Role of p38 MAP kinase in diperoxovanadate-induced phospholipase D activation in endothelial cells

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reactive oxygen species; p38 mitogen-activated protein kinase; vascular endothelium; phospholipase D; phosphorylation; peroxovanadate

PEROXOVANADIUM COMPOUNDS such as pervanadate, prepared by reacting H2O2 and vanadate, are potent modulators of signal transduction pathways in mammalian cells and are useful agents to the study of protein phosphorylation/dephosphorylation in cell proliferation, differentiation, and apoptosis. For example, pervanadate is an insulin mimic that modulates glucose transport, glycogen metabolism, and lipolysis and activates the insulin receptor tyrosine kinase (10, 31, 45, 50). Emerging evidence suggests that pervanadate promotes insulin receptor tyrosine phosphorylation by inhibiting phosphotyrosine phosphatase associated with the insulin receptor (49). Furthermore, pervanadate activates signal transducer and activator of transcription (STAT) proteins (15), mitogen-activated protein kinases (MAPKs; see Refs. 5, 36, 55), myosin light chain kinase (14), and focal adhesion kinase (11, 14), elevates intracellular Ca2+ (36), and enhances hydrolysis of phospholipids (3, 20, 53). Recently, we have shown that diperoxovanadate (DPV), a potent inhibitor of protein tyrosine phosphatases and activator of tyrosine kinases (21), stimulates extracellular signal-regulated kinases (ERKs), caveolin, and focal adhesion kinase (14, 36, 51) in endothelial cells (ECs). In addition, DPV activates phospholipasases A2, C, and D (PLD) as evidenced by enhanced generation of arachidonic acid, diacylglycerol, and phosphatidic acid (PA), respectively (30, 36). Regulation of PLD activation by a wide variety of stimuli is complex and involves changes in intracellular Ca2+, protein kinase C (PKC), heterotrimeric G proteins, small molecular weight G proteins, intracellular thiols, and tyrosine kinase/protein tyrosine phosphatases (7, 12, 13, 34, 39, 40, 44), suggesting multiple pathways of PLD activation (9, 38) or perhaps PLD isoform-specific regulation (16, 19, 46).

Activation of PLD results in the generation of PA, which is subsequently metabolized to lysyPA by phospholipase A2/A1 (2). Both PA and lysyPA have been implicated in cellular processes such as proliferation, differentiation, protein trafficking, and secretion (9, 16). The physiological relevance of PLD activation and role of PA/lysoPA in vascular function is unclear. We have previously demonstrated that exogenous PA but not lysoPA enhanced endothelial monolayer perme-
ability to albumin and altered levels of intracellular free calcium (8). Furthermore, the effects of ectopic PA on endothelial barrier dysfunction were mimicked by PA confined to neutrophil plasma membranes. In porcine aortic ECs, it was shown that lysoPA-mediated stimulation of actin stress fiber formation was dependent on PLD activation and PA generation (5). Overexpression of PLD1 in ECs potentiated oxidant-induced permeability to albumin, suggesting a potential role for PLD1 in barrier dysfunction (Natarajan, unpublished results). These data indicate that PA generated by the PLD signaling pathway in response to oxidative stress disrupts integrity of the vascular endothelium, an early event in the pathophysiology of lung injury.

In view of our earlier observation that DPV activated MAPKs, e.g., ERK1/2 in ECs (36, 41), and ectopic PA altered barrier function of the endothelium (8), we investigated the role of MAPKs in regulating DPV-induced PLD activation. We found that p38 MAPK activation regulates DPV-induced PLD stimulation in ECs. We also show that PLD1 and PLD2 are phosphorylated by p38 MAPK in vitro and in vivo and that DPV treatment enhances PLD1 and PLD2 activities associated with p38 MAPK immunoprecipitates. Furthermore, PLD1 and PLD2 immunoprecipitates from DPV-treated ECs exhibited p38 MAPK activity and were associated with p38 MAPK. Also, association between PLD1/PLD2 and p38 MAPK occurred in vitro with glutathione S-transferase (GST) fusion protein. These results, for the first time, provide evidence for p38 MAPK-dependent regulation of PLD via direct association in basal and DPV-stimulated ECs.

METHODS

Materials. Bovine pulmonary artery ECs (CCL-209, passage 16) were obtained from the American Type Culture Collection (Manassas, VA). MEM, nonessential amino acids, trypsin, FBS, 12-O-tetradecanoylphorbol 13-acetate (TPA), penicillin-streptomycin, DMEM-phosphate-free modified medium, and DMSO were purchased from Sigma Chemical (St. Louis, MO). Phosphatidylbutanol (PBT), dipalmitoylphosphatidylcholine (DPPC), dioleoyl phosphatidylethanolamine (DOPE), and phosphatidylglycerol (PG) were from Avanti Polar Lipids (Alabaster, AL). [32P]orthophosphate (carrier-free), [3H]myristic acid (sp. act. 64.7 Ci/mmol), and [γ-32P]ATP (sp. act. 5 Ci/mmole) were from New England Nuclear (Wilmington, DE). Phosphatidylcholine and 1-O-[choline-methyl-3H]-diomylglycerol (sp. act. 30–60 Ci/mmol) were purchased from American Radiolabeled Chemicals (St. Louis, MO). SB-203580, SB-202474, and PD-98059 were obtained from Calbiochem (San Diego, CA). EC growth factor, affinity-purified 203580, SB-202474, and PD-98059 were obtained from Calbiochem (San Diego, CA). EC growth factor, affinity-purified 203580, SB-202474, and PD-98059 were obtained from Calbiochem (San Diego, CA). EC growth factor, affinity-purified

[32P]orthophosphate (5 μCi/ml) or [3H]myristic acid (1 μCi/ml) in DMEM-phosphate-free medium containing 2% FBS for 18–24 h (37, 42). Cells were washed in MEM and incubated with 1 ml of MEM or 1 ml of MEM plus DPV and 0.05% butanol for various time periods as indicated at 37°C. In some experiments, cells were pretreated with various MAPK inhibitors for 1–2 h before addition of DPV (1–5 μM). The incubations were terminated by addition of methanol-concentrated HCl (100:1 vol/vol). Lipids were extracted essentially according to the method of Bligh and Dyer (42) as described previously. [32P]PBT or [3H]PBT formed as a result of PLD activation and transphosphatidylation reaction, an index of in vivo PLD stimulation (32), was separated by TLC in 1% potassium iodide vapors. Radioactivity associated with the PBt was during the lipid separation by TLC and was visualized under NMR as described earlier (48), was kindly provided by Dr. T. Ramasarma from the Department of Biochemistry, Indian Institute of Science (Bangalore, India).

Preparation of cell lysates and Western blotting. After stimulation with DPV, cells were rinsed two times with ice-cold PBS, scraped in 1 ml of lysis buffer containing 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Nonidet P-40 (NP-40), 0.25% sodium deoxycholate, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM Na3VO4, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 μg/ml pepstatin. Cell lysates were used for immunoprecipitation with PLD1 or PLD2 antibodies.

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p38 MAPK (1:2,000 dilution), anti-JNK (1:2,000 dilution), anti-phospho-ERK1/ERK2 (1:2,000 dilution), anti-phospho-p38 MAPK (1:500 dilution), anti-PLD1 (1:500 dilution), anti-PLD2 (1:500 dilution), or anti-phosphotyrosine 4G10 (1:2,000 dilution) overnight at 4°C. The membranes were washed at least three times with Tris-buffered saline containing 0.1% Tween 20 (TBS-T) and were incubated for 2–4 h at room temperature in horseradish peroxidase-conjugated goat anti-rabbit (1:1,000 dilution in TBS-T containing 5% BSA) or goat anti-mouse secondary antibodies (1:5,000 dilution in TBS-T containing 5% nonfat milk). The immunobLOTS were developed with ECL according to the manufacturer’s recommendation.

In vitro PLD assay. Immunoprecipitates of PLD1, PLD2, and p38 MAPK from control and DPV-treated bovine pulmonary artery ECs were assayed for PLD activity by the head group release assay (4). Liposomal substrate (DOPE-PIP2-DPPC in a molar ratio of 16:1.4:1) was prepared by sonication overnight at 4°C. The lipid mixture with a probe sonicator at a setting of six for DPPC in a molar ratio of 16:1.4:1) was prepared by sonicating (MO to immunoprecipitation with anti-ERK1 plus ERK2 (2 centrifuged in a microfuge at 10,000 rpm for 5 min at 4°C for 12 h. Protein A/G (20 μg) was added, incubated for an additional 2–4 h at 4°C, and centrifuged at 5,000 g for 5 min at 4°C. Aliquots of equal protein were subjected to immunoprecipitation with anti-PLD1 or anti-PLD2 antibodies (5 μg/ml each of internal plus NH₂-terminal antibodies) overnight at 4°C. Protein A/G plus agarose (20 μg) was added, incubated for an additional 2–4 h at 4°C, and centrifuged at 5,000 g for 5 min. The immunocomplexes were washed three times with ice-cold PBS and dissociated by boiling in 1× SDS sample buffer. The samples were subjected to SDS-PAGE on 10% gels, dried in a gel dryer, analyzed by autoradiography, and quantified by image analysis.

Transient expression of dominant negative mutant of p38 MAPK. Bovine pulmonary artery ECs were cultured in 35-mm dishes until they reached 80% confluence. Each well was transfected with 1.5 μg of vector or a plasmid DNA of the dominant negative mutant of p38αa cloned in pCMV-5 cDNA in 5 μl of LipofectAMINE reagent in MEM without serum according to the manufacturer’s recommendation. After 5 h of transfection, the medium was replaced by complete medium. After 24 h of transfection, the cells were labeled with [3H]myristic acid (1 μCi/ml) in complete MEM with 20% serum for 18–24 h, and the effect of DPV or TPA on PLD activity was determined. Transiently transfected cells were analyzed for expression of p38 MAPK by Western blotting, and p38 MAPK activity was determined by ATF-2 phosphorylation using a p38 kinase assay kit.

p38 MAPK/PLD binding assays in vitro. GST alone or GST-p38 fusion protein was incubated with 20 μl of glutathione-Sepharose in 250 μl of PBS containing 0.1% NP-40, 1 mM PMSF, 5 mM DTT, 1 μg/ml leupeptin, 1 μg/ml aprotinin, and 1 μg/ml pepstatin for 3 h at 4°C. The beads were washed three times with 200 μl of binding assay buffer [20 mM HEPES (pH 7.6), 500 mM NaCl, 0.5 mM EDTA, and 0.1% NP-40] and incubated with SX lysates from control or PLD1a/PLD2-overexpressed cells. The beads were washed with PBS, eluted with 20 μl of SDS sample buffer [75 mM Tris·HCl (pH 6.8), 0.5% glycerol, 1% SDS, 4% β-mercaptoethanol, and 0.01% bromophenol blue], and boiled for 3 min before separation on 8% SDS-PAGE. PLD1/PLD2 were immunodetected by Western blotting with anti-PLD1 or anti-PLD2 antibodies.

RESULTS

DPV activates ERK, p38, and JNK MAPKs in ECs. To investigate the role of MAPKs in DPV-induced PLD activation, bovine pulmonary artery ECs were treated...
with DPV (5 μM) for various time periods, and total cell lysates were analyzed for ERK1/2, p38 MAPK, and JNK activation by Western blotting with phospho-specific antibodies. As shown in Fig. 1, A–C, DPV stimulated ERK1/2, p38, and JNK activities in a time-dependent manner as evidenced by enhanced phosphorylation of threonine/tyrosine residues. The activation of ERK1/2 and p38 by DPV was dose dependent, with increased phosphorylation being detected with concentrations as low as 1 μM (Table 1). Figure 2 shows that DPV also increased tyrosine phosphorylation of the three MAPKs detected by immunoblotting with anti-phosphotyrosine antibody. Also, it was observed that DPV treatment of ECs resulted in increased phosphorylation of myelin basic protein or ATF-2 in in vitro kinase assays (Fig. 2).

DPV-induced PLD activation in ECs requires p38 MAPK activation. To further elucidate the role of MAPKs in mediating DPV-induced PLD stimulation, we used selective and known chemical inhibitors for ERK1/ERK2 and p38 MAPKs. As shown in Table 2, pretreatment of ECs for 2 h with PD-98059 (25 μM), a specific inhibitor of MAPK kinase (MEK) 1/MEK2 (1), did not affect the DPV-induced [32P]PBt formation. In contrast, pretreatment of ECs with selective p38 MAPK inhibitor SB-203580 (25 μM), SB-202190 (25 μM), or PD-169316 (25 μM; see Ref. 54) for 2 h before DPV challenge partially mitigated DPV-induced PLD activation without altering basal PLD activity (Table 2). In addition, SB-202474 (25 μM), a negative control for SB-203580 and SB-202190, did not significantly alter the DPV-induced [32P]PBt formation. The effect of SB-202190 on DPV-induced [32P]PBt formation was dose and time dependent (Fig. 3, A and B). In parallel experiments, we also examined the influence of SB-202190 on DPV-induced p38 MAPK activation. As shown in Fig. 5C, SB-202190 in a dose-dependent manner attenuated p38 activity as determined by phosphorylation of ATF-2. SB-202190 had no effect on DPV-induced phosphorylation of p38 MAPK (Fig. 3C) as determined with phospho-specific p38 antibodies, since SB-202190 directly binds to p38 MAPK and alters its kinase activity. Furthermore, the effect of SB-202190 in inhibiting p38 MAPK-dependent phosphorylation of ATF-2 was specific, since no effect on phosphorylation of ERK1/2 was observed (data not shown). These data suggest that p38 MAPK activation is part of the signaling cascade involved in DPV-induced PLD stimulation.

Fig. 1. Time course of diperoxovanadate (DPV)-induced mitogen-activated protein kinase (MAPK) activation. Bovine pulmonary artery endothelial cells (ECs; 3 × 10^6 cells/100-mm dish) were treated with DPV (5 μM) for the indicated times at 37°C. Cell extracts were prepared as described in METHODS and were assayed for total and phosphorylated (Phospho) extracellular signal-regulated kinase (ERK; A), p38 MAPK (B), or c-Jun NH2-terminal kinase (JNK; C) by Western blotting with specific antibodies. Results shown are representative blots of at least 3 independent experiments.
### Table 1. Concentration-dependent phosphorylation of MAPK by DPV

<table>
<thead>
<tr>
<th>DPV Addition, μM</th>
<th>MAP Kinase Phosphorylation</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Phospho-ERK/ERK</td>
</tr>
<tr>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>1</td>
<td>6.9</td>
</tr>
<tr>
<td>2</td>
<td>9.3</td>
</tr>
<tr>
<td>5</td>
<td>5.8</td>
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</table>

Bovine pulmonary artery endothelial cells (ECs; 3 × 10⁶ cells/100-mm dish) were incubated with MEM or MEM containing indicated concentrations of diperoxovanadate (DPV; 1–5 μM) for 30 min at 37°C. Cell lysates were prepared, and equal proteins were loaded on 10% SDS-PAGE and Western blotted for total extracellular signal-regulated kinase (ERK) and phosphorylated (phospho) ERK, total p38 mitogen-activated protein kinase (MAPK) and phosphorylated p38 MAPK, or total c-Jun NH₂-terminal kinase (JNK) and phosphorylated (phospho) JNK as described in METHODS. Blots were scanned in a densitometer and quantified by image analysis. MAPK phosphorylation is expressed as phospho-ERK/ERK, phospho-p38/p38, or phospho-JNK/JNK. The ratios were normalized to basal phosphorylation of 1 without DPV. Results shown are from 2 independent experiments.

### Table 2. Effect of MAPK inhibitors on DPV-induced PLD activation

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>Treatment</th>
<th>[³²P]PBl</th>
<th>Formed, dpm/ dish</th>
<th>Control, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>Vehicle</td>
<td>504 ± 26</td>
<td>100</td>
<td></td>
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<tr>
<td>Vehicle</td>
<td>DPV</td>
<td>3,418 ± 105</td>
<td>678</td>
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<tr>
<td>PD-98059</td>
<td>Vehicle</td>
<td>596 ± 74</td>
<td>118</td>
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<td>PD-98059</td>
<td>DPV</td>
<td>3,576 ± 52</td>
<td>710</td>
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<tr>
<td>SB-203580</td>
<td>Vehicle</td>
<td>540 ± 36</td>
<td>107</td>
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<tr>
<td>SB-203580</td>
<td>DPV</td>
<td>2,411 ± 60*</td>
<td>478</td>
<td></td>
</tr>
<tr>
<td>SB-209190</td>
<td>Vehicle</td>
<td>474 ± 31</td>
<td>94</td>
<td></td>
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<tr>
<td>SB-209190</td>
<td>DPV</td>
<td>1,817 ± 78†</td>
<td>560</td>
<td></td>
</tr>
<tr>
<td>PD-169316</td>
<td>Vehicle</td>
<td>604 ± 44</td>
<td>120</td>
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<tr>
<td>PD-169316</td>
<td>DPV</td>
<td>2,310 ± 82*</td>
<td>458</td>
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<tr>
<td>SB-202474</td>
<td>Vehicle</td>
<td>470 ± 25</td>
<td>93</td>
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<tr>
<td>SB-202474</td>
<td>DPV</td>
<td>378 ± 146</td>
<td>750</td>
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Bovine pulmonary artery ECs (5 × 10⁶ cells/35-mm dish) were prelabeled with [³²P]orthophosphate (5 μCi/dish) in DMEM-phosphate-free medium for 24 h. After removal of the labeled medium, cells were pretreated with MEM or MEM containing either PD-98059 (25 μM), SB-203580 (25 μM), SB-209190 (25 μM), or PD-169316 (25 μM), or the negative control SB-202474 (25 μM) for 2 h at 37°C, after which cells were incubated in MEM or MEM plus DPV (5 μM) for 30 min in the presence of 0.05% butanol. Lipids were extracted under acidic conditions as described in METHODS, and [³²P]phosphotybutanol (PBl) formed, an index of phospholipase D (PLD) activation, was quantified by TLC and liquid scintillation counting. Results are expressed as means ± SE from 3 individual experiments in triplicate. Counts in [³²P]PBl were normalized to 10⁶ dpm in total lipid extracts. *Significantly different at P < 0.05 compared with vehicle. †Significantly different at P < 0.001 compared with vehicle. Not shown.

Effect of SB-202190 on TPA-, bradykinin-, and ATP-induced PLD activation in ECs. To further characterize the specificity of the p38 MAPK inhibitor SB-202190 on agonist-mediated PLD activation, ECs were preincubated with SB-202190 (25 μM) for 2 h before challenge with TPA (25 nM), bradykinin (1 μM), ATP (100 μM), or DPV (5 μM), and [³²P]PBl accumulation was measured. As shown in Table 3, 25 μM SB-202190 attenuated DPV-induced PLD activation by ~50% but failed to mitigate either TPA- or bradykinin- or ATP-mediated PLD stimulation. In addition to SB-202190, we also examined the effect of the MEK1/MEK2 inhibitor PD-98059 on TPA- or DPV-induced PLD stimulation, which, unlike the p38 MAPK inhibitor, failed to alter either TPA- or DPV-induced [³²P]PBl formation (data not shown). Under similar incubation conditions, PD-98059 partially blocked activation of ERK1/2 by TPA (25 nM; data not shown). These results further confirm the specificity of SB-202190 in attenuating DPV-induced p38 MAPK and PLD activation in ECs.

Expressing a dominant negative mutant of p38 MAPK attenuates DPV-induced PLD activation. To further confirm a role for p38 MAPK in DPV-induced PLD activation, was quantified by TLC and liquid scintillation counting. Results are expressed as means ± SE from 3 individual experiments in triplicate. Counts in [³²P]PBl were normalized to 10⁶ dpm in total lipid extracts. *Significantly different at P < 0.05 compared with vehicle. †Significantly different at P < 0.001 compared with vehicle. Not shown.

Expressing a dominant negative mutant of p38 MAPK attenuates DPV-induced PLD activation. To further confirm a role for p38 MAPK in DPV-induced PLD activation, was quantified by TLC and liquid scintillation counting. Results are expressed as means ± SE from 3 individual experiments in triplicate. Counts in [³²P]PBl were normalized to 10⁶ dpm in total lipid extracts. *Significantly different at P < 0.05 compared with vehicle. †Significantly different at P < 0.001 compared with vehicle. Not shown.
activation, we transiently transfected ECs with a dominant negative mutant of p38 MAPK or a vector control. Cell lysates from vector or dominant negative p38 MAPK-transfected ECs were subjected to immunoprecipitation with anti-p38 MAPK antibody, and the immunoprecipitates were analyzed for the expression of p38 MAPK protein and assayed for kinase activity in vehicle- or DPV-treated cells by measuring phosphorylation of ATF-2. Western blotting of the lysates from vector or dominant negative p38 MAPK-transfected cells revealed overexpression of the p38 MAPK protein (Fig. 4A). However, p38 MAPK immunoprecipitates from cells transfected with the dominant negative mutant of p38 MAPK exhibited lower kinase activity as determined by phosphorylation of ATF-2 with and without DPV treatment. Transfection of ECs with the dominant negative mutant of p38 MAPK had no effect on DPV-induced activation of ERK1/2 or JNK (data not shown). Under similar experimental conditions, transfection with vector plasmid alone had no effect on DPV-induced activation of p38 MAPK (Fig. 4A). Expression of the dominant negative mutant of p38 attenuated DPV-induced [3H]PBt formation by 40% (Fig. 4B) without altering TPA-mediated PLD activation. These results further confirm participation of p38 MAPK in DPV-induced PLD stimulation in ECs.

Association of PLD1 and PLD2 with p38 MAPK immunoprecipitates. Because the above data suggested the importance of p38 MAPK in the activation of PLD by DPV, we investigated whether direct interaction from cells transfected with the dominant negative mutant of p38 MAPK exhibited lower kinase activity as determined by phosphorylation of ATF-2 with and without DPV treatment. Transfection of ECs with the dominant negative mutant of p38 MAPK had no effect on DPV-induced activation of ERK1/2 or JNK (data not shown). Under similar experimental conditions, transfection with vector plasmid alone had no effect on DPV-induced activation of p38 MAPK (Fig. 4A). Expression of the dominant negative mutant of p38 attenuated DPV-induced [3H]PBt formation by 40% (Fig. 4B) without altering TPA-mediated PLD activation. These results further confirm participation of p38 MAPK in DPV-induced PLD stimulation in ECs.

**Table 3. Effect of SB-202190 on agonist-induced or DPV-induced PLD activation**

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>Treatment</th>
<th>[32P]PBt Formed, dpm/dish</th>
<th>Control, %</th>
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<tr>
<td>Vehicle</td>
<td>Vehicle</td>
<td>960 ± 109</td>
<td>100</td>
</tr>
<tr>
<td>Vehicle</td>
<td>DPV</td>
<td>4,896 ± 294</td>
<td>510</td>
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<tr>
<td>Vehicle</td>
<td>TPA</td>
<td>3,634 ± 453</td>
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<tr>
<td>Vehicle</td>
<td>BRK</td>
<td>1,790 ± 225</td>
<td>186</td>
</tr>
<tr>
<td>Vehicle</td>
<td>ATP</td>
<td>1,835 ± 28</td>
<td>191</td>
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<tr>
<td>SB-202190</td>
<td>Vehicle</td>
<td>825 ± 73</td>
<td>86</td>
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<td>SB-202190</td>
<td>DPV</td>
<td>2,528 ± 146</td>
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<td>TPA</td>
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<td>362</td>
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<td>SB-202190</td>
<td>BRK</td>
<td>1,897 ± 142</td>
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<tr>
<td>SB-202190</td>
<td>ATP</td>
<td>2,009 ± 118</td>
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Values are means ± SE of 3 independent experiments in triplicate and were normalized to 10^6 dpm in total lipid extracts. Bovine pulmonary artery ECs (5 × 10^5 cells/dish) were labeled with [32P]orthophosphate (5 μCi/dish) in DMEM-phosphate-free medium for 18 h. Cells were rinsed in MEM and pretreated with serum-free MEM or MEM containing SB-202190 (25 μM) for 2 h. Subsequently, cells were challenged with DPV (5 μM), 12-O-tetradecanoylphorbol 13-acetate (TPA, 25 nM), bradykinin (Brk, 1 μM), or ATP (100 μM) in the presence of 0.05% butanol for 30 min. Lipids were extracted under acidic conditions and separated by TLC. Radioactivity associated with PBt was quantified by scintillation spectroscopy as described in Methods.
extracted under acidic conditions, and [3H]PBt was quantified as for 30 min at 37°C in the presence of 0.05% butanol. Lipids were normalized to 106 dpm in total lipid extracts. Values are means ± SE of 3 independent experiments in triplicate. *Significant at P < 0.05 compared with vector-transfected cells.

exists between p38 MAPK and PLD. ECs were exposed to medium alone or medium containing DPV (5 μM) for 5 min, and cell lysates were subjected to immunoprecipitation under native conditions with anti-p38 MAPK antibody plus protein A/G Sepharose. Equal volumes of the p38 MAPK immunoprecipitate were assayed for activity with ATP-2 as substrate. Total p38 MAPK in the immunoprecipitate and phospho-ATF-2 were detected by Western blotting. Densitometric analysis of the p38 MAPK immunoprecipitates against p38 MAPK antibodies showed an immunodetectable protein band on SDS-PAGE in the range of 38 kDa, suggesting the possible presence of p38 MAPK in PLD immunoprecipitates (Fig. 6, A and B). Also, the hPLD1 and hPLD2 immunoprecipitates from control cells phosphorylated ATF-2, which was enhanced after DPV treatment (Fig. 6, A and B). These results further confirm an association between p38 MAPK and hPLD1/hPLD2 under basal and stimulated conditions.

Association of p38 MAPK with hPLD1 and hPLD2 immunoprecipitates. Based on the above data, we next investigated the possible association of p38 MAPK in PLD1 and PLD2 immunoprecipitates. Western blot analysis of hPLD1 and hPLD2 immunoprecipitates from control and DPV-treated ECs with anti-p38 MAPK antibodies showed an immunodetectable protein band on SDS-PAGE in the range of 38 kDa, suggesting the possible presence of p38 MAPK in PLD immunoprecipitates (Fig. 6, A and B). Also, the hPLD1 and hPLD2 immunoprecipitates from control cells phosphorylated ATF-2, which was enhanced after DPV treatment (Fig. 6, A and B). These results further confirm an association between p38 MAPK and hPLD1/hPLD2 under basal and stimulated conditions.

Binding of p38 MAPK to PLD in vitro. To further study the interaction between p38 MAPK and PLD, we tested the binding of an active p38-GST fusion protein to PLD1 and PLD2 in vitro. Lysates from control SF9 cells or SF9 cells overexpressing hPLD1 or hPLD2 were incubated with GST beads or p38 MAPK-GST fusion protein with physiological salt concentrations. Samples were subjected to SDS-PAGE, and PLD1 and PLD2 were immunodetected by Western blotting. As shown in Fig. 7, A and B, PLD1 and PLD2 were detected bound to p38 MAPK-GST fusion protein in lysates from SF9 cells overexpressing hPLD1 or hPLD2. No binding of PLD1 to GST was detected when cell lysates were incubated with GST beads in the absence of p38 MAPK-GST fusion protein. Compared with PLD1, low binding of PLD2 to GST was detected (Fig. 7). In addition to Western blotting, the GST and p38-GST plus PLD pull-down assays were evaluated for PLD1 and PLD2 activities. As shown in Fig. 7C, incubation of SF9 cell lysates overexpressing PLD1 and PLD2 with GST beads alone exhibited activities of 20.8 and 112.0 pmol of substrate hydrolyzed in 30 min, respectively, compared with 4.7 pmol of substrate hydrolyzed by cell lysates from control SF9 cells. However, in the presence of p38-GST fusion protein, the beads in the pull-down assay expressed PLD1 and PLD2 activities of 50.4 and 310.2, respectively. These in vitro protein-protein binding assays further suggest interaction between p38 MAPK and PLD1/PLD2.

PLD1 and PLD2 are phosphorylated in vitro by p38 MAPK. The above data suggest that p38 MAPK activation is involved in DPV-induced PLD stimulation in ECs. At present, it is not clear whether PLD is a substrate for phosphorylation by p38 MAPK. To test this, we incubated p38 MAPK immunoprecipitates prepared from control and DPV-treated ECs with hPLD1 or hPLD2 immunoprecipitates from control cells that
served as substrates for phosphorylation. As shown in Fig. 8A, incubation of hPLD1 or hPLD2 immunoprecipitates with \([\gamma-^{32}P]ATP\) in the absence of exogenously added p38 MAPK resulted in slight phosphorylation of both substrates, suggesting association of p38 MAPK with PLD. Interestingly, addition of p38 MAPK immunoprecipitates obtained from DPV-treated cells (as determined by increased phosphorylation of p38 MAPK and ATF-2 phosphorylation) significantly enhanced the phosphorylation of hPLD1 and hPLD2. Maximum phosphorylation of hPLD1 or hPLD2 was observed at 5 min and declined at 15 and 30 min of DPV treatment (Fig. 8A). A commercial preparation of activated p38 MAPK also phosphorylated hPLD1 and hPLD2 obtained from Sf9-overexpressed cells, thus confirming the ability of p38 MAPK to use PLD1 or PLD2 as substrates (Fig. 8B). These data suggest that both PLD1 and PLD2 are phosphorylated by activated p38 MAPK in vitro and that p38 MAPK is constitutively associated with PLD1 and PLD2.

**SB-202190 attenuates DPV-induced in vivo phosphorylation of PLD.** To examine whether PLD is a physiological substrate of p38 MAPK, ECs prelabeled with \([3^{2}P]orthophosphate\) were incubated with 25 \(\mu\)M SB-202190 for 2 h before DPV challenge. In vivo PLD1 and PLD2 phosphorylation increased by 3.5 (Fig. 9A) and 4.8-fold (Fig. 9B), respectively, after 5 min of DPV treatment. Interestingly, some basal phosphorylation of PLD1 and PLD2 was observed in control cells without DPV stimulation. The DPV-induced PLD1 and PLD2 phosphorylation was partially attenuated by the p38 inhibitor SB-202190. These results demonstrate a
Fig. 6. Association of p38 MAPK in PLD1 and PLD2 immunoprecipitates. Bovine pulmonary artery ECs (3 × 10⁶ cells/100-mm dish) were challenged with MEM or MEM containing DPV (5 μM) for 5 min at 37°C. Cell lysates were subjected to immunoprecipitation with human (h) PLD1 (A) or hPLD2 (B). The PLD1 (A) or PLD2 (B) immunoprecipitates from control and DPV-treated cells were Western blotted for PLD1 or PLD2 or phospho-ATF-2. Western blots from 3 independent experiments were scanned and quantified by image analysis and are expressed as ratios of phospho-ATF-2/PLD1 and phospho-ATF-2/PLD2. Data shown are representative blot from 3 independent experiments.

Fig. 7. PLD1 and PLD2 interact with p38 MAPK-glutathione S-transferase (GST) fusion protein in vitro. Total cell lysates of Spodoptera frugiperda (Sf) 9 control and Sf9-overexpressing hPLD1a and hPLD2 were prepared as described in METHODS. Glutathione-Sepharose beads containing GST or p38 MAPK-GST fusion protein were mixed with cell lysates from control Sf9 or Sf9 cells overexpressing hPLD1a or hPLD2 (5 μg of cell lysate protein). The beads were incubated overnight with the cell lysates, centrifuged, washed in lysis buffer three times, and subjected to Western blotting for detection of PLD1 (A) or PLD2 (B) as described in METHODS. Blot shown is representative of 3 independent experiments. C: beads from A or B were assayed for PLD1 or PLD2 activity. Values are means ± SE from those independent experiments in triplicate.
p38 MAPK-dependent in vivo phosphorylation of PLD1 and PLD2 after stimulation by DPV but do not exclude involvement of other kinases, such as Src, receptor tyrosine kinases, or protein kinase C (PKC).

Effect of p38 phosphorylation on PLD activity in vitro. The physiological relevance of p38 MAPK-dependent phosphorylation of PLD was examined by determining PLD activity in the presence of PIP2. With the use of a commercial preparation of active p38 MAPK, phosphorylation of PLD1 from Sf9 cells overexpressing hPLD1 using \[^{32}\text{P}\]ATP and PLD1 activity at various time periods of phosphorylation was followed. As shown in Fig. 10A, p38 MAPK phosphorylated PLD1 in a time-dependent fashion, and no phosphorylation of PLD1 was observed in the absence of p38 or PLD1.

DISCUSSION

We have demonstrated for the first time the involvement of p38 MAPK in DPV-induced PLD activation in vascular ECs. This conclusion is based on the following experimental evidence: 1) the p38 MAPK inhibitor SB-202190 but not the MEK1/MEK2 inhibitor PD-98059 mitigated PLD activation by DPV without altering TPA, bradykinin, and ATP responses; 2) a rapid and early onset (2 min) of p38 MAPK activation by DPV correlated with the in vitro phosphorylation of PLD1 and PLD2 by p38 MAPK immunoprecipitation from DPV-treated cells and \[^{32}\text{P}\]ATP [10 \(\mu\text{M}\), sp. act. 200 counts·min\(^{-1}\)·cpm·pmol\(^{-1}\)] in a final volume of 100 \(\mu\text{l}\) as described in METHODS. Incubations were carried out for 30 min at 30°C, reactions were terminated by addition of 6× Laemmli buffer, and substrate phosphorylation was detected by autoradiography after SDS-PAGE on 7% gels. B: commercial preparation of activated p38 MAPK (250 ng) was incubated with hPLD1a or hPLD2 immunoprecipitates from Sf9 cells and \[^{32}\text{P}\]ATP (10 \(\mu\text{M}\), sp. act. 200 cpm/pmol) for 30 min at 30°C as described above. Phosphorylation of PLD1 and PLD2 by p38 MAPK was detected by autoradiography after SDS-PAGE on 7% gels. Shown is a representative autoradiogram from 3 independent experiments.
TPA-induced PLD activation; 5) in vitro binding of PLD1 and PLD2 to p38 MAPK was demonstrated using p38-GST fusion protein; 6) DPV enhanced phosphorylation of PLD1 and PLD2 in vivo, which was attenuated by SB-202190; and 7) in vitro phosphorylation of PLD1 by p38 MAPK had no effect on the PLD activity.

In a variety of mammalian cells and tissues, PLD is activated by hormones, growth factors, neurotransmitters, cytokines, and reactive oxygen species (ROS; see Refs. 9, 12, 34). Depending upon the stimulus, activation of PLD is dependent on PKC, changes in intracellular calcium, heterotrimeric G proteins, modulation of protein tyrosine kinases/phosphatases, and low molecular weight G proteins of the Rho family of GTPases (9, 12). Earlier studies in ECs have shown that ROS- and DPV-induced PLD stimulation was not attenuated by PKC inhibitors or downregulation of PKC by prolonged

![Image](http://ajplung.physiology.org/)

Fig. 9. SB-202190 attenuates DPV-induced phosphorylation of PLD1 (A) and PLD2 (B) in vivo. Bovine pulmonary artery ECs (3 x 10^6 cells/100-mm dish) were labeled with [32P]orthophosphate (25 Ci/ml) in DMEM-phosphate-free medium containing 2% FBS for 4 h at 37°C. Cells were rinsed in MEM and treated with SB-202190 (25 μM) for 2 h before challenge with 5 μM DPV for 5 min. Lysates (1 mg/ml) prepared in lysis buffer as described in METHODS were subjected to immunoprecipitation with PLD1 and PLD2 antibodies, SDS-PAGE, and autoradiography and were quantified by densitometric scanning and image analysis. Data shown are representative of 2 independent experiments. Phosphorylation of PLD1 and PLD2 after DPV challenge in the absence or presence of SB-202190 is expressed as the degree of increase normalized to basal phosphorylation from 2 independent experiments.

![Image](http://ajplung.physiology.org/)

Fig. 10. Effect of p38 phosphorylation on PLD activity in vitro. PLD1 immunoprecipitates from Sf9 cells overexpressing hPLD1 were prepared as described in METHODS. PLD1 (5 μl) immunoprecipitates obtained from 1 mg of total Sf9 cell lysate protein was reconstituted in 50 μl of 50 mM HEPES buffer (pH 7.4) and incubated in a buffer containing 100 μM ATP, 8 μCi of [γ-32P]ATP, and active p38-MAP kinase (250 ng) in a final volume of 50 μl for various time periods. At the end of the incubation, one set of samples was treated with Laemmli buffer, boiled for 5 min, and subjected to SDS-PAGE on 10% polyacrylamide gels and autoradiography (A), and another set was assayed for PLD activity (B) at two different PIP2 concentrations (5 and 7 μM). Values are means ± SE of 3 independent experiments in triplicate.
exposure to TPA (40–42), whereas thrombin-, bradykinin-, or ATP-mediated PLD stimulation was highly dependent on PKC activation (13, 34). However, the ROS- or DPV-induced PLD stimulation in ECs was partially blocked by the Src kinase inhibitor PP-1, indicating a role for protein tyrosine phosphorylation in regulating PLD activation either directly or indirectly (9, 12). Although PLD is phosphorylated at serine/threonine residues in intact cells in response to TPA (24) or at tyrosine residues by pervanadate (28, 47), it is still unclear whether this phosphorylation of PLD in response to a stimulus is of physiological relevance to enhanced hydrolysis of phosphatidylcholine or other phospholipid substrates.

p38 MAPK belongs to subfamilies of mammalian MAPKs that also include ERKs, JNK/stress-activated protein kinases (SAPKs), and ERK5. p38 MAPKs are generally activated via phosphorylation of both threonine and tyrosine residues by distinct and dual specific serine/threonine MAPK kinases (MKK3, MKK4, and MKK6), which in turn are phosphorylated and activated by upstream MKK kinases (43). In bovine pulmonary artery ECs, DPV activated all three MAPK families, as determined by enhanced phosphorylation and activity against exogenous substrates. It was apparent that there were some differences between activity measurements and Western blots with anti-phosphotyrosine antibodies. Although MBP activity or ATF-2 phosphorylation reflects enhanced phosphorylation at threonine/tyrosine residues, the Western blots with anti-phosphotyrosine antibody represent increased phosphorylation at tyrosine residues of ERK or p38 MAPK. Also, dephosphorylation of threonine/tyrosine residues by specific phosphatases may explain the apparent differences between Western blots and activity measurements. These data demonstrate that DPV enhances the phosphorylation and activity of MAPKs in ECs.

In contrast to DPV, treatment with TPA resulted only in the activation of ERK1 and ERK2. Our studies with PD-98059 showed that this MEK1/MEK2 inhibitor specifically attenuated TPA- or DPV-induced ERK phosphorylation but had no effect on TPA- or DPV-induced PLD activation. Similarly, SB-202190, which inhibits by binding to phosphorylated p38 MAPK (and not upstream of MKK3, MKK4, or MKK6), specifically blocked DPV-induced phosphorylation of ATF-2 (54). Furthermore, a 40% reduction in DPV-induced PLD stimulation was observed after transient transfection of ECs with a dominant negative mutant of p38 MAPK, confirming a role for p38 MAPK in the regulation of PLD activation. Under similar experimental conditions of transfection, the expressed p38 MAPK mutant protein exhibited ~10% phosphorylation of ATF-2 in vitro compared with vector controls. This suggests that in addition to p38 MAPK, other pathways involving Src or receptor tyrosine kinases may be involved in DPV-induced PLD activation in ECs.

Although ERK activation regulates PLD activity in PC-12 cells (22), neutrophils (6), and smooth muscle cells (33), our results do not suggest the participation of ERK1/2 in DPV-induced PLD activation. In rat pheochromocytoma PC-12 cells, the H$_2$O$_2$-induced PLD activation and MAPK phosphorylation were attenuated dose dependently by PD-98059 (22). Similarly, PD-98059 blocked formyl-Met-Leu-Phe-mediated activation of ERK and PLD, with a concomitant reduction in respiratory burst in human neutrophils (6). Recent studies in rabbit aortic smooth muscle cells demonstrated that norepinephrine-mediated PLD activation was attenuated by farnesyltransferase inhibitors and by PD-98059, suggesting the Ras/MAPK pathway in regulating PLD activity via a phosphorylation-dependent mechanism (33). In contrast to ERK-dependent activation of PLD in rabbit smooth muscle cells, data reported here confirm the earlier studies showing that vasopressin-induced PLD activation was not dependent on ERK in A7r5 rat vascular smooth muscle cells (23). Thus, depending on the cell type and stimulus, activation of ERK or p38 MAPK regulates PLD either directly or indirectly. Although JNK is also activated by DPV, the present study did not address the role of JNK in DPV-induced PLD stimulation.

We have performed preliminary studies to identify possible mechanism(s) of PLD regulation by p38 MAPK. Interestingly, our data show a physical association between PLD1/PLD2 and p38 MAPK in control and DPV-treated ECs. Furthermore, both PLD1 and PLD2 were phosphorylated in vitro by p38 MAPK immunoprecipitates from control and DPV-treated cells and a commercial preparation of activated p38 MAPK, suggesting PLD as a substrate for the kinase. Also, it was observed that DPV treatment of ECs enhanced in vivo phosphorylation of PLD1 and PLD2, which was partially attenuated by the p38 MAPK inhibitor SB-202190. Earlier studies have identified several other downstream substrates of p38 MAPK. These include ATF-1, ATF-2, cAMP response element binding protein, MAPK activated protein kinase 2/3, myocyte enhancer factor 2C, CCAAT/enhancer binding protein, p38-regulated/activated protein kinase, p53 tumor suppressor protein, and cytosolic phospholipase A$_2$ (cPLA$_2$; see Ref. 43). Recently, it has been demonstrated that cPLA$_2$ is a physiological target of p38 MAPK in thrombin-stimulated platelets. However, the p38 MAPK-dependent phosphorylation of cPLA$_2$ appears not to be involved in thrombin-stimulated release of arachidonic acid (26). We have shown that both PLD1 and PLD2 are substrates for p38 MAPK-mediated phosphorylation in vivo and in vitro in ECs; however, in vitro phosphorylation of PLD1 did not affect the PLD1 activity measured in the presence of PIP$_2$ and Arf. Further studies are necessary to understand the physiological role of PLD phosphorylation relative to secretion, interaction with other proteins, or translocation in response to external stimuli. DPV treatment increased tyrosine phosphorylation of PLD1 in HL-60 granulocytes (28) and ECs (47).

Although a variety of agonists, including TPA, activate PLD via a PKC-dependent pathway, the mechanism of PLD stimulation by PKC is still controversial. Inhibitors of PKC that block its catalytic activity can also alter agonist-induced PLD activation, suggesting...
a phosphorylation-dependent regulation (27). However, in vitro studies examining the mechanism of PKC-dependent activation of PLD have been varied. PKC-α and -β isoenzymes in a phorbol ester-dependent manner enhance PLD activity in membrane preparations, partially purified PLD, and baculovirus-expressed PLD isoforms (9). This activation occurs in the absence of ATP, indicating a non-phosphorylation-dependent mechanism of PLD stimulation such as protein-specific interaction. In additional studies, it has been shown that PLD1 is associated with PKC-α immunoprecipitates after TPA challenge of fibroblasts (29), and it was suggested that possible protein-protein interaction between PKC and PLD1 may be involved in the activation (12). In Rat1 fibroblasts, communoprecipitates of PKC-α and recombinant PLD1 were associated with an unidentified 220-kDa protein in response to TPA (12), which may represent a scaffolding protein (9, 29). However, other studies point out that PKC-mediated phosphorylation of PLD1 in vitro results in an inhibition of PLD activity (12). In the present study, DPV treatment of ECs for 30 min compared with 5 min resulted in an inhibition of PLD1 activity measured in the p38 MAPK immunoprecipitates (Fig. 5B). At this time, it is not known if this phosphorylation also occurs in vivo or if this phosphorylation is important for activation of the enzyme. Only recently, direct evidence for in vivo phosphorylation of PLD1 by PKC after TPA stimulation in 3Y1 cells and in COS-7 cells transiently expressing PLD1 was reported (24). Inhibitors of PKC or downregulation of PKC by phorbol esters attenuated TPA-induced phosphorylation and activation of PLD1. In vitro, in the presence of TPA, purified PLD1 was phosphorylated by PKC-α at serine-2, threonine-147, and serine-561 residues. Mutation of serine-2, threonine-147, or serine-157 reduced TPA-induced PLD1 activity in vitro, suggesting involvement of phosphorylation of PLD1 in regulation of its activation (24).

The physiological significance of p38 MAPK-mediated phosphorylation of PLD1 or PLD2 is unclear. Although in vitro phosphorylation of PLD1 and PLD2 by a commercial preparation of p38 MAPK had no effect on the catalytic activity, further studies on the role of in vivo phosphorylation of PLD are essential to determine a link between phosphorylation and activation of the enzyme. Because PLD is known to regulate protein trafficking and secretion, it is possible that serine/threonine and/or tyrosine phosphorylation of PLD1 or PLD2 may be critical for its interaction with other Src homology (SH) 2- or SH3-containing proteins and not for activity. Further studies on mapping the p38 MAPK-dependent phosphorylation sites combined with mutation of specific amino acids involved in phosphorylation should provide information to further define the physiological relevance between phosphorylation and function.

In conclusion, our data indicate that p38 MAPK is involved in DPV-induced PLD activation in bovine pulmonary artery ECs. In ECs, both PLD1 and PLD2 were constitutively associated with p38 MAPK, and a dominant negative mutant of p38 MAPK partially mitigated DPV-mediated PLD activation without affecting the TPA response. DPV treatment of ECs enhanced phosphorylation of PLD1 and PLD2 in vivo, which was partially attenuated by SB-202190. However, in vitro phosphorylation of PLD1 by p38 MAPK did not enhance PLD1 activity, suggesting that either phosphorylation may not be directly involved in the activation of the enzyme or that other factors in addition to p38 MAPK-mediated phosphorylation are required for the enhanced enzymatic activity. Further studies are in progress to characterize the binding sites between p38 MAPK and PLD, sites of phosphorylation by p38 MAPK, and a possible physiological role of phosphorylation of PLD activation and generation of PA in EC function.

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