

FIG. 10. Effect of NAC on diamide-induced changes in cellular thiols. BPAECs in 60-mm dishes were pretreated with MEM or MEM containing varying concentrations of NAC for 60 min. The cells were extensively washed with ice-cold PBS and were challenged with MEM or MEM containing diamide (1 mM) for 30 min. Cellular GSH **(A)** and protein thiols **(B)** were determined as described under Materials and Methods. Thiols were expressed as μg/mg protein. Each value is an average of three replicates with ±SD.

with H_2O_2, whereas BPAECs pretreated with BSO showed no change in TPA-induced PLD stimulation (Table 3). No signs of cytotoxicity in BPAECs due to BSO pretreatment for 18 hr were apparent. These results suggest that NAC blocks depletion of intracellular GSH and PLD activation by diamide or BSO. Furthermore, BSO pretreatment renders the BPAECs more sensitive to H_2O_2-mediated PLD activation.

Effect of NAC on diamide-induced protein tyrosine phosphorylation

Having established that pretreatment of BPAECs with diamide resulted in depletion of intracellular GSH and activation of PLD, we investigated the effect of diamide on protein tyrosine phosphorylation. As shown in Fig. 12A, diamide enhanced tyrosine phosphorylation of

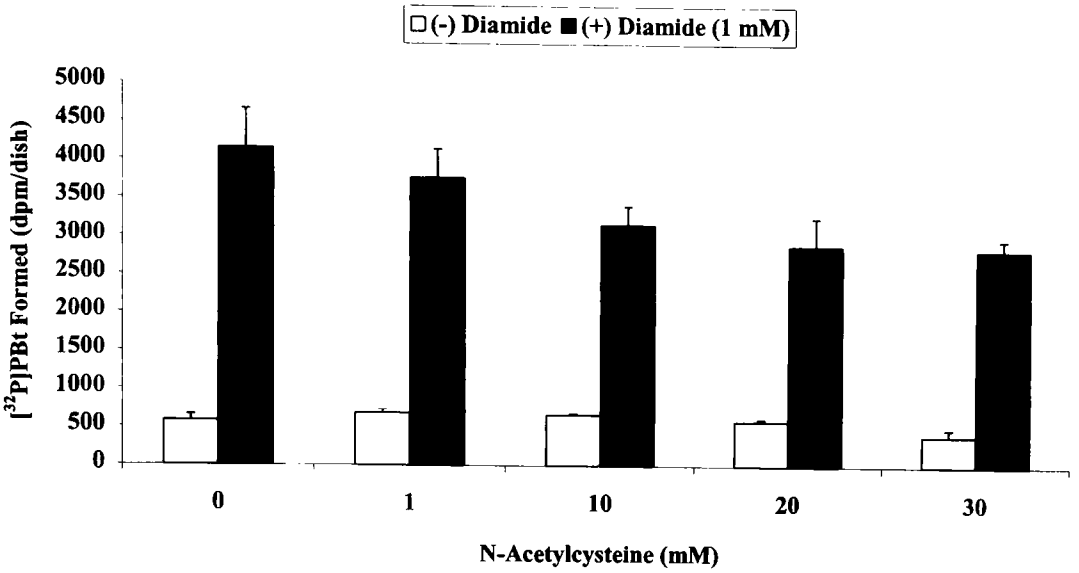

FIG. 11. Effect of NAC on diamide-mediated PLD activation. BPAECs in 35-mm dishes were labeled with [^{32}P]orthophosphate (5 μCi/dish) in DMEM phosphate-free medium containing 2% FBS for 18 hr. The radioactive medium was aspirated and cells were pretreated with varying concentrations of NAC for 60 min. The cells were rinsed extensively with MEM and challenged with MEM or MEM containing diamide (1 m*M*) for 30 min in presence of 0.05% butanol. Lipids were extracted under acidic conditions, and [^{32}P]PBt formed was quantified by TLC as described under Materials and Methods. Values are averages ± of two independent experiments in triplicates.

EC proteins in a dose-dependent manner as determined by western blotting. These prominent protein tyrosine bands at 69–118 KDa were immunodetected using monoclonal antiphosphotyrosine antibodies. Pretreatment of BPAECs with NAC (30 m*M*) specifically attenuated the tyrosine phosphorylation band at 69 KDa (Fig. 12B). These results suggest that diamide-mediated protein tyrosine phosphorylation is sensitive to redox-state of the ECs.

DISCUSSION

Intracellular thiols, including GSH and protein thiols, play a crucial role in signal transduction pathways and regulation of gene transcription, and perturbation of the cellular thiol-redox state is known to be associated with several pathological conditions including AIDS, cancer, and atherosclerosis (Sun and Oberley, 1996; Nakamura *et al.*, 1997; Sen *et al.*,

TABLE 3. EFFECT OF BSO ON H_2O_2-INDUCED PLD ACTIVATION

Pretreatment (120 min)	*Treatment (30 min)*	*PLD Activation [^{32}P] PBt formed (dpm/dish)*	*Δ dpm*	*% Control*
Vehicle	Vehicle	641 ± 86	—	100
Vehicle	H_2O_2 (100 μ*M*)	722 ± 71	81	113
Vehicle	H_2O_2 (1 m*M*)	1,236 ± 101	595	193
Vehicle	TPA (100 n*M*)	8,854 ± 345	8,213	1,381
BSO (1 m*M*)	Vehicle	1,181 ± 115	540	184
BSO (1 m*M*)	H_2O_2 (100 μ*M*)	1,701 ± 95	1,060	265
BSO (1 m*M*)	H_2O_2 (1 m*M*)	2,616 ± 137	1,975	408
BSO (1 m*M*)	TPA (100 n*M*)	9,597 ± 190	8,956	1,497

BPAECs in 35-mm dishes were labeled with [^{32}P] orthophosphate (5 μCi/dish) in DMEM phosphate-free medium for 18 hr. Dishes were also pretreated with BSO (1 m*M*) along with [^{32}P] orthophosphate for 18 hr wherever indicated. Cells were washed and treated with H_2O_2 (100 μ*M* or 1 m*M*) or TPA (100 n*M*) in the presence of 0.05% butanol for 30 min. Lipids were extracted under acidic conditions and [^{32}P]PBt was separated by TLC and quantified as described under Materials and Methods. Values are means ± SD (n = 3).

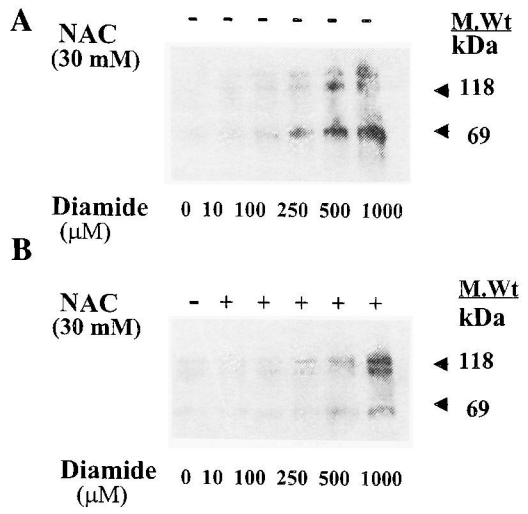

FIG. 12. Effect of NAC on diamide-induced protein tyrosine phosphorylation. BPAECs in 60-mm dishes were pretreated with MEM **(A)** or MEM + NAC (30 μM) **(B)** for 60 min. The cells were washed extensively with MEM and challenged with MEM or MEM containing diamide (10 μM–1 mM) for 30 min. Cell lysates (equal protein) were subjected to SDS-PAGE and tyrosine phosphorylated proteins were immunodetected as described under Materials and Methods.

1998). Although several studies with antioxidants and redox-sensitive molecules have focused on the intracellular redox state and regulation of gene expression, the possible mechanism(s) of regulation of redox-sensitive signaling pathways is unclear.

In this report, we have investigated the redox regulation of ROS-induced PLD activation in vascular endothelial cells. Stimulation of PLD by ROS and other agonists results in the generation of PA, which is subsequently metabolized to lysophosphatidic acid (LPA) or DAG by phospholipase A_1/A_2 (PLA_1/A_2) (Billah *et al.*, 1981) or lipid phosphate phosphatase (Brindley and Waggoner, 1998). PA, LPA, and DAG have been recognized as intracellular second-messengers involved in signal transduction pathways (Nishizuka, 1992; Moolenaar *et al.*, 1997; English *et al.*, 1996). Thiol agents such as NAC, MPG, or DMTU, which possess redox-regulating activity, free radical scavenging capability, and antioxidant properties, attenuated ROS-induced PLD activation. Our data on ROS-modulated intracellular thiols, including GSH levels, are consistent with earlier reports (Chang *et al.*, 1992), and pretreatment of ECs with NAC or MPG restored to certain extent the cellular GSH and protein thiols and under similar experimental conditions, NAC blocked the DPV- and H_2O_2-induced [^{32}P]PBt accumulation, an index of PLD activation. Although both NAC and MPG restored intracellular thiols in the absence or presence of ROS, MPG inhibited the H_2O_2- but not DPV-induced [^{32}P]PBt formation.

These results suggest that the mechanisms of DPV- and H_2O_2-induced PLD activation may have additional component(s) that are distinctly different. The effect of NAC was specific toward ROS-induced PLD activation as compared to TPA- or bradykinin-mediated [^{32}P]PBt accumulation. However, in differentiated HL-60 cells, H_2O_2 failed to activate PLD whereas NAC inhibited fMLP-induced PLD activation (Nakamura *et al.*, 1998). In these studies, a temporal- and concentration-dependent inhibitory profile of protein tyrosine phosphorylation correlated with fMLP-induced PLD activation, suggesting NAC-dependent blockage of protein tyrosine phosphorylation. NAC blocked ROS-mediated protein tyrosine phosphorylation in ECs similar to HL-60 cells and neutrophils (Fialkow *et al.*, 1993a; Nakamura *et al.*, 1998), suggesting redox-regulation of tyrosine kinases. As reported earlier in ECs, both DPV- and H_2O_2-enhanced tyrosine phosphorylation of several proteins (21–130 KDa) (Fig. 5); however, it is not clear which of the tyrosine phosphorylated proteins are redox sensitive. It has been advocated that GSH, in addition to acting as an antioxidant, also participates in the cell signaling and NAC elevates cellular GSH levels (Sen, 1997). It was also established that treatment with NAC resulted in elevation of intracellular GSH levels in BPAECs (Phelps *et al.*, 1992), diminished H_2O_2-augmented transcription of immune genes in giant silkmoth (Sun and Faye, 1995), reduced nitric oxide-mediated cellular injury in pulmonary type II cells (Gow *et al.*, 1998), prevented oxidized low-density lipoprotein-induced apoptosis in human umbilical vein ECs (Dimmeler *et al.*, 1997), and inhibited 12(R)-hydroxyeicosatrienoic acid-induced increase in binding activity of nuclear factor (NF-κB) in rabbit coronary microvessel ECs (Stoltz *et al.*, 1996). Pretreatment of bovine carotid artery ECs with GSH remarkably inhibited 15-hydroperoxyeicosatetraenoic acid-

induced cytotoxicity (Ochi *et al.*, 1992). Also it was shown that MPG at nontoxic doses effectively inhibited the oxidation of thiols during ischemia/reperfusion injury in lung (Ayene *et al.*, 1993), and redox-regulation of mitogen-activated protein kinases in neutrophils and NIH-3T3 fibroblast activation has been reported (Fialkow *et al.*, 1993b; Guyton *et al.*, 1996). It should be of interest to investigate further the effect of thiol agents on ROS-induced phosphorylation of ERK-1/ERK-2 and p38 mitogen-activated protein kinases in ECs.

A role for the intracellular thiols in ROS-induced PLD activation was further confirmed by depletion of cellular GSH and protein thiols by diamide or BSO. These two agents deplete thiols by different mechanisms; diamide depletes GSH by oxidizing it to its disulfide, GSSG and BSO inhibits γ-glutamylcysteine synthetase and thus blocks synthesis of GSH (Meister, 1983; Harian *et al.*, 1984; Sies, 1985). Depletion of endogenous GSH and protein thiols by diamide or BSO enhanced the formation of [^{32}P]PBt in the absence of any exogenous addition of ROS to ECs, and the diamide- or BSO-induced PLD activation was blocked by NAC. NAC not only inhibited DPV- or H_2O_2-induced PLD activation, but also attenuated diamide-mediated [^{32}P]PBt formation and blocked tyrosine phosphorylation of proteins between 69 and 118 KDa, which are yet to be identified. In HL-60 cells, NAC blocked in a concentration-dependent manner the fMLP-induced tyrosine phosphorylation of 64- and 138-KDa proteins (Nakamura *et al.*, 1998). A similar role for intracellular GSH in cytokine-mediated induction of ceramide formation in astrocytes was observed which was blocked by NAC and BSO (Singh *et al.*, 1998). In a recent study, nitric oxide and NAC inhibited ERK activation by angiotensin II in rat cardiac fibroblasts, consistent with redox-sensitive steps in ERK activation (Wang *et al.*, 1998).

Several studies indicate that ROS-mediated PLD activation involves tyrosine kinases (Bourgoin and Grinstein, 1992; Natarajan *et al.*, 1996a,b, 1998a; Ito *et al.*, 1997; Min *et al.*, 1998; Nakamura *et al.*, 1998). At present, it is unclear whether PKC directly participates in ROS-induced PLD activation in ECs. In Swiss 3T3 fibroblasts, the H_2O_2 + vanadate-induced PLD stimulation was sensitive to PKC and tyrosine kinase inhibitors (Min *et al.*, 1998); however, in ECs the H_2O_2-, 4-HNE- or DPV-induced PLD activation was insensitive to PKC inhibitors and down-regulation of PKC by TPA (Natara-

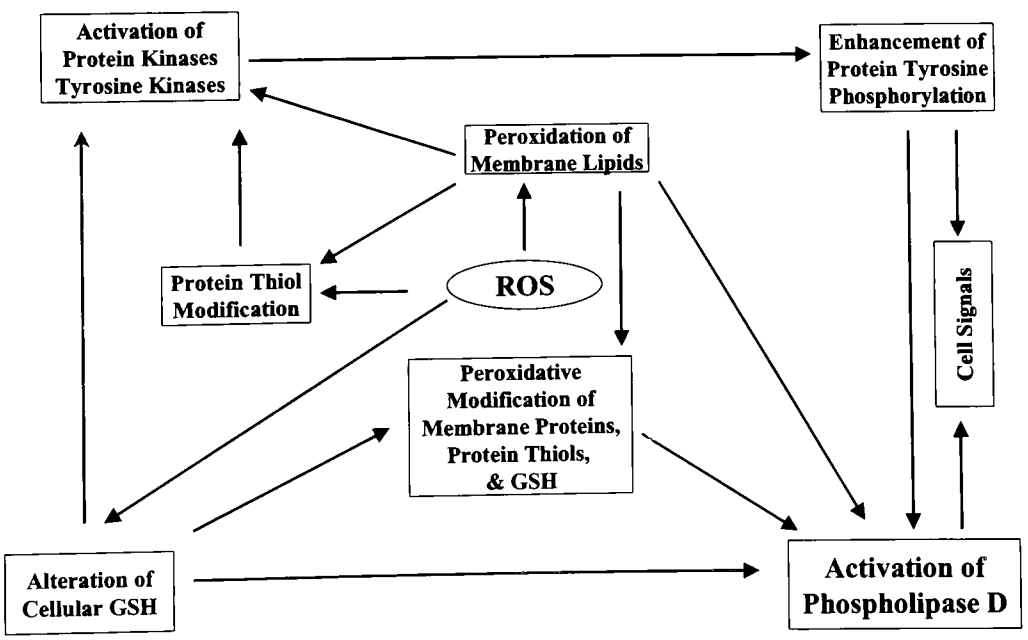

FIG. 13. Schematic representation of ROS-mediated alteration of thiol-redox status, PLD activation, and signaling in ECs.

jan *et al.*, 1996b) but was sensitive to tyrosine kinase inhibitors. In ECs, similar to other mammalian cells, PLD_1 and PLD_2 are the two major isoforms of PLD present (V. Natarajan, unpublished) and this could very well hold true for these isoforms being sensitive to ROS with regards to their activation. At this time there is no information available on which type(s) of PLD isoenzyme(s) is activated by ROS and which isoform is redox sensitive. As oxidative and nitrosative agents alter the functions of proteins by modifying SH groups in cysteine residues, it is likely that the modulation of PLD activity by ROS involves modification of cysteine residues. Interestingly, activation of Ras in the human T-cell line Jurkat by nitrosative and oxidative stress was redox sensitive (Lander *et al.*, 1996). These observations clearly suggest that perturbation of the thiol balance by ROS or other agents lead to the modulation of signal transduction pathways.

In summary, we conclude that ROS-mediated changes in the intracellular thiols contribute to activation of PLD in ECs (Fig. 13). Thiol agents such as NAC and MPG restore, at least partially, the depleted GSH and protein thiols and block the ROS-mediated protein tyrosine phosphorylation and PLD activation. As ROS have been implicated in the pathophysiology of several vascular disorders including ischemia/reperfusion, hypertension, adult respiratory distress syndrome (ARDS), and atherosclerosis, elucidation of redox-sensitive signaling pathways will help in better understanding of the cellular and biochemical responses in disease processes.

ACKNOWLEDGMENTS

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ABBREVIATIONS

ANOVA, analysis of variance; ARDS, adult respiratory distress syndrome; BHT, butylated hydroxytoluene; BPAECs, bovine pulmonary artery endothelial cells; BSA, bovine serum albumin; BSO, L-buthionine-(*S,R*)-sulfoximine; DAG, diacylglycerol; DMEM, Dulbecco's modified Eagle's medium; DMTU, dimethylthiourea; DTNB, 5,5'-Dithio-*bis*(2-nitrobenzoic acid); DPV, diperoxovanadate; ECs, endothelial cells; ECL, enhanced chemoluminescence; FBS, fetal bovine serum; FMLP, N-Formyl-Met-Leu-Phe; GSSG, oxidized glutathione; H_2O_2, hydrogen peroxide; 4-HNE, 4-hydroxynonenal; LPA, lysophosphatidic acid; LPP, lipid phosphate phosphatase; MEM, minimal essential medium; MPG, 2-mercaptopropionylglycine; NAC, *N*-Acetyl-L-cysteine; NF-κB, nuclear factor κB; NMR, nuclear magnetic resonance; PA, phosphatidic acid; PBS, phosphate-buffered saline; PBt, phosphatidylbutanol; PC, phosphatidylcholine; PIP2, phosphatidylinositol 4,5-biphosphate; PKC, protein kinase C; PLA_1/A_2, phospholipase A_1/A_2; PLD, phospholipase D; PMSF, phenylmethylsulfonyl fluoride; PVDF, polyvinylidene difluoride; ROS, reactive oxygen species; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TLC, thin-layer chromatography, TPA, 12-*O*-tetradecanoyl phorbol-13-acetate.

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