

# Reactive Oxygen Species Signaling through Regulation of Protein Tyrosine Phosphorylation in Endothelial Cells

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Tyrosine phosphorylation of proteins, controlled by tyrosine kinases and protein tyrosine phosphatases, plays a key role in cellular growth and differentiating. A wide variety of hormones, growth factors, and cytokines modulate cellular tyrosine phosphorylation to transmit signals across the plasma membrane to the nucleus. Recent studies suggest that reactive oxygen species (ROS) also induce cellular protein tyrosine phosphorylation through receptor or nonreceptor tyrosine kinases. To determine whether protein tyrosine phosphorylation by ROS regulates endothelial cell (EC) metabolism and function, we exposed vascular ECs to H<sub>2</sub>O<sub>2</sub> or H<sub>2</sub>O<sub>2</sub> plus vanadate. This resulted in a time- and dose-dependent increase in protein tyrosine phosphorylation of several proteins (*M<sub>r</sub>* 21–200 kDa), as determined by immunoprecipitation and Western blot analysis with antiphosphotyrosine antibody. Immunoprecipitation with specific antibodies identified increased tyrosine phosphorylation of mitogen-activated protein kinases (42–44 kDa), paxillin (68 kDa), and FAK (125 kDa) by ROS. An immediate signaling response to increased protein tyrosine phosphorylation by ROS was activation of phospholipases such as A<sub>2</sub>, C, and D. Suramin pretreatment inhibited ROS stimulation of phospholipase D (PLD), suggesting a role for growth factor receptors in this activation. Further, PLD activation by ROS was attenuated by *N*-acetylcysteine, indicating that intracellular thiol status is critical to ROS-mediated signal transduction. These results provide evidence that ROS modulate EC signal transduction via a protein tyrosine phosphorylation-dependent mechanism. — *Environ Health Perspect* 106(Suppl 5):1205–1212 (1998). <http://ehpnet1.niehs.nih.gov/docs/1998/Suppl-5/1205-1212natarajan/abstract.html>

Key words: reactive oxygen species, hydrogen peroxide, diperoxovanadate, protein tyrosine phosphorylation, tyrosine kinases, protein tyrosine phosphatases, phospholipases, vascular permeability

## Introduction

Reactive oxygen species (ROS), generated at relatively high levels by activated leukocytes, or by inhalation of environmental toxins such as asbestos, silica, and ozone, have been

implicated in cell damage through oxidative modification of cellular macromolecules (1,2). Several studies have implicated ROS in the pathophysiology of vascular disorders

including atherosclerosis, pulmonary hypertension, vasculitis, and ischemia-reperfusion (3–6). However, the mechanisms of ROS-induced vascular disorders are poorly defined. Recent studies suggest that the effects of ROS in the vascular endothelium are not entirely mediated through damage to cellular components but may involve modulation of signal transduction pathways (7,8). This concept is supported by a number of recent observations that ROS stimulate protein kinase C, tyrosine kinases, mitogen-activated protein (MAP) kinases, Ca<sup>2+</sup> signaling pathways, phospholipases, and regulate transcription factors. Furthermore, some of the toxic effects mediated by environmental toxins and ROS are blocked by free radical scavengers and antioxidants (7).

Tyrosine phosphorylation of proteins, a balance between tyrosine kinases and protein tyrosine phosphatases (PTPs), is modulated by a variety of hormones, growth factors, and cytokines (9,10). Recent reports indicate that ROS induce cellular protein tyrosine phosphorylation through receptor and nonreceptor tyrosine kinases (11,12). H<sub>2</sub>O<sub>2</sub> alone or in combination with vanadate, which generates peroxovanadium compounds, modulates intracellular calcium (13), and activates phospholipases (14–16) and mitogen-activated protein kinases (17) through modulation of protein kinase/phosphatases. However, the physiologic significance of protein tyrosine phosphorylation by ROS in endothelial cell (EC) metabolism and function is not well understood. This study was undertaken to examine the ability of ROS to modulate EC function through protein tyrosine phosphorylation-dependent signaling pathways. Our data show that H<sub>2</sub>O<sub>2</sub> and H<sub>2</sub>O<sub>2</sub> plus vanadate (diperoxovanadate [DPV]) stimulate tyrosine phosphorylation of several EC proteins. Furthermore, our results suggest that ROS-induced stimulation of protein tyrosine phosphorylation alters downstream signaling pathways such as Ca<sup>2+</sup> signaling and activation of phospholipases A<sub>2</sub> (PLA<sub>2</sub>), phospholipase C (PLC), and phospholipase D (PLD), and generation of lipid-derived second messengers.

## Materials and Methods

### Materials

Minimal essential medium (MEM), H<sub>2</sub>O<sub>2</sub>, sodium orthovanadate, nonessential amino

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Abbreviations used: AA, arachidonic acid; AM, acetoxymethyl ester; BPAEC, bovine pulmonary artery endothelial cell; BCA, bicinchoninic acid; BRK, bradykinin; DAG, diacylglycerol; DMEM, Dulbecco's modified Eagle's medium; DPV, diperoxovanadate; EC, endothelial cell; ECL, enhanced chemiluminescence; FAK, focal adhesion kinase; IB, immunoblotting; IP, immunoprecipitation; IP<sub>3</sub>, inositol 1,4,5-trisphosphate; PA, phosphatidic acid; PBS, phosphate-buffered saline; PBT, phosphatidylbutanol; PKC, protein kinase C; PLA<sub>2</sub>, phospholipase A<sub>2</sub>; PLC, phospholipase C; PLD, phospholipase D; PMSF, phenylmethylsulfonyl fluoride; PTP, protein tyrosine phosphatase; PY, phosphotyrosine; RIPA, radioimmunoprecipitation assay; ROS, reactive oxygen species; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TLC, thin-layer chromatography; TPA, 12-O-tetradecanoylphorbol-13-acetate; 6-keto PGF<sub>1α</sub>, 6-keto prostaglandin F<sub>1α</sub>.

acids, genistein, 12-*O*-tetradecanoylphorbol-13-acetate (TPA), and fetal bovine serum were obtained from Sigma Chemical Co. (St. Louis, MO). [<sup>32</sup>P]Orthophosphate (carrier-free) was purchased from New England Nuclear (Wilmington, DE). [<sup>3</sup>H]Arachidonic acid ([<sup>3</sup>H]AA) (sp act 18 Ci/mmol) and [<sup>3</sup>H]myoinositol (sp act 10–20 Ci/mmol) were from ARC (St. Louis, MO). Bovine pulmonary artery endothelial cells (BPAECs) (CCL-209) were from American Type Culture Collection (Rockville, MD). Antiphosphotyrosine antibody (4G10) and EC growth factor were procured from Upstate Biotechnology Inc. (Lake Placid, NY). Diperoxovanadate (potassium salt), a gift from T. Ramasarma (Indian Institute of Science, India), was prepared by bubbling SO<sub>2</sub> gas through a solution of triperoxovanadate and characterized as described earlier (18). Enhanced chemiluminescence (ECL) kit for the detection of tyrosine phosphorylated proteins was from Amersham (Arlington Heights, IL). Phosphatidylbutanol (PBt) was purchased from Avanti Polar Lipids (Alabaster, AL).

## Methods

**Cell Culture.** BPAECs (passage 16) were cultured in MEM as described previously (19). Confluent cells showed cobblestone morphology and stained positive for Factor VIII. All experiments were performed at 90 to 95% confluency and at passage 19 or 20.

## Measurement of Phospholipase D

BPAECs in 35-mm dishes (5 × 10<sup>5</sup> cells/dish) were incubated with [<sup>32</sup>P]orthophosphate (5 μCi/ml) in phosphate-free Dulbecco's modified Eagle's medium (DMEM) containing 2% fetal calf serum for 18 to 24 hr at 37°C (19,20). About 2% of the added radioactivity was incorporated into total phospholipids. Labeled cells were washed in serum-free MEM and were incubated with MEM or MEM containing ROS or other agents, at concentrations and time periods as indicated, in the presence of 0.05% butanol. Incubations were terminated by the addition of 1 ml of methanol:HCl (100:1 v/v) followed by extraction of lipids in chloroform:methanol (19). [<sup>32</sup>P]PBt formed as a result of PLD activation (19) was separated by thin-layer chromatography (TLC) and quantified by scintillation spectrometry.

## Measurement of Phospholipase C

Determination of diacylglycerol (DAG) and inositol trisphosphate (IP<sub>3</sub>) served as

an index of PLC activation. BPAECs grown in T-75 cm<sup>2</sup> flasks were labeled with [<sup>3</sup>H]myoinositol (sp act 10–20 Ci/mmol) (5 μCi/flask) for 48 hr. Cells were washed in MEM and challenged with ROS for indicated time periods; at the end of the incubation, lipids were extracted under acidic conditions (19). The methanol-water phase of the lipid extract containing the [<sup>3</sup>H]inositol phosphates was subjected to anion exchange chromatography in AG 1 × 8 column as described earlier (21) and [<sup>3</sup>H]IP<sub>3</sub> formed was quantified by liquid scintillation counting. For the measurement of DAG formed, BPAECs in T-75 cm<sup>2</sup> flasks were challenged with ROS and reaction was terminated by the addition of 2 ml of ice-cold methanol. Lipids were extracted by addition of chloroform, methanol, and water, and DAG levels were determined by measuring incorporation of [<sup>γ</sup>-<sup>32</sup>P] ATP into DAG using DAG kinase from *Escherichia coli* (22). Appropriate sample blanks and 25 to 250 pmol dioleoylglycerol served as control and standards. DAG levels in the lipid extract were expressed as nmoles of [<sup>32</sup>P]phosphatidic acid (PA) formed per milligram of protein.

## Measurement of Arachidonic Acid Released and 6-Keto Prostaglandin F<sub>1α</sub>

Confluent BPAECs in 35-mm dishes (5 × 10<sup>5</sup> cells/dish) were incubated in 1 ml of MEM containing 0.5 μCi of [<sup>3</sup>H]AA (18 Ci/mmol) for 24 hr at 37°C. The cells were then washed and incubated with ROS or other agents for indicated time periods. The medium was removed and radioactivity was quantified by scintillation counting. In some experiments the media containing the [<sup>3</sup>H]AA metabolites were acidified with formic acid to pH 3.0 and extracted 3 times with 4.5 ml of ethylacetate. The ethylacetate fractions were evaporated under nitrogen and 6-keto prostaglandin F<sub>1α</sub> (6-keto PGF<sub>1α</sub>), the stable metabolite of prostacyclin, was separated by TLC using the upper phase of ethylacetate:isooctane:glacial acetic acid:water (90:50:20:100 by vol). Areas corresponding to free AA and 6-keto PGF<sub>1α</sub> were visualized under iodine vapors using standards and radioactivity was determined. About 40% of the added [<sup>3</sup>H]AA was incorporated into the total lipids of the ECs. TLC analysis showed that 70 to 80% of the released radioactivity comigrated with authentic 6-keto PGF<sub>1α</sub> and 10 to 20% with AA. In some experiments, the cells were treated with MeOH:HCl (100:1 v/v) and lipids were extracted as described earlier (19).

The chloroform phase was dried under N<sub>2</sub> and radioactivity in [<sup>3</sup>H]AA and DAG was determined after TLC, using hexane:diethylether:glacial acetic acid (50:50:1 by vol) as the developing solvent.

## Immunoprecipitation, SDS-PAGE and Western Blot Analysis

Cells treated with MEM or MEM containing DPV or vanadate were rinsed 3 times in ice-cold phosphate-buffered saline (PBS) containing 1 mM vanadate. Cells were scraped in 250 μl of lysis buffer (20 mM Tris-HCl, pH 7.4; 1% NP-40, 137 mM NaCl, 0.5% Triton X-100) supplemented with 2 μg/ml leupeptin, 2 μg/ml pepstatin, 1 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 1 mM vanadate. Cell lysates were sonicated and centrifuged at 14,000 × g for 15 min at 4°C. An aliquot of the supernatant was used for protein estimation by the Pierce bicinchoninic acid assay. The cell lysates (equal protein of about 0.5–1 mg) were subjected to immunoprecipitation with anti-FAK or antipaxillin or anti-ERK-1 plus ERK-2 (1–2 μg/ml) at 4°C for 4 to 12 hr. Protein A/G (20 μl) was then added and incubated for an additional 4 to 6 hr at 4°C. The antibody complex was pelleted and one portion of the immunocomplex was dissociated by boiling in 1 × sodium dodecyl sulfate (SDS) sample buffer for 5 min. Another aliquot of the immunocomplex was washed twice with kinase buffer (50 mM PIPES, pH 7.0; 10 mM MgCl<sub>2</sub>; 3 mM MnCl<sub>2</sub>, and 0.1 mM dithiothreitol). The kinase assays were initiated by the addition of 1 μg myelin basic protein and 50 μM [<sup>γ</sup>-<sup>32</sup>P]ATP (10 Ci/mmol) in a final volume of 100 μl. The reaction was terminated after various time periods at 30°C by the addition of 10 mM ATP and Laemmli sample buffer. The phosphorylation of myelin basic protein was examined by SDS-polyacrylamide gel electrophoresis (PAGE) followed by autoradiography. For Western blot analysis, 40 μl of 6 × Laemmli SDS-PAGE buffer was added to 200 μl of the lysate (20,23), and samples were boiled for 5 min and stored at –20°C. Cell lysates adjusted to equal protein were subjected to SDS-PAGE on 8 or 14% gels and were electrotransferred onto polyvinylidene difluoride (PVDF) membranes for Western blot analysis. Membranes were blocked with blocking buffer (GIBCO-BRL, Gaithersburg, MD) for 1 hr, followed by incubation with 4G10 monoclonal antiphosphotyrosine antibody (1:1000 dilution) for 2 hr. The blots were washed

with TBST (50 mM Tris base, 200 mM NaCl, and 0.1% Tween 20) and were then incubated with goat antimouse IgG heavy and light chains horseradish peroxidase (1:3000 dilution) for 1 hr. Subsequently, the blots were washed in TBST and the phosphotyrosine-containing proteins were immunodetected using ECL.

### Determination of Tyrosine Kinase and PTP Activities

Tyrosine kinase activity in control and ROS-treated BPAEC lysates was determined as described previously (24) using Raytide peptide and [ $\gamma$ - $^{32}$ P]ATP. The tyrosine kinase activity was expressed as picomoles of [ $^{32}$ P] incorporated into Raytide per minute per milligram of protein. PTP activity was assayed by following the dephosphorylation of [ $^{32}$ P]-labeled Raytide peptide. [ $^{32}$ P]-Labeled Raytide peptide was prepared by tyrosine phosphorylation of Raytide peptide with src kinase and [ $\gamma$ - $^{32}$ P]ATP (25). [ $^{32}$ P]-Labeled Raytide peptide was incubated with the cell lysates prepared in 10 mM HEPES, pH 7.4; 1 mM PMSF; 0.5  $\mu$ g/ml leupeptin; 5 mM EDTA; 10 mM NaF; 0.5  $\mu$ g/ml pepstatin) in an assay buffer containing 20 mM amidazole, 5 mM EGTA, 20 mM NaF, and 0.2% 2-mercaptoethanol in a final volume of 200  $\mu$ l. The reaction was carried out for 30 min at 30°C in the absence or presence of 0.2 mM sodium vanadate to determine vanadate-sensitive tyrosine phosphatase activity and terminated by the addition of 1.0 ml of a 2% slurry of activated charcoal. The reaction mixture was centrifuged and free radioactivity in the supernatant [ $^{32}$ P]<sub>i</sub> was counted. Results are expressed as free [ $^{32}$ P]phosphate released per minute per milligram of protein.

### Changes in [Ca<sup>2+</sup>]<sub>i</sub>

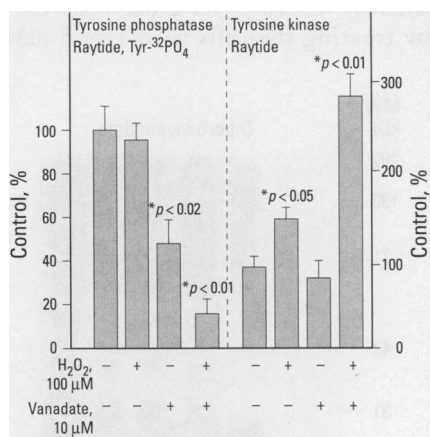
BPAECs grown on glass cover slips as monolayers were loaded with 10  $\mu$ M Fura-2 acetoxymethylester (AM) in the dark for 45 min as described earlier (16). The loaded cells were rinsed 3 times and incubated for an additional 30 min in the dark in buffer containing: 4.8 mM KCl, 130 mM NaCl, 1.0 mM MgCl<sub>2</sub>, 1.5 mM CaCl<sub>2</sub>, 1.0 mM Na<sub>2</sub>HPO<sub>4</sub>, 15 mM glucose, and 10 HEPES (pH 7.4) without albumin. The Fura-2 loaded cells were inserted diagonally in the 1.0-cm acryl cuvettes filled with 2 ml buffer. The cells were challenged with varying concentrations of DPV and Fura-2 fluorescence was measured with an Aminco-Bowman

Series 2 luminescence spectrometer (SLM/Aminco, Urbana, IL) at excitation wavelengths of 340 and 380 nm and an emission wavelength of 510 nm. The 340/380 ratio was taken every half second and measurements were corrected for autofluorescence by measuring the fluorescence of the cells not loaded with Fura-2 AM. Intracellular free calcium [Ca<sup>2+</sup>]<sub>i</sub> was calculated with software provided by the manufacturer.

## Results

### ROS Modulates Tyrosine Kinase and PTP Activities

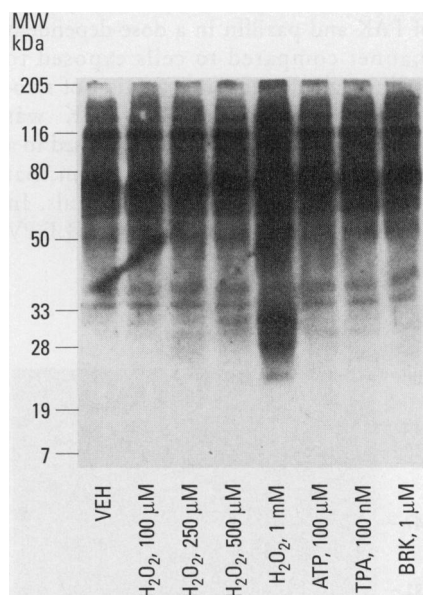
As ROS are potent inhibitors of PTPs (13,26), it was necessary to determine the effect of H<sub>2</sub>O<sub>2</sub> and H<sub>2</sub>O<sub>2</sub> plus vanadate on tyrosine kinase and phosphatase activities in ECs. As shown in Figure 1, H<sub>2</sub>O<sub>2</sub> treatment of BPAECs increased tyrosine kinase activity without altering the PTP activity. However, 10  $\mu$ M vanadate inhibited the PTP activity without altering the tyrosine kinase activity. A combination of vanadate plus H<sub>2</sub>O<sub>2</sub> was a potent inhibitor of PTP (>80% inhibition) and activator of tyrosine kinase (3-fold over control). These data suggest that modulation of tyrosine kinase and PTP activities in ECs is dependent on the nature of ROS used.



**Figure 1.** Effect of ROS on protein tyrosine phosphatase and tyrosine kinase activities *in vitro*. BPAECs ( $5 \times 10^6$  cells/flask) were challenged with MEM or MEM containing 100  $\mu$ M H<sub>2</sub>O<sub>2</sub>, 10  $\mu$ M vanadate, or 100  $\mu$ M H<sub>2</sub>O<sub>2</sub> plus 10  $\mu$ M vanadate for 60 min at 37°C. Cells were washed in ice-cold PBS, scraped into 1 ml lysis buffer (10 mM HEPES, pH 7.4, 1 mM PMSF; 0.5  $\mu$ g/ml leupeptin; 5 mM EDTA; 10 mM NaF; 0.5  $\mu$ g/ml pepstatin) and sonicated. Cell lysates were centrifuged and supernatants were assayed for PTP and tyrosine kinase activities as described under "Materials and Methods." Data are mean  $\pm$  SE of 3 independent determinations.

### ROS Increases Tyrosine Phosphorylation of Proteins in ECs

Modulation of tyrosine kinases/PTPs by ROS may increase protein tyrosine phosphorylation of ECs in a manner similar to that in other cell types (27). Treatment of BPAECs with varying concentrations of H<sub>2</sub>O<sub>2</sub> resulted in a dose-dependent increase in tyrosine phosphorylation of several proteins, as determined by immunoblotting with antiphosphotyrosine antibody (Figure 2). At lower concentrations of H<sub>2</sub>O<sub>2</sub>, the only prominent tyrosine phosphorylated band that was immunodetected was 110 to 130 kDa. Under similar experimental conditions, treatment of BPAECs with ATP, bradykinin (BRK), or TPA showed no significant increase in tyrosine phosphorylation of proteins. Similarly, treatment of BPAECs with 1 to 5  $\mu$ M vanadate showed no increase in tyrosine phosphorylation of EC proteins. However, vanadate at 10  $\mu$ M exhibited a small increase in tyrosine phosphorylation of proteins at 20 to 35 kDa and 110 to 180 kDa (Figure 3). As reported for neutrophils, a combination of

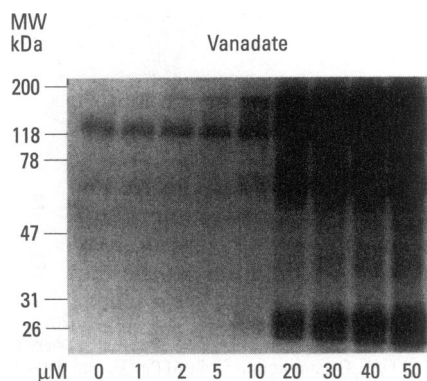


**Figure 2.** Stimulation of protein tyrosine phosphorylation by H<sub>2</sub>O<sub>2</sub> and agonists. BPAECs (35-mm dishes,  $5 \times 10^5$  cells/dish) were challenged with varying concentrations of H<sub>2</sub>O<sub>2</sub> for 1 hr or with ATP, TPA, or bradykinin. Cells were washed in ice-cold PBS containing 1 mM vanadate and scraped with 200  $\mu$ l radio-immunoprecipitation assay (RIPA) buffer. Cell lysates (10  $\mu$ g protein) were subjected to SDS-PAGE (8% gel) followed by transfer to PVDF membrane and immunoblotted with antiphosphotyrosine antibody (1:200 dilution). Tyrosine phosphorylated proteins were detected by ECL using anti-IgG horseradish peroxidase.

vanadate plus H<sub>2</sub>O<sub>2</sub> in a dose-dependent manner caused a marked increase in tyrosine phosphorylation of several proteins in the molecular weight range of 25 to 180 kDa (Figure 4). These results clearly suggest a direct correlation between ROS-mediated modulation of tyrosine kinase/PTP activities and protein tyrosine phosphorylation of EC proteins.

### ROS Enhances Tyrosine Phosphorylation of FAK and MAP Kinases

We previously demonstrated that ROS stimulates tyrosine phosphorylation of caveolin, a 22-kDa marker protein of caveolin in ECs (24). To further examine the effect of ROS on protein tyrosine phosphorylation in ECs, BPAECs were exposed to varying concentrations of 50 to 200 μM H<sub>2</sub>O<sub>2</sub> for 30 min. Cell lysates from control and H<sub>2</sub>O<sub>2</sub>-treated cells were subjected to immunoprecipitation with anti-FAK or antipaxillin monoclonal bodies and the immunoprecipitates were separated by SDS-PAGE and probed with A-PY (or anti-FAK) or antipaxillin. As shown in Figures 5 and 6, H<sub>2</sub>O<sub>2</sub> significantly increased tyrosine phosphorylation of FAK and paxillin in a dose-dependent manner compared to cells exposed to medium alone. Similar activation of tyrosine phosphorylation of FAK was observed when BPAECs were exposed to a xanthine/xanthine oxidase system that generates superoxide anion radicals. In addition to FAK and paxillin, 5 μM DPV

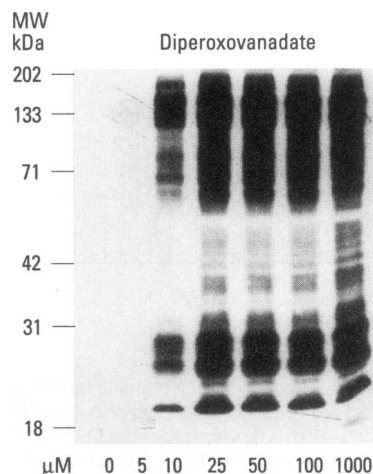


**Figure 3.** Effect of vanadate on EC protein tyrosine phosphorylation. BPAECs (35-mm dishes,  $5 \times 10^5$  cells/dish) were challenged with varying concentrations of vanadate for 60 min. Cells were washed in ice-cold PBS and cell lysates were prepared in 200 μl RIPA buffer. Cell lysates (10 μg protein) were subjected to SDS-PAGE and tyrosine phosphorylated proteins were detected as indicated in Figure 2.

activated ERK-1 and ERK-2, as determined by immunoprecipitation and immunoblotting with antiphosphotyrosine antibodies (Figure 7). Furthermore, measurement of the kinase activity in the ERK-1 and ERK-2 immunocomplexes using myelin basic protein as a substrate showed enhanced incorporation of [<sup>32</sup>P] from [ $\gamma$ -<sup>32</sup>P]ATP into myelin basic protein. These results show activation of focal adhesion proteins and ERK-1 and ERK-2 members of the MAP kinase family by ROS in ECs.

### Role of Ca<sup>2+</sup> in ROS-Induced Protein Tyrosine Phosphorylation

To investigate the role of Ca<sup>2+</sup> in DPV-induced protein tyrosine phosphorylation, BPAECs were challenged with 1 to 10 μM DPV. As shown in Figure 8, DPV increased [Ca<sup>2+</sup>]<sub>i</sub> in a dose-dependent manner. The time-course of DPV-induced [Ca<sup>2+</sup>]<sub>i</sub> showed an initial increase of [Ca<sup>2+</sup>]<sub>i</sub> that was followed by a second phase of gradual decrease. Having demonstrated that DPV caused an increase in [Ca<sup>2+</sup>]<sub>i</sub>, we investigated the role of Ca<sup>2+</sup> in DPV-induced protein tyrosine phosphorylation. Pretreatment of BPAECs with BAPTA/AM (25 μM), a chelator of [Ca<sup>2+</sup>]<sub>i</sub>, attenuated DPV-induced protein tyrosine phosphorylation (Figure 9), as demonstrated by Western blot analysis and probing with antiphosphotyrosine antibody. Chelating the extracellular Ca<sup>2+</sup> by treating the cells with 1 to 5 mM

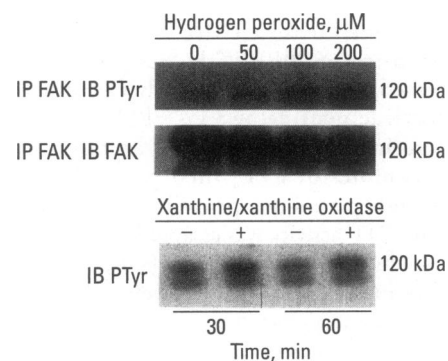


**Figure 4.** Dose-dependent stimulation of proteins tyrosine phosphorylation by DPV in BPAECs. BPAECs (35 mm dish,  $5 \times 10^5$  cells/dish) were challenged with varying concentrations of DPV for 30 min and cell lysates were prepared as described in Figure 2. Cell lysates (5 μg protein) were subjected to SDS-PAGE and tyrosine phosphorylated proteins were immunodetected as described under "Materials and Methods."

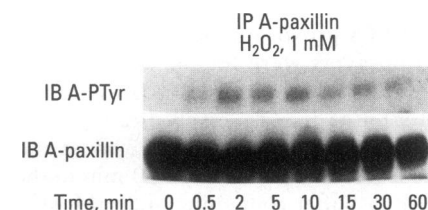
EGTA had no effect on DPV-induced protein tyrosine phosphorylation. These results suggest that DPV-induced changes in [Ca<sup>2+</sup>]<sub>i</sub> modulate protein tyrosine phosphorylation in BPAECs.

### ROS Activates PLA<sub>2</sub>, PLC, and PLD

Stimulation of BPAECs with H<sub>2</sub>O<sub>2</sub> (100 μM) resulted in accumulation of [<sup>3</sup>H]AA



**Figure 5.** ROS stimulates tyrosine phosphorylation of focal adhesion kinase in ECs. Abbreviations: IP, immunoprecipitation; IB, immunoblotting; PTyr, phosphotyrosine. BPAECs ( $5 \times 10^6$  cells/T-75 cm<sup>2</sup> flask) were challenged with MEM or MEM containing varying concentrations of H<sub>2</sub>O<sub>2</sub> or xanthine/xanthine oxidase (100 μM/10 U per ml). Cells were washed in ice-cold PBS and cell lysates were prepared in 2 ml RIPA buffer (denaturing condition). About 1 mg protein was subjected to immunoprecipitation with anti-FAK antibody for 12–18 hr. At the end of the time period, 20 μg protein A/G Sepharose was added and incubations continued for an additional 4 hr. The samples were microcentrifuged and immunoprecipitates were washed 3 times in ice-cold RIPA buffer and reconstituted with sample loading buffer. Equal portions of immunoprecipitates were subjected to SDS-PAGE, followed by transfer to PVDF membrane, and immunoblotted with antiphosphotyrosine antibody and anti-FAK antibody. Tyrosine phosphorylated proteins were detected by ECL (Amersham).



**Figure 6.** ROS stimulates tyrosine phosphorylation of paxillin in ECs. BPAECs ( $5 \times 10^6$  cells/T-75 cm<sup>2</sup> flask) were challenged with MEM or MEM containing 1 mM H<sub>2</sub>O<sub>2</sub> for varying time periods. Cell lysates (1 mg protein) were subjected to immunoprecipitation with antipaxillin antibody and SDS-PAGE as described in Figure 5. Immunoblots were probed with antiphosphotyrosine (A-PTyr) or antipaxillin antibodies using ECL (Amersham).

metabolites in the medium, characterized as 6-keto PGF<sub>1α</sub>, a stable derivative of prostacyclin (Table 1). To further confirm that ROS-mediated increases in [<sup>3</sup>H]AA metabolites involve PLA<sub>2</sub> activation, [<sup>3</sup>H]AA-labeled BPAECs were treated with mepacrine, a PLA<sub>2</sub> inhibitor, before challenging with ROS. As shown in Table 2, mepacrine attenuated the ROS and ionomycin-induced release of [<sup>3</sup>H]AA and accumulation of [<sup>3</sup>H]AA metabolites. These data also suggest that activation of PLA<sub>2</sub> by ROS is an essential step in the release of [<sup>3</sup>H]AA and its conversion to [<sup>3</sup>H]AA metabolites. Also, treatment of BPAECs with H<sub>2</sub>O<sub>2</sub> (100 μM) caused an accumulation of [<sup>3</sup>H]IP<sub>3</sub> and [<sup>32</sup>P]PBt, indicating activation of PLC and PLD pathways. Interestingly, vanadate (10 μM) induced a slight increase in the generation of IP<sub>3</sub> without affecting the generation of [<sup>3</sup>H]AA metabolites or [<sup>32</sup>P]PBt (Table 1). Under similar experimental conditions, H<sub>2</sub>O<sub>2</sub> (100 μM) caused activation of tyrosine kinases, as evidenced by increased protein tyrosine phosphorylation (Western blot analysis). In addition to IP<sub>3</sub>, an accumulation of DAG was observed with H<sub>2</sub>O<sub>2</sub> (100 μM) (2- to 5-fold over control) and H<sub>2</sub>O<sub>2</sub> (100 μM) plus vanadate (10 μM) (7-fold over control). However, vanadate (10–100 μM) failed to enhance DAG levels. These results suggest that ROS activate PLA<sub>2</sub>, PLC, and PLD pathways that may involve stimulation of tyrosine kinases.

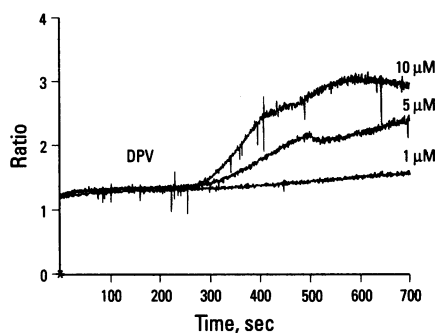
As protein tyrosine phosphorylation is a balance between tyrosine kinases and PTPs, inhibition of phosphatases should

upregulate tyrosine kinase-mediated activation of phospholipases and protein tyrosine phosphorylation. To investigate whether PTP inhibitors modulate H<sub>2</sub>O<sub>2</sub>-induced stimulation of phospholipases and protein tyrosine phosphorylation, BPAECs were challenged with MEM, MEM containing H<sub>2</sub>O<sub>2</sub>, or H<sub>2</sub>O<sub>2</sub> plus vanadate. As shown in Table 3, vanadate potentiated H<sub>2</sub>O<sub>2</sub>-induced accumulation of [<sup>3</sup>H]6-keto PGF<sub>1α</sub>, [<sup>3</sup>H]IP<sub>3</sub>, and [<sup>32</sup>P]PBt as well as protein tyrosine phosphorylation. A similar modulation of the phospholipases and protein tyrosine phosphorylation was observed with other phosphatase inhibitors such as phenylarsineoxide or diamide (data not shown). To further confirm that H<sub>2</sub>O<sub>2</sub>-induced activation of phospholipases and protein tyrosine phosphorylation was tyrosine kinase dependent, the effect of tyrosine kinase inhibitor, genistein, was investigated. Genistein, at a concentration of 100 μM, attenuated the H<sub>2</sub>O<sub>2</sub>-induced release of [<sup>3</sup>H]AA metabolite, and [<sup>32</sup>P]PBt

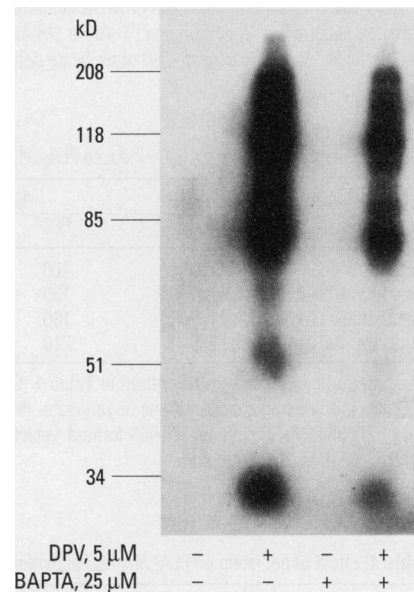
accumulation as well as protein tyrosine phosphorylation (Table 4). These results suggest a role for tyrosine kinase/PTP in ROS-mediated activation of signal transduction pathways in ECs.

**Role of Growth Factor Receptors in ROS-Mediated PLD Activation**

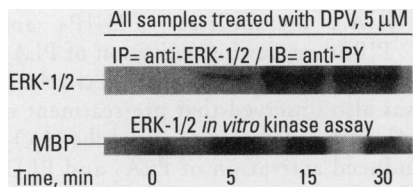
Because H<sub>2</sub>O<sub>2</sub> or H<sub>2</sub>O<sub>2</sub> plus vanadate mimics the action of insulin and growth



**Figure 8.** Changes in [Ca<sup>2+</sup>]<sub>i</sub> by DPV. BPAECs were loaded with Fura-2 and treated with 1–10 μM DPV. Changes in [Ca<sup>2+</sup>]<sub>i</sub> were monitored using an SLM-AMINCO fluorospectrometer as described under "Materials and Methods." Results are expressed as ratio of 340/380 nm and the tracing is representative of three independent experiments.



**Figure 9.** Effect of BAPTA on DPV-induced protein tyrosine phosphorylation. BPAECs were pretreated with medium alone or medium containing 25 μM BAPTA for 30 min. Cells were washed and challenged with medium or medium containing 5 μM DPV for 15 min. Cell lysates were prepared as described under "Materials and Methods." Cell lysates from each of the treatments (10 μg protein) were subjected to SDS-PAGE, transferred onto PVDF membrane and immunoblotted with antiphosphotyrosine antibody 4G10. Tyrosine phosphorylated proteins were detected using ECL.



**Figure 7.** Time course of DPV-induced tyrosine phosphorylation of ERK-1 and ERK-2. BPAECs. (5 × 10<sup>6</sup> cells/T-75 cm<sup>2</sup> flask) were challenged with MEM or MEM containing 5 μM DPV for different time periods as indicated. Cell lysates were subjected to immunoprecipitates with anti-ERK-1 plus anti-ERK-2 polyclonal antibodies (1 μg/mg protein), followed by immunoblotting of equal amounts of immunoprecipitates with antiphosphotyrosine antibody as described under "Materials and Methods." An aliquot of ERK-1 plus 2 immunophosphate was also assayed for MAP kinase activity using 5 μg myelin basic protein and (γ-<sup>32</sup>P-ATP). Tyrosine phosphorylated proteins were detected by ECL and <sup>32</sup>P-labeled myelin basic protein was detected on X-ray film after SDS-PAGE (5% gel).

**Table 1.** Effect of ROS on phospholipases A<sub>2</sub>, C, and D activities and protein tyrosine phosphorylation.

Treatment	Activity, % control			Protein tyrosine phosphorylation, % control
	PLA <sub>2</sub> <sup>a</sup>	PLC <sup>b</sup>	PLD <sup>c</sup>	
Vehicle	100	100	100	100
H <sub>2</sub> O <sub>2</sub> , 100 μM	160	220	325	205
Vanadate, 10 μM	110	140	126	104

BPAECs (5 × 10<sup>5</sup> cells/dish) was pre-labeled with [<sup>3</sup>H]AA (0.5 μCi/dish) or [<sup>32</sup>P]orthophosphate (5 μCi/dish) in phosphate-free DMEM for 24 hr. For PLC assay, cells (5 × 10<sup>6</sup> cells/T-75 cm<sup>2</sup> flask) were pre-labeled with [<sup>3</sup>H]myoinositol (5 μCi/flask) for 48 hr. Cells were washed in MEM and challenged with MEM or MEM containing 100 μM H<sub>2</sub>O<sub>2</sub> or 10 μM vanadate for 30 min at 37°C. Release of [<sup>3</sup>H]AA metabolites, generation of IP<sub>3</sub>, or accumulation of [<sup>32</sup>P]PBt was measured as described in "Materials and Methods." For determination of protein tyrosine phosphorylation, unlabeled cells were treated as above and cell lysates were subjected to SDS-PAGE and Western blot analysis with antiphosphotyrosine antibody. Following ECL, the relative intensities were quantified by a gel scanner. Shown is the average of data obtained in 3 independent experiments. <sup>a</sup>PLA<sub>2</sub> activity represents [<sup>3</sup>H]AA metabolites including 6-keto PGF<sub>1α</sub>. <sup>b</sup>PLC activity represents [<sup>3</sup>H]IP<sub>3</sub> released. <sup>c</sup>PLD activity represents [<sup>32</sup>P]PBt generated. <sup>d</sup>Protein tyrosine phosphorylation was determined by Western blot analysis with antiphosphotyrosine antibody.

**Table 2.** Effect of mepacrine on ROS- and ionomycin-mediated [<sup>3</sup>H]AA and metabolites.

Pretreatment	Treatment	[ <sup>3</sup> H]dpm, AA	[ <sup>3</sup> H]dpm, AA metabolites
Vehicle	Vehicle	1020 ± 82	1920 ± 244
Vehicle	H <sub>2</sub> O <sub>2</sub> , 100 μM	2332 ± 102	3576 ± 94
Vehicle	Ionomycin, 1 μM	3736 ± 146	18400 ± 356
Mepacrine, 100 μM	Vehicle	1428 ± 134	1748 ± 80
Mepacrine, 100 μM	H <sub>2</sub> O <sub>2</sub> , 100 μM	1688 ± 104	2140 ± 76
Mepacrine, 100 μM	Ionomycin, 1 μM	1802 ± 78	4915 ± 106

BPAECs (35-mm dishes; 5 × 10<sup>5</sup> cells/dish) were labeled with [<sup>3</sup>H]AA (0.5 μCi/dish) for 24 hr. At the end of labeling, cells were washed and pretreated with MEM or MEM containing 100 μM mepacrine for 60 min. Cells were rinsed in MEM and challenged with MEM or MEM containing 100 μM H<sub>2</sub>O<sub>2</sub> or 1 μM ionomycin for 30 min. Lipids were extracted under acidic condition and [<sup>3</sup>H]AA or [<sup>3</sup>H]AA metabolites were quantified as described under "Materials and Methods." Values are mean ± SD of triplicate determination.

**Table 3.** Effect of vanadate on H<sub>2</sub>O<sub>2</sub>-induced PLA<sub>2</sub>, PLC, PLD activities, and protein tyrosine phosphorylation.

Addition	% over H <sub>2</sub> O <sub>2</sub> -induced activation			
	PLA <sub>2</sub> <sup>a</sup>	PLC <sup>b</sup>	PLD <sup>c</sup>	Protein tyrosine phosphorylation
None	100	100	100	100
Vanadate, 10 μM	120	135	320	220
Vanadate, 50 μM	160	180	460	640
Vanadate, 100 μM	225	210	580	920

Experimental conditions are described in Table 1. Cells were challenged with MEM or MEM containing H<sub>2</sub>O<sub>2</sub> (100 μM) plus varying concentrations of vanadate. <sup>a</sup>PLA<sub>2</sub> activity—[<sup>3</sup>H]AA metabolite formed: vehicle, 1460 dpm; H<sub>2</sub>O<sub>2</sub>, 3200 dpm. <sup>b</sup>PLC activity—[<sup>3</sup>H]IP<sub>3</sub> formed: vehicle, 640 dpm; H<sub>2</sub>O<sub>2</sub>, 1600 dpm. <sup>c</sup>PLD activity—[<sup>32</sup>P]PBt formed: vehicle, 306 dpm; H<sub>2</sub>O<sub>2</sub>, 826 dpm.

**Table 4.** Effect of genistein on PLA<sub>2</sub> and PLD activities and protein tyrosine phosphorylation.

Addition	% over H <sub>2</sub> O <sub>2</sub> -induced activation		
	PLA <sub>2</sub> <sup>a</sup>	PLD <sup>b</sup>	Protein tyrosine phosphorylation
None	100	100	100
Genistein, 100 μM	65	52	58

BPAECs (5 × 10<sup>5</sup> cells/dish) were prelabeled with [<sup>3</sup>H]AA (0.5 μCi/dish) or [<sup>32</sup>P]orthophosphate (5 μCi/dish) for 24 hr. Cells were washed in MEM and preincubated with 100 μM genistein for 60 min. Cells were rinsed again and challenged with 100 μM H<sub>2</sub>O<sub>2</sub> for 30 min at 37°C. PLA<sub>2</sub> and PLD activities were measured as described in "Materials and Methods." For protein tyrosine phosphorylation, unlabeled cells were preincubated with genistein (100 μM) for 30 min. Cell lysates were subjected to SDS-PAGE and Western blot analysis with antiphosphotyrosine antibody as described in "Materials and Methods." <sup>a</sup>[<sup>3</sup>H]AA metabolite formed: vehicle, 1600 dpm; H<sub>2</sub>O<sub>2</sub>, 3426 dpm. <sup>b</sup>[<sup>32</sup>P]PBt formed: vehicle, 346 dpm; H<sub>2</sub>O<sub>2</sub>, 768 dpm.

**Table 5.** Effect of suramin on ROS- and TPA-mediated PLD activation.

Pretreatment	Treatment	[ <sup>32</sup> P]PBt formed, dpm/dish	Activity, % control
Vehicle	Vehicle	356 ± 79	100
Vehicle	DPV, 2 μM	2953 ± 379	829
Vehicle	TPA, 100 μM	3146 ± 242	884
Suramin, 100 μM	Vehicle	520 ± 105	146
Suramin, 100 μM	DPV, 2 μM	1462 ± 260	410
Suramin, 100 μM	TPA, 100 μM	3266 ± 175	917

BPAECs (5 × 10<sup>5</sup> cells/dish) were prelabeled with [<sup>32</sup>P]orthophosphate (5 μCi/dish) in phosphate-free DMEM containing 2% fetal calf serum for 24 hr. Cells were washed in MEM and then were incubated at 37°C with MEM or MEM containing 100 μM suramin. After 60 min, the cells were washed again in MEM and challenged with 2 μM DPV or 100 nM TPA in the presence of 0.05% butanol for 30 min at 37°C. Lipids were extracted under acidic condition as described in "Materials and Methods." [<sup>32</sup>P]PBt was separated by TLC. Values are mean ± SD of triplicate determination.

factors, we explored the role of growth factor receptors in ROS-mediated activation of PLD. Suramin blocks agonist-growth factor receptor interactions and therefore inhibits PLD activation by ROS when growth factor receptors are involved. As shown in Table 5, suramin pretreatment of BPAECs blocked DPV-induced [<sup>32</sup>P]PBt formation. The effect of suramin was specific for DPV-induced PLD activation, as it had no effect on TPA-mediated [<sup>32</sup>P]PBt formation (Table 5). Also, suramin by itself had minimal stimulatory effect on the basal [<sup>32</sup>P]PBt formation. The attenuating effect of suramin on DPV-induced [<sup>32</sup>P]PBt formation was dose dependent, with 50% inhibition at 100 μM suramin (Table 6). These results suggest that growth factor receptors may be involved in ROS-mediation PLD activation.

**Effect of Antioxidants on ROS-Mediated PLD Activation**

As proteins and lipids are likely targets through which ROS can modulate cell signaling, we investigated the effect of antioxidants on DPV-induced PLD activation. Enhancing the EC redox state by pretreatment with *N*-acetylcysteine abolished the ability of DPV to activate PLD activity (Table 7). These results suggest that modulation of sulfhydryl reactivity by DPV is blocked by *N*-acetylcysteine.

**Discussion**

In this study, the effects of ROS on activation of PLA<sub>2</sub>, PLC, and PLD and protein tyrosine phosphorylation in ECs were examined. Our results show that H<sub>2</sub>O<sub>2</sub>-stimulated the generation of [<sup>3</sup>H]AA metabolites, [<sup>3</sup>H]IP<sub>3</sub> and [<sup>32</sup>P]PBt, suggesting activation of PLA<sub>2</sub>, PLC, and PLD pathways, respectively. It was also observed that pretreatment of ECs with vanadate potentiated the H<sub>2</sub>O<sub>2</sub>-induced activation of PLA<sub>2</sub> and PLD. Vanadate, a known inhibitor of phosphatases, also interacts with H<sub>2</sub>O<sub>2</sub> to generate peroxovanadium compounds (11,12). A major product generated by mixing equimolar amounts of H<sub>2</sub>O<sub>2</sub> and vanadate under neutral pH condition was identified as DPV (18). Our data also indicate that ROS-induced stimulation of PLA<sub>2</sub> and PLD was attenuated by genistein, an inhibitor of tyrosine kinases. These results suggest that ROS-induced activation of phospholipases involves protein tyrosine phosphorylation. This finding further supports the earlier observation that H<sub>2</sub>O<sub>2</sub> plus vanadate mimics insulin (12),



**Table 6.** Suramin inhibits ROS-induced activation of PLD.

Treatment	<sup>32</sup> P]Pbt formed, % over DPV-induced activation
2 μM DPV	100
50 μM suramin ± 2 μM DPV	71
100 μM suramin ± 2 μM DPV	50
250 μM suramin ± 2 μM DPV	30

BPAECs ( $5 \times 10^5$  cells/dish) were prelabeled with [<sup>32</sup>P] orthophosphate (5 μCi/dish) in phosphate-free DMEM containing 2% fetal calf serum for 24 hr. Cells were washed in MEM and then were incubated at 37°C with MEM or MEM containing varying concentrations of suramin. After 60 min, the cells were washed again in MEM and challenged with 2 μM DPV containing 0.05% butanol for 30 min at 37°C. Lipids were extracted under acidic condition and [<sup>32</sup>P]PBT was quantified after TLC as described in "Materials and Methods." Radioactivity associated with Pbt after DPV treatment was  $2746 \pm 172$  dpm/dish. Values are the average of 3 independent experiments in triplicate.

**Table 7.** Effect of *N*-acetylcysteine on ROS- and TPA-induced PLD activation.

Addition	<sup>32</sup> P]Pbt, dpm/dish			
	(-) DPV	(+) DPV	(-) TPA	(+) TPA
—	250 ± 26	1246 ± 155	215 ± 12	4886 ± 755
<i>N</i> -acetylcysteine, 10 mM	158 ± 26	486 ± 47	336 ± 35	4116 ± 475

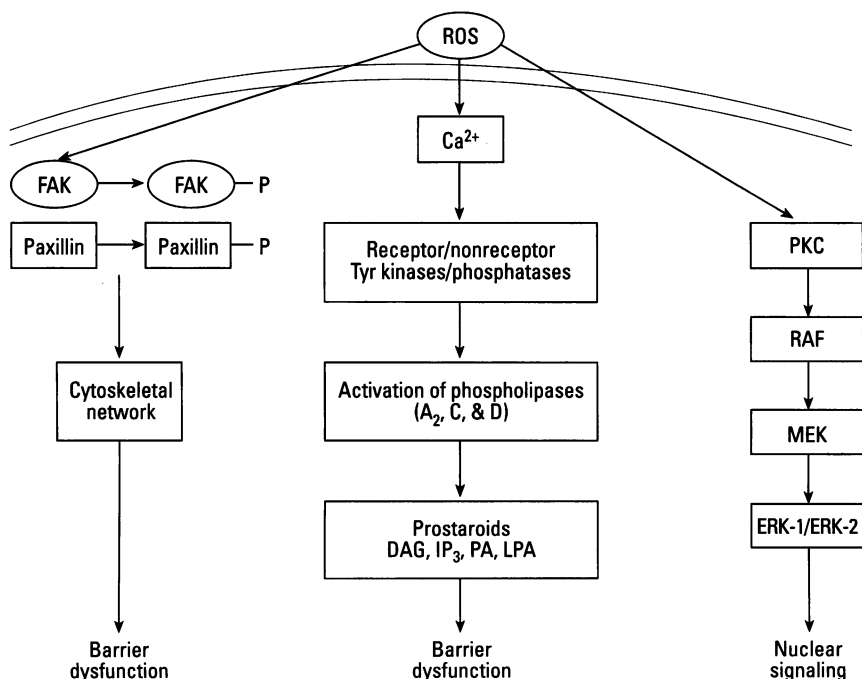
BPAECs ( $5 \times 10^5$  cells/dish) were prelabeled with [<sup>32</sup>P]orthophosphate (5 μCi/dish) in phosphate-free DMEM containing 2% fetal calf serum for 24 hr. Cells were washed in MEM and then were incubated with 10 mM *N*-acetylcysteine for 60 min. At the end of the preincubation, cells were washed in MEM and challenged with 2 μM DPV or 100 nM TPA and 0.05% butanol for 30 min at 37°C. Lipids were extracted under acidic conditions and [<sup>32</sup>P]PBT was quantified after TLC as described in "Materials and Methods." Values are mean ± SD of triplicate determination.

stimulates PI3 kinase (28), and enhances protein tyrosine phosphorylation (29).

The mechanism(s) involved in tyrosine kinase-mediated activation of PLA<sub>2</sub>, PLC, and PLD is unclear. There is increasing evidence for the involvement of protein tyrosine phosphorylation in growth factor-, oxidant-, and IgE-mediated PLD activities. However, the nature of tyrosine phosphorylated proteins involved in PLD activation has not been identified. Earlier studies by Vepa et al. (24) indicate that caveolin and FAK (125 kDa) are targets for H<sub>2</sub>O<sub>2</sub> in ECs. In this study, we have identified ERK-1 and ERK-2 and focal adhesion proteins (FAK and paxillin) as potential targets for ROS-induced tyrosine phosphorylation. Exogenous addition of oxidants induced tyrosine phosphorylation of ERK-1 and ERK-2, and this activation was mediated in

part by MEK (30,31). Similarly, exposure of NIH3T3 cells to H<sub>2</sub>O<sub>2</sub> differentially activated ERK-2, JNK, and p38 MAP kinases (32). Activation of MAP kinases culminates in phosphorylation of downstream targets including enzymes and nuclear factors (33). Although the data presented here do not implicate a direct role for MAP kinases in the activation of EC phospholipases, recent studies suggest that p38 MAP kinase and phosphorylation of heat shock protein HSP27 involve MAPKAPK-2 (34). Suramin, a known inhibitor of ligand-receptor interaction, attenuated ERK-2 activation by epidermal growth factor (EGF) (32). A similar inhibitory effect of suramin on DPV-induced PLD activation was observed, suggesting a role for growth factor receptors rich in cysteine in ROS-mediated cell signaling. Indeed, a role for sulfhydryl groups in ROS-mediated signal transduction was confirmed by the inhibitory effect of *N*-acetylcysteine on DPV-induced PLD activation (Table 7) and protein tyrosine phosphorylation (16). ROS increase [Ca<sup>2+</sup>]<sub>i</sub> through IP<sub>3</sub>-dependent release, enhanced Ca<sup>2+</sup> transport, and through Ca<sup>2+</sup>-dependent channels (7). Although there are several studies to indicate that oxidative stress induces cell toxicity (2–8), recent evidence, including the results of this study, suggests that ROS modulate EC function by altering Ca<sup>2+</sup> signaling, generation of second messengers and protein kinases/phosphatases. Although the precise mechanism(s) and regulation of ROS-mediated activation of phospholipases remain to be clearly established, this study provides support for changes in [Ca<sup>2+</sup>]<sub>i</sub> and redox status of the cell in ROS-mediated protein tyrosine phosphorylation and EC signaling pathways (Figure 10).

Although it is well known that exposure of mammalian cells including ECs to elevated levels of ROS induces toxicity, there is no direct correlation between ROS-induced alterations in signaling pathways and ROS-mediated toxicity. It has been hypothesized that ROS-mediated signaling alterations precede toxicity and by blocking changes in cell signaling by free radical scavengers and antioxidants, the ROS-mediated toxicity can be partially blocked. Further studies on ROS-mediated cellular signaling and toxicity should provide a better understanding of the complexities of cell signaling mechanisms under normal and pathologic conditions.


**Figure 10.** Modulation of EC signaling pathways by ROS. Abbreviations: LPA, lysophosphatidic acid; RAF.

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