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Activation of epidermal growth factor receptor is responsible for pervanadate-induced phospholipase D activation

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Abbreviations: EGFR, epidermal growth factor receptor; ERK, extracellular signal-regulated kinase; JNK, c-Jun NH_2-terminal kinase; MAPK, mitogen-activated protein kinase; PLD, phospholipase D; PtdBut, phosphatidylbutanol; ROS, reactive oxygen species

Abstract

Pervanadate, a complex of vanadate and H_2O_2, has an insulin mimetic effect, and acts as an inhibitor of protein tyrosine phosphatase. Pervanadate-induced phospholipase D (PLD) activation is known to be dependent on the tyrosine phosphorylation of cellular proteins and protein kinase C (PKC) activation, and yet underlying molecular mechanisms are not clearly understood. Here, we investigated the signaling pathway of pervanadate-induced PLD activation in Rat2 fibroblasts. Pervanadate increased PLD activity in dose- and time-dependent manner. Protein tyrosine kinase inhibitor, genistein, blocked PLD activation. Interestingly, AG-1478, a specific inhibitor of the tyrosine kinase activity of epidermal growth factor receptor (EGFR) blocked not only the PLD activation completely but also phosphorylation of p38 mitogen-activated protein kinase (MAPK). However, AG-1295, an inhibitor specific for the tyrosine kinase activity of pletlet drived growth factor receptor (PDGFR) did not show any effect on the PLD activation by pervanadate. We further found that pervanadate increased phosphorylation levels of p38, extracellular signal-regulated kinase (ERK) and c-Jun NH_2-terminal kinase (JNK). SB203580, a p38 MAPK inhibitor, blocked the PLD activation completely. However, the inhibitions of ERK by the treatment of PD98059 or of JNK by the overexpression of JNK interacting peptide JBD did not show any effect on pervanadate-induced PLD activation. Inhibition or down-regulation of PKC did not alter the pervanadate-induced PLD activation in Rat2 cells. Thus, these results suggest that pervanadate-induced PLD activation is coupled to the transactivation of EGFR by pervanadate resulting in the activation of p38 MAP kinase.

Keywords: mitogen-activated protein kinases; phospholipase D; proto-oncogene protein pp60 (c-src); receptor, epidermal growth factor; vanadates

Introduction

Hydrolysis of membrane phosphatidylcholine by phospholipase D (PLD) has been recognized as a mechanism of signal transduction in mammalian cells (Exton, 1997; Liscovitch *et al.*, 2000). PLD can be activated by stimulation of G-protein coupled receptors, receptor tyrosine kinases, cytokines and reactive oxygen species (ROS). PLD activity is believed to be involved in vesicle transport, proliferation and death (Kim *et al.*, 2003).

Reactive oxygen species such as hydrogen peroxide (H_2O_2) and superoxide is recognized as a second messenger (Bae *et al.*, 1997). It was reported that the level of cellular protein tyrosine phosphorylations increases by oxidative stress by H_2O_2 and is strongly potentiated by addition of vanadate (Heffetz *et al.*, 1990). The underlying molecular mechanism involves the inhibitions of protein-tyrosine phosphatase (Heffetz *et al.*, 1990) or increased tyrosine kinase activity (Koshio *et al.*, 1988) or both. Pervanadate promotes insulin receptor tyrosine phosphorylation by inhibiting phosphotyrosine phosphatase associated with the insulin receptor (Swarup *et al.*, 1982). Furthermore, pervanadate activates signal transducer and activator of transcription (STAT) proteins (Ruff *et* al., 1997), extracellular signal-regulated kinase (ERK) (Gudmundsdottir *et al.*, 2001; Natarajan *et al.*, 2001), p38 (Gudmundsdottir *et al.*, 2001; Natarajan *et al.*, 2001), c-Jun NH_2-terminal kinase (JNK) (Gudmundsdottir *et al.*, 2001; Natarajan *et al.*, 2001), and phos-

pholipase D (Natarajan *et al.*, 2001).

Upon binding of specific ligand, EGFR undergoes dimerization and activation of its intrinsic tyrosine kinase activity. These events lead to the autophosphorylation of multiple tyrosine residues in the COOH-terminal tail of the molecule that serve as binding sites for cytosolic signaling proteins containing Src homology 2 (SH2) domains and phosphotyrosine binding domains (Hackel *et al.*, 1999). EGFR also participates in the signaling networks transactivated by stimuli that do not directly interact with this receptor. This transactivation occurs by agonists that specifically bind to other membrane receptors, membrane depolarization agents, and environmental stressors (Carpenter *et al.*, 1999). Cellular Src functions as a co-transducer of transmembrane signals emanating from a variety of growth factor receptors, including EGFR (Luttrell *et al.*, 1988). Evidence indicates that EGFR and the non-receptor tyrosine kinase Src cooperate in both mitogenesis and transformation (Luttrell *et al.*, 1988).

Pervanadate stimulates PLD activity by G protein-independent (Bourgoin and Grinstein, 1992), or G protein-dependent (Dubyak *et al.*, 1993) mechanisms or p38 MAPK dependent manner (Natarajan *et al.*, 2001; Min *et al.*, 2002). In response to stimulation by pervanadate, PLD1 was tyrosine-phosphorylated and associated with several undefined, tyrosine-phosphorylated proteins in HL-60 cell (Marcil *et al.*, 1997). p38 MAPK and Src are associated with PLD and involved in pervanadate-induced PLD activation in endothelial cells (Natarajan *et al.*, 2001; Parinandi *et al.*, 2001). However, the upstream event mediating p38-dependent PLD activation by pervanadate was not determined. Here, we demonstrate for the first time that the transactivation of EGFR is involved in pervanadate-induced PLD activation in Rat2 cells through p38 MAPK activation.

Materials and Methods

Materials

FBS, Hepes-buffered DMEM with L-glutamine, penicillin and streptomycin were purchased from Life Technologies (Rockville, MD). Sodium orthovanadate, hydrogen peroxide and PMA were from Sigma (St. Louis, MO). Pervanadate was prepared by mixing 1 ml of 100 mM H_2O_2 with 1 ml of 100 mM Na_3VO_4. Anti-PLD antibody was a generous gift from Dr. Ryu, S.H. (Pohang University, Korea). Antibodies against phospho-ERK, phospho-JNK and phospho-p38 were from Cell Signaling (Beverly, MA). [9,10-^{3}H]Myristic acid (10 Ci/mmol) was purchased from DuPont NEN. Ro31-8220 was from Roche. SB203580, PD98059, AG1295, PP2, AG1478 and GF109203X were from CalBiochem (La Jolla, CA). Nitrocellulose filter and ECL detection kit were from Amersham. Reagents for protein assay were from BioRad (Hercules, CA). Silica gel 60 plate was from Whatman. Phosphatidylbutanol (PtdBut) was a generous gift from Dr. M. U. Choi (Seoul National University, Korea).

Cell culture

Rat-2 fibroblast cells were cultured in Hepes-buffered DMEM supplemented with 4 mM L-glutamine, 10% (v/v) FBS, 100 units/ml of penicillin, and 100 μg/ml of streptomycin at 37°C in a humidified, CO_2-controlled (5%) incubator. For all experiments, cells were grown to 70-80% of confluency.

Cell stimulation and PLD assay

PLD activity was determined by measuring the formation of phosphatidylbutanol (PtdBut), a reaction product of PLD in the presence of n-butanol. Rat-2 cells (2×10^5 cells per 35 mm plate) were cultured in high glucose DMEM overnight at 37°C in humidified 5% CO_2 incubator. Cells were starved in DMEM for 9 h, then labeled with [^{3}H]myristic acid (1 μCi/ml) for 3 h. Unincorporated [^{3}H]myristic acid was removed by washing with PBS, and cells were incubated in 3 ml of PBS for 1 h. Butanol was then added to a final concentration of 0.3% (vol/vol). After 10 min, cells were treated with genistein, PP2, AG1478, AG1295, PD98059, or SB203580, and stimulated with pervanadate or PMA. Cells were then incubated for time period as indicated in Figures. Radioactivities incorporated into total phospholipids and PtdBut were measured. The data were presented as a PtdBut per total count.

Immunoblot analysis

Cells were lysed with 20 mM Hepes (pH 7.2) containing 1% Triton X-100, 1% sodium deoxycholate, 10% glycerol, 150 mM NaCl, 50 mM NaF, 1 mM Na_3VO_4, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 1 mM PMSF. Cell lysates were boiled for 5 min in SDS sample buffer and subjected to 10% SDS-polyacrylamide gel electrophoresis. Proteins were transferred to a nitrocellulose membrane, and blots were incubated for 30 min with 20 mM Tris (pH 7.6), 150 mM NaCl, and 0.1% (v/v) Tween-20 containing 5% (w/v) nonfat dried milk. The membrane was incubated with antibodies recognizing ERK, p38, phosphorylated ERK or phosphorylated p38. Blots were washed in 20 mM Tris (pH 7.6), 150 mM NaCl, and 0.1% (v/v) Tween-20. Depending on the origin of the primary antibodies, anti-mouse or anti-rabbit IgG antibodies coupled with horseradish peroxidase were used for detection of corresponding proteins using enhanced chemiluminescence reagent.

JNK assay

Cells were washed with cold PBS and lysed in kinase lysis buffer (20 mM Tris-HCl, pH 7.4, 25 mM β-glycerophosphate, 2 mM sodium pyrophosphate, 1 mM Na_3VO_4, 2 mM EDTA, 0.5 mM dithiothreitol, 50 μg/ml leupeptin, 50 μg/ml aprotinin and 1 mM PMSF). The 200 μg protein of cell extract and 10 μg GST-c-Jun protein were mixed and incubated in kinase reaction buffer (25 mM Hepes, pH 7.4, 1 nM ATP, 25 mM β-glycerophosphate, 25 mM $MgCl_2$, 0.1 mM Na_3VO_4, 0.5 mM dithiothreitol, I mM PMSF containing 10 μCi [γ-^{32}P]ATP) for 30 min at 30°C. Following incubation, the reaction was stopped by addition of 4× Laemmli's sample buffer followed by boiling. The samples were resolved by electrophoresis, and phosphorylation levels of GST-c-Jun were determined by autoradiography.

Results

Pervanadate-induced PLD activation in Rat-2 cell is dependent not on PKC but protein tyrosine phosphorylation

Treatment of Rat2 cells with 1 mM pervanadate activated PLD in a time-dependent manner (Figure 1A) and PLD activity was increased in a dose-dependant manner reaching maximal activation at 1 mM pervanadate (Figure 1B). Rat2 cells did not show any effect up to 5 mM H_2O_2 (data not shown). Since pervanadate greatly induced tyrosine phosphorylation of cellular proteins (Figure 2A), the involvement of protein tyrosine kinase was examined in a pervanadate-mediated PLD activation. Pretreatment of cells with genistein, a general protein tyrosine kinase inhibitor, and an antioxidant NAC blocked pervanadate-induced PLD activation in a concentration dependent manner (Figure 2B, C). Possible involvement of PKC activity was also checked in pervanadate-induced PLD activation. PKC inhibitors GF-109203X and Ro-31-8220 induced slight increase in pervanadate-induced PLD activation (Figure 3) while blocking PMA-induced PLD activation completely (data not shown). These results are in contrast to an earlier report of Min *et al.* (2002). Moreover, down-regulation of PKC by prolonged treatment of PMA did not inhibit the pervanadate-induced PLD activation. These results demonstrate that protein tyrosine kinase, but not PKC is involved in pervanadate-induced PLD activation in Rat2 cells.

Pervanadate-induced PLD activation involves EGFR and Src tyrosine kinase activity

Cellular stresses such as H_2O_2, UV and osmotic shock can activate growth factor receptors to maintain a homeostatic cellular environment and consequential cellular signaling (Carpenter, 1999). Possible involvement of EGFR was explored in pervanadate-induced PLD activation. Pretreatment of Rat2 cells with EGFR tyrosine kinase inhibitor, AG-1478 showed attenuation of PLD activation by pervanadate in a dose-dependent manner (Figure 4A). However, AG-1295 a specific PDGF inhibitor did not show any effect on the PLD activation.

In addition, an antioxidant NAC blocked the tyrosine phosphorylation of EGFR (Figure 2D), but H_2O_2 did not (data not shown). Src is known to transactivate EGFR, and also involved in PLD activation. Pretreatment of cells with PP-2, a specific Src inhibitor resulted in complete inhibition of pervanadate-induced PLD activation (Figure 4B). These data suggest that pervanadate-induced stimulation of PLD activity was likely mediated through the transactivation of EGFR and Src activation.

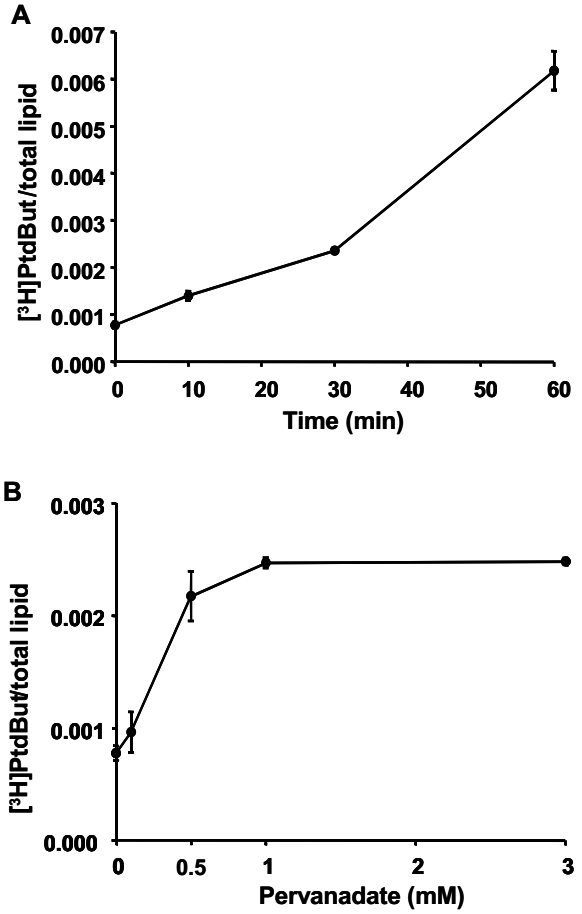

Figure 1. Time- and dose-dependence of pervanadate-stimulated PLD activation in Rat-2 cells. Serum-starved Rat2 cells were stimulated with 1 mM pervanadate for the indicated times (A) or with various concentrations of pervanadate for 30 min (B). The radioactivity incorporated into PtdBut was measured. Data represent means±SEM of three separate experiments.

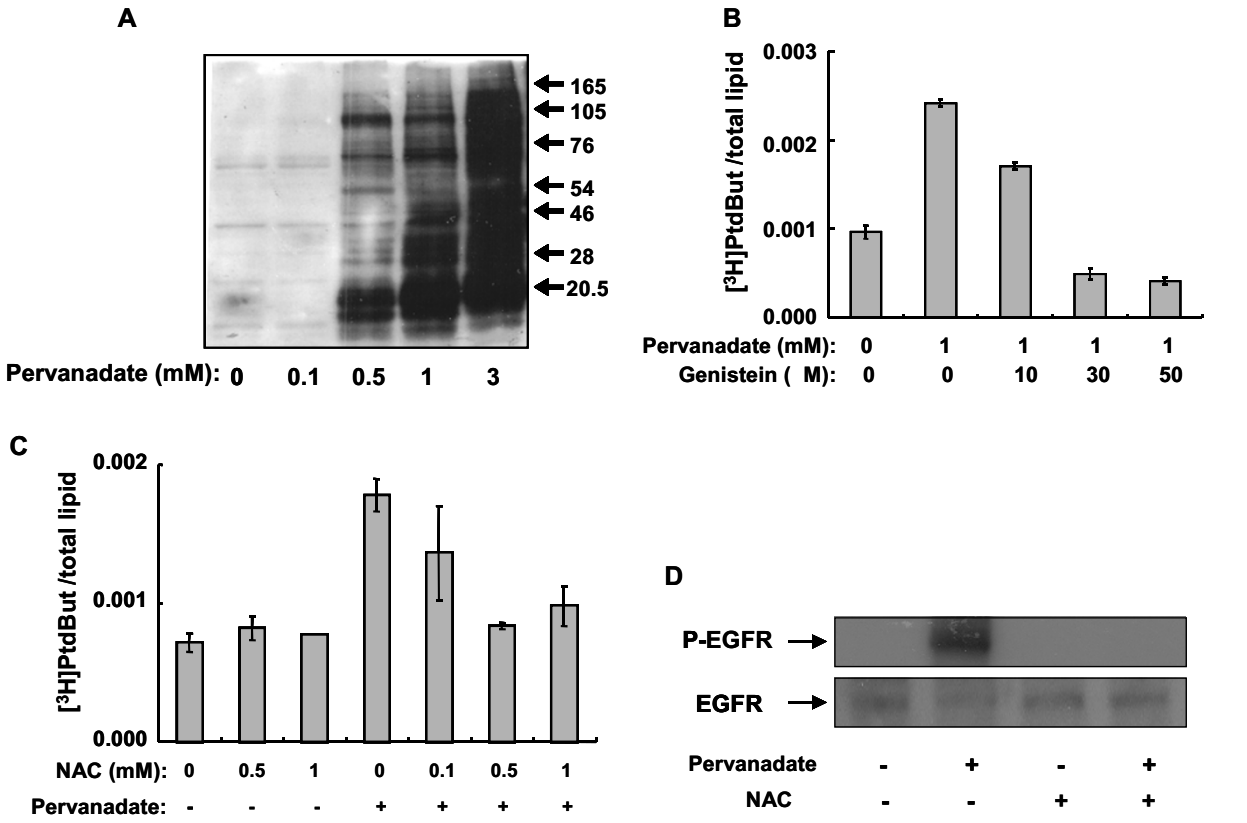

Figure 2. Effects on pervanadate-stimulated PLD activations by tyrosine kinase inhibitor and antioxidant. Serum-starved Rat-2 cells were treated with various concentrations of pervanadate. Protein tyrosine phosphorylations of cellular proteins were probed with anti-phosphotyrosine antibody (4G10) (A). Also, inhibitions of pervanadate-induced PLD activation by genistein (B) or antioxidant *N*-acetylcysteine (C) were investigated in the presence or the absence of One millimolar pervanadate for 30 min. D: The phosphorylation of EGFR was assayed by Western blotting with anti- phospho-EGFR (Y1173) antibody. One millimolar *N*-acetylcysteine was pretreated to Rat2 cells for 30 min, and then 1 mM pervanadate was treated for 30 min. The radioactivity incorporated into PtdBut was measured. Data represent means±SEM of three independent experiments.

Pervanadate stimulates PLD activity through p38 activation

Pervanadate is known to induce activations of PLD as well as MAPK. Possible singal cascade between PLD activation with MAPK was examined by using specific p38 MAPK inhibitor, SB-203580. As shown in Figure 5, pervanadate activated ERK1/2, p38, and JNK MAPK. The profiles of the p38 MAPK and PLD activations by pervanadate were very similar (compare Figure 5 with Figure 1B). Moreover, SB-203580 specifically inhibited pervanadate-induced PLD activation in a concentration dependent manner (Figure 6A). Overexpression of dominant negative p38 MAPK inhibited pervanadate-induced PLD activation (Figure 6B). Finally, blockage of EGFR kinase activity by AG-1478 attenuated p38 phosphorylation (Figure 6C) and PLD activation (Figure 4A).

However, Rat2 cells pretreated with PD98059, a specific ERK inhibitor did not affect the activation of PLD activity induced by pervanadate (data not shown). Moreover, 0.1 mM concentration of pervanadate that

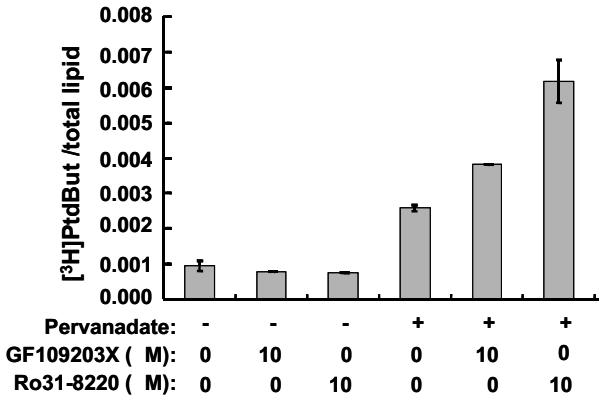

Figure 3. Effects of pervanadate on PLD activations by PKC inhibitors, GF 109203X and Ro 31-8220. Serum-starved Rat-2 cells were pretreated with various concentrations of GF 109203X and Ro31-8220, then PLD activities were assayed by the treatment of 1 mM pervanadate for 30 min. The radioactivity incorporated into PtdBut was measured. Data represent the means±SEM of three separate experiments.

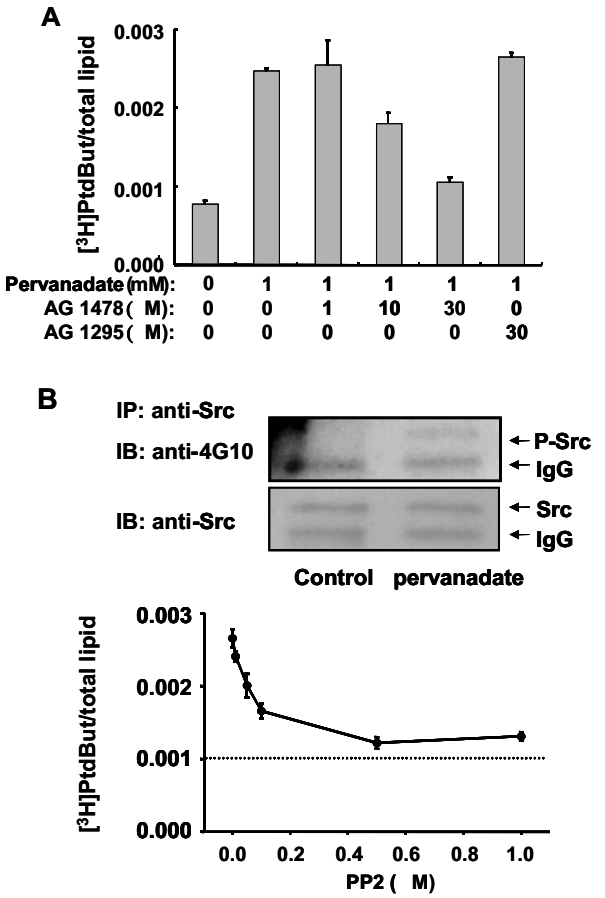

Figure 4. Regulation of pervanadate-induced PLD activity by EGFR tyrosine kinase and Src tyrosine kinase. Serum-starved Rat2 cells were treated with various concentration of AG 1478, AG 1295 (A) and PP-2 (B) for 30 min, then stimulated with 1 mM pervanadate for 30 min. The radioactivity incorporated into PtdBut was measured. Data represent the means±SEM of three separate experiments.

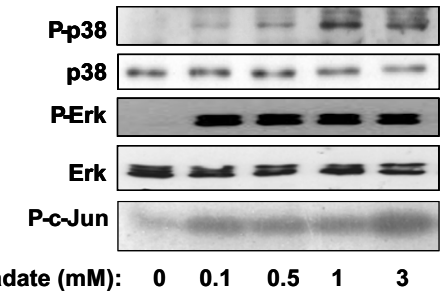

Figure 5. Pervanadate-induced PLD activation by MAPKs. Serum-starved Rat2 cells were treated with various concentrations of pervanadate for 30 min. Phosphorylations of ERK and p38 MAPKs were determined by Western blotting with their specific antibodies. JNK activation was assessed by phosphorylation of GST-c-Jun protein. The autoradiograms shown here are the representative results of three independent experiments.

had no affect in PLD activation, caused maximal stimulation of ERK1/2 phosphorylations (Figure 5 and 1B), suggesting that ERK is not involved in pervanadate-induced PLD activation in Rat2 cells.

Although JNK is strongly activated by pervanadate (Figure 5), pervanadate concentrations required for maximal stimulation of the PLD activity differ from those for the JNK activation. Also overexpression of JBD, a negative regulator of JNK, did not affect the PLD activation (data not shown).

These results suggest that in Rat2 cells, stimulation of p38, but not JNK or ERK kinases precedes PLD activation in the signaling pathway of EGFR transactivation by pervanadate.

Discussion

The object of the present study was to examine possible signaling pathway in pervanadate-mediated PLD activation. We demonstrated that pervanadate-induced PLD activation in Rat2 cells is mediated by EGFR transactivation including Src and p38 activation, not by PKC.

H_2O_2 stimulates PLD activity in a PKC-dependent manner in leukemic L1210 cell, Swiss 3T3 fibroblast cell, pheochromocytoma PC12 cell and brain slice (Min *et al.*, 1998; Lee *et al.*, 2000; Oh *et al.*, 2000; Servitja *et al.*, 2000), and in a PKC-independent manner in bovine pulmonary artery endothelial cells (Natarajan *et al.*, 1993). Pervanadate-induced PLD activation in HL-60 cells is mainly dependent on tyrosine phosphorylation (Bourgoin and Grinstein, 1992). In this study, we found that pervanadate-induced PLD activation in Rat-2 cells was completely dependent on tyrosine phosphorylation pathway. Pervanadate-induced PLD activation was inhibited by the pretreatment of antioxidant, NAC (Figure 2C), and H_2O_2 concentration required for PLD activation was higher than pervanadate. Although H_2O_2 and pervanadate are oxidizing agents and inhibit phosphotyrosine phosphatase, they have different effects on cells. In this study, H_2O_2 did not stimulate PLD activity and tyrosine phosphorylation of EGFR (data not shown) while pervanadate stimulated them. These results demonstrate that H_2O_2 and pervanadate have different effects on cells although they enhance tyrosine phosphorylations of proteins. Previous report showing that the activation mechanism of NF-κB activation by H_2O_2 is different from the activation mechanism by pervanadate (Krejsa *et al.*, 1997) supports our data.

There are reports that blocking of p38 MAPK attenuates oxidant-stimulated PLD activation in endothelial cells by 30% (Natarajan *et al.*, 2001) and PC-12 cells by 50% (Banno *et al.*, 2001). ERK is also involved in PLD activation in PC-12 cells (Ito *et al.*, 1997), neutrophils (Djerdjouri *et al.*, 1999) and smooth

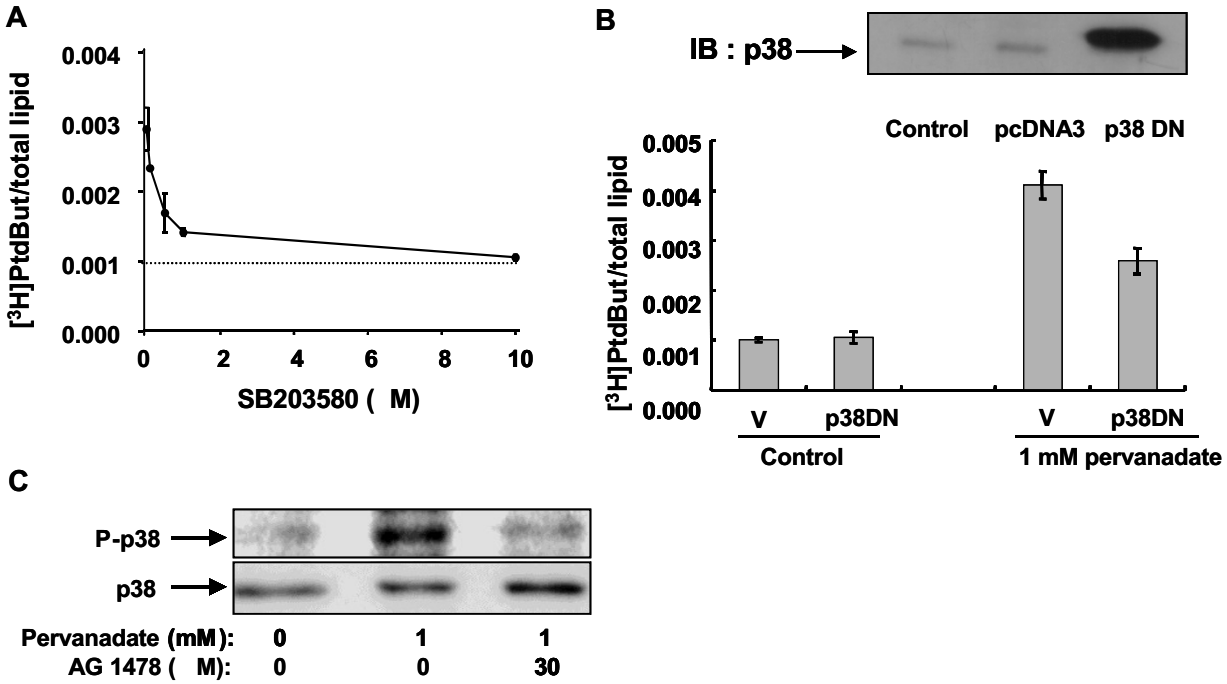

Figure 6. Involvement of the p38 MAP kinase activation in pervanadate-induced PLD activation. A, Serum starved Rat-2 cells were treated with various concentration of SB-203580 for 30 min, then stimulated with 1 mM pervanadate for 30 min. B, Dominant negative mutant of p38 MAP kinase was expressed in Rat 2 cells by gene transfection, then pervanadate-induced PLD activations were measured. C, The phosphorylation of p38 MAP kinase was assayed by Western blotting with anti-phospho-p38 antibody and with anti-p38 antibody. AG 1478 was pretreated to Rat2 cells for 30 min, and then 1 mM pervanadate was treated for 30 min. The radioactivity incorporated into PtdBut was measured. Data represent the means±SEM of three independent experiments.

muscle cells (Muthalif *et al.*, 2000). Blocking of both p38 and ERK inhibited PLD activation almost completely in PC-12 cells (Banno *et al.*, 2001). We observed that pervanadate stimulated p38 and ERK strongly (Figure 5). However, p38 inhibition by SB-203580 almost completely removed the pervanadate-induced PLD activation in Rat-2 cells (Figure 6A), whereas blocking of ERK or JNK by the treatment of PD98059 or expression of JBD did not. Our finding is consistent with the previous notion that activations of ERK and PLD are concurrent but independent responses to vasopressin in A7r5 cells (Jones *et al.*, 1994). AG-1478, the specific inhibitor for EGFR kinase activity inhibited both pervanadate-stimulated p38 and PLD activation (Figure 6C and 4A). These results suggest that the transactivation of EGFR by pervanadate is coupled to PLD activation through p38 MAPK pathway.

Nonreceptor tyrosine kinase Src is implicated in nonclassical use of EGFR and stress mediated signaling (Carpenter, 1999) and also involved in PLD activation (Jiang *et al.*, 1995). It was recently reported that Src is associated with pervanadate-induced PLD activation in endothelial cells (Parinandi *et al.*, 2001). Our data demonstrating that the specific Src inhibitor PP-2 inhibited the pervanadate-induced PLD activation (Figure 4B) are consistent with the observation in endothelial cells (Parinandi *et al.*, 2001). Because Src is implicated in stress-activated transactivation of EGFR (Carpenter, 1999) and pervanadate-mediated PLD activation (Figure 4B), Src may be involved in pervanadate-induced PLD activation.

Acknowledgement

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