Vitamin C-induced activation of phospholipase D in lung microvascular endothelial cells: Regulation by MAP kinases

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Abstract

Our earlier studies have shown that vitamin C at pharmacological doses (mM) induces loss of redox-dependent viability in bovine lung microvascular endothelial cells (BLMVECs) that is mediated by oxidative stress. Therefore, here, we investigated the vitamin C-induced activation of the lipid signaling enzyme, phospholipase D (PLD) in BLMVECs. Monolayer cultures of BLMVECs were treated with vitamin C (0–10 mM) for different time periods (0–2 h) and the activity of PLD was determined. Vitamin C induced activation of PLD in BLMVECs in a time- and dose-dependent fashion that was significantly attenuated by antioxidants, p38 mitogen-activated protein kinase (p38 MAPK)-specific inhibitor (SB203580), extracellular signal-regulated protein kinase (ERK)-specific inhibitor (PD98059), and transient transfection of cells with dominant-negative (DN)-p38 MAPK and DN-ERK1/ERK2. Vitamin C also induced phosphorylation and enhanced the activities of p38 MAPK and ERK in BLMVECs in a time-dependent fashion. It was also evident that vitamin C induced translocation of PLD1 and PLD2, association of p38 MAPK and ERK with PLD1 and PLD2, threonine phosphorylation of PLD1 and PLD2 and SB203580- and PD98059-inhibitable threonine phosphorylation of PLD1 in BLMVECs. Transient transfection of BLMVECs with DN-p38 MAPK and DN-ERK1/ERK2 resulted in marked attenuation of vitamin C-induced phosphorylation of threonine in PLD1 and PLD2. We, for the first time, showed that vitamin C at pharmacological doses, activated PLD in the lung microvascular ECs through oxidative stress and MAPK activation.
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Keywords: Ascorbic acid; Endothelial cell; Phospholipase D; MAPK signaling; Oxidative stress

1. Introduction

Vitamin C (ascorbic acid), an essential water-soluble vitamin well known for its antiscorbutic and antioxidant functions in humans, has been recognized as an important therapeutic agent for several pathological states, including cardiovascular diseases [1–5]. Recent clinical trials have provided compelling evidences that vitamin C at pharmacological concentrations (mg – ~g), upon infusion into circulation, raises the circulating levels of the vitamin to mM concentration, and modulates vasodilation and vascular tone in humans [5]. Cigarette smoke products have been implicated in the alterations of pulmonary vascular endothelium leading to pulmonary hypertension in chronic obstructive pulmonary disease [6]. Vitamin C, upon infusion into blood at a dose of 10 mg/min for 120 min, improved the impairment of endothelial function in smokers [7]. Vitamin C, in addition to functioning as an antioxidant, acts as a prooxidant, generates reactive oxygen species (ROS), and causes oxidative stress [5]. The endothelium plays a pivotal role in the maintenance of vascular integrity and function, and is a prime target for the high levels of circulating
prooxidant vitamin C. Therefore, in our earlier study, we have used the bovine lung microvascular endothelial cells (BLMVECs) as the most appropriate model EC system and have shown that vitamin C at mM pharmacological doses induces oxidative stress and loss of redox-dependent cell viability [5].

Phospholipases specifically hydrolyze the membrane phospholipids and generate bioactive lipid second messengers, which play a vital role in cell signaling [8]. Phospholipase D (PLD), one such lipid signaling enzyme, ubiquitously present in all mammalian cells, preferentially hydrolyzes phosphatidylcholine (PC) and generates phosphatidic acid (PA) and choline [9]. PA is further metabolized to either 1,2-diacylglycerol (DAG) by phosphatidate phosphohydrolase or to lysophosphatidic acid (LPA) by phospholipase A1/A2 [9–11]. Agonist-mediated activation of PLD plays a crucial role in signal transduction in mammalian cells that has been attributed to the signaling actions of PA/LPA [9,12–14]. Two major forms of PLD, hPLD1, and hPLD2, have been cloned in mammalian cells which are selectively activated by various cofactors such as Arf, Rho, Cdc42, and phosphatidylinositol 4,5-bisphosphate (PIP2), and detergents in cell-free preparations [15–21].

Our earlier studies have shown that reactive oxygen species (ROS) stimulate PLD in vascular ECs, smooth muscle cells, and fibroblasts [13,22–32]. Our previous studies have also demonstrated that oxidants stimulate several signaling cascades including p38 mitogen-activated protein kinase (p38 MAPK), extracellular signal-regulated kinases (ERKs), and Src kinase which in turn regulate oxidant-induced activation of PLD in bovine pulmonary artery ECs (BPAECs) [31,33,34,35,36,37]. However, the regulation of PLD activation by a wide variety of stimuli is complex and involves changes in intracellular Ca2+ and protein kinase C (PKC), heterotrimeric G proteins, small molecular weight G proteins, and protein tyrosine kinases/protein tyrosine phosphatases [9,20,36,38].

As we have observed earlier that vitamin C at mM pharmacological concentrations causes loss of redox-dependent viability through oxidative stress in BLMVECs [5] and also, no attempts have been made so far on vitamin C-induced activation of PLD in mammalian cells, including ECs, in the present study, we investigated the vitamin C-induced activation of PLD and its regulation by MAPKs in lung microvascular ECs. For the first time, the results of the present study revealed that vitamin C at mM concentrations induced the activation of PLD in BLMVECs in a time- and dose-dependent fashion through prooxidant mechanism and upstream regulation by p38 MAPK and ERK1/ERK2.

2. Materials and methods

2.1. Materials

BLMVECs (passage 4) were purchased from VEC Technologies (NY). Phosphate-buffered saline (PBS) was obtained from Biofluids Inc. (Rockville, MD). Minimal essential medium (MEM), nonessential amino acids, trypsin, fetal bovine serum (FBS), penicillin/streptomycin, DMEM, phosphate-free modified medium, L-aspartic acid, tissue culture reagents, and analytical reagents of highest purity were all purchased from Sigma Chemical Co. (St. Louis, MO). Phosphatidylbutanol (PBt) was obtained from Avanti Polar Lipids (Alabaster, AL). [32P] Orthophosphate (carrier-free) was obtained from New England Nuclear (Wilmington, DE). Dekydroascorbic acid, desferal, SB203580, and PD98059 were obtained from Calbiochem (San Diego, CA). Endothelial cell growth factor was obtained from Upstate Biotechnology (Lake Plack, NY). Polyclonal rabbit antibodies against PLD, and PLD2 were purchased from Biosource (Camarillo, CA). Polyclonal rabbit and mouse antibodies against p38 MAPK, ERK1/ERK2, phospho-p38 MAPK, and phospho-ERK1/ERK2, phospho-PLD, Thr[147], and p38 MAPK and p44/42 MAPK assay kits were obtained from Cell Signaling (Beverly, MA). Mouse primary anti-phosphothreonine antibodies were purchased from Zymed Laboratories, Invitrogen (Carlsbad, CA). Secondary anti-rabbit AlexaFluor 488-conjugated and anti-mouse AlexaFluor 586-conjugated antibodies were obtained from Molecular Probes (Eugene, OR). Protein A/G plus sepharose were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). HRP-conjugated anti-rabbit secondary antibody and the enhanced chemiluminescence kit for the detection of proteins by Western blots were obtained from Amersham (Arlington Heights, IL).

2.2. Cell culture

BLMVECs were grown to confluence in MEM supplemented with 10% fetal bovine serum, 100 units/ml penicillin and streptomycin, 5 μg/ml endothelial cell growth factor and 1% nonessential amino acids at 37 °C under a humidified 95% air–5% CO2 atmosphere as described earlier [5,37]. BLMVECs, from passages 7 to 15, were used in the experiments. ECs cultured in 35-mm or 60-mm sterile dishes or T-75 cm sterile flasks to confluence. ECs were washed with MEM and incubated at 37 °C in a 5% CO2 atmosphere as described earlier [5,37]. Cells were washed with MEM and incubated under a humidified 95% air–5% CO2 atmosphere for different lengths of time to achieve confluence under a humidified 95% air–5% CO2 atmosphere for treatments with vitamin C (L-aspartic acid) and desired pharmacological agents. MEM containing vitamin C and other pharmacological agents were carefully adjusted to pH 7.4 for cellular treatments.

2.3. Phospholipase D activation in intact ECs

ECs in 35-mm dishes (5×105 cells/dish) were prelabelled with [32P] orthophosphate (5 μCi/ml) in DMEM phosphate-free medium containing 2% fetal bovine serum for 12–14 h [25,37,39]. Cells were washed with MEM and incubated at 37 °C in 1 ml of MEM containing 0.05% butanol in absence and presence of desired concentrations of vitamin C for different lengths of time under a humidified 95% air–5% CO2 atmosphere. In some experiments, wherever required, ECs were pretreated for 1 h with selected pharmacological inhibitors prior to exposure to vitamin C or co-treated with vitamin C and pharmacological inhibitors for the desired lengths of incubation time. The incubations were terminated by addition of methanol/cone. HCl (100:1 v/v). Lipids were extracted essentially according to the method of Bligh and Dyer, with slight modifications [25]. [125I]-Labeled phosphatidylbutanol (PBt) formed as a result of PLD activation and transphosphatidylation reaction, as an index of PLD activity in intact cells, was separated by thin-layer chromatography (TLC) [21,25,37]. Radioactivity associated with the [125I]-PBt was quantified by liquid scintillation counting and data were expressed as DPM normalized to 106 counts in the total cellular lipid extract or as % of control (vehicle-treated cells).

2.4. Preparation of cell lysates and Western blotting

Preparation of cell lysates and immunoprecipitates (IPs), SDS–polyacrylamide gel electrophoresis (PAGE), and Western blotting were done according to Natarajan et al. [36]. Following exposure to vitamin C, ECs were rinsed twice with ice-cold PBS, scraped in 1 ml of lysis buffer containing 20 mM Tris–HCl (pH, 7.4), 150 mM NaCl, 2 mM EGTA, 5 mM glycerophosphate, 1 mM MgCl2, 1% Triton X-100, 1 mM sodium orthovanadate, 10 μg/ml protease inhibitors, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μM phenylmethylsulfonyl fluoride, and 1 μg/ml pepstatin, incubated at 4 °C for 30 min and were cleared by centrifugation in a microfuge at 10,000×g for 5 min at 4 °C. After determination of the total protein in the lysates, 6% Laemmli sample buffer was added to cell lysates and boiled for 5 min. Proteins were separated on 12% gels by SDS–PAGE, transferred onto polyvinylidene difluoride (PVDF) membranes and subjected to...
immunoblotting with either anti-ERK1/ERK2 (1:1000 dilution) or anti-p38 MAPK (1:2000 dilution) or anti-phospho ERK1/ERK2 (1:2000 dilution) or anti-phospho p38 MAPK (1:500 dilution) overnight at 4 °C. For preparation of PLD1 and PLD2 IPs, cell lysates containing equal amounts of protein (1 mg/ml) were incubated with 10 μl of rabbit polyclonal anti-PLD, or anti-PLD, (against N-terminal and internal sequences) antibodies for 12 h at 4 °C. Protein A/G agarose (20 μl) was then added following incubation with the primary anti-PLD antibodies and the mixture was incubated for 4 h at 4 °C. The mixture was centrifuged at 5000 g for 5 min, agarose bead precipitates were washed 3 times with ice-cold lysis buffer, dissociated by boiling in 1× Laemmli buffer for 5 min, subjected to SDS–PAGE on 8% gels, proteins were electrotransferred onto PVDF membranes, and membranes were incubated with TBST containing 3% milk for 12 h at 4 °C with rabbit primary anti-PLD or anti-PLD antibodies (against N-terminal and internal sequences, 1:1000 dilution) or with mouse primary anti-phosphothreonine antibodies (1:1000 dilution). The membranes were washed 3 times with Tris-buffered saline containing 0.1% Tween-20 (TBST) and incubated for 1–2 h at room temperature in horseradish peroxidase-conjugated goat anti-rabbit (1: 2000 dilution in TBST containing 5% BSA) or goat anti-mouse secondary antibodies (1:5000 dilution in TBST containing 3% or 5% non-fat milk). The immunoblots were then developed with enhanced chemiluminescence (ECL) reagents according to manufacturer’s recommendation.

2.5. Assay of MAPK activity

The activities of p38 MAPK and ERK1/ERK2 were assayed using the p38 MAPK and p44/42 MAPK assay kits (Cell Signaling, Beverly, MA), respectively, according to the manufacturer’s recommendations with slight modifications. BLMVECs, grown in T-75 flasks (4×106 cells/flask) were incubated in MEM containing vitamin C (5 mM) for 0–120 min at 37 °C under a humidified 95% air–5% CO2 atmosphere. At the end of the incubation period, cell lysates were prepared, protein content was determined, and IPs of p38 MAPK and ERK1/ERK2 were prepared from the cell lysates with the immobilized phospho-p38 MAPK (Thr180/Tyr184) and phospho-p44/42 MAPK (Thr202/Tyr204) monoclonal antibodies (15 μl/200 μg of protein), respectively. For the kinase assays, IPs were resuspended in 50 μl of kinase buffer supplemented with 200 μM ATP and 2 μg of ATF-2 fusion protein (for p38 MAPK) or Elk-1 fusion protein (for ERK1/ERK2) and were incubated for 30 min at 37 °C. Following incubation, reaction mixture was subjected to SDS–PAGE on 10% gels, proteins were transferred onto PVDF membranes, and Western blotting was performed with phospho-ATF2 antibody (1:1000 dilution) and HRP-conjugated anti-rabbit secondary antibody (1:2000) and HRP-conjugated anti-biotin antibody (1:1000). Proteins on PVDF membranes were detected using 1× LumiGLO solution and images were captured on X-ray film. Images were processed digitally with Unscanit Software, intensities of protein bands were calculated, and MAPK activity was expressed as % 0 min treatment with vitamin C.

2.6. Immunofluorescence microscopy

BLMVECs cultured on sterile cover slips (Harvard Apparatus, 22 mm2) in 35-mm sterile dishes at a density of 105 cells/dish were treated with MEM alone, MEM containing vitamin C, and pharmacological inhibitors for designated lengths of time under a humidified atmosphere of 95% air–5% CO2 at 37 °C. At the end of the incubation period, cells attached to cover slips were washed with 1× PBS and fixed with 3.7% of para-formaldehyde for 10 min, permeabilized with 0.25% Triton X-100 in TBST containing 0.01% Tween-20 for 5 min, and blocked for 30 min with 1% BSA in 0.01% TBST and incubated with (1) rabbit primary anti-PLD1 (N-terminal and Internal) or anti-PLD2 (N-terminal and Internal) antibodies at a dilution of 1:200 for visualization of PLD isoforms or (2) rabbit primary anti-p38 MAPK or anti-ERK1/ERK2 or anti-phospho-p38 MAPK or anti-phospho-ERK1/ERK2 antibodies, at a dilution of 1:500, for visualization of p38 MAPK or ERK1/ERK2, in 0.01% TBST containing 1% BSA for 1 h at room temperature. For visualization of PLD1, cells on cover slips were incubated with rabbit primary antiphosphothreonine PLD2 antibody (Thr147), at a dilution of 1:500, in 0.01% TBST containing 1% BSA for 1 h at room temperature. Following treatment of cells with the chosen primary antibodies, they were incubated with secondary anti-rabbit AlexaFluor 488-conjugated and secondary anti-mouse AlexaFluor 586-conjugated antibodies (1:100 dilution), wherever necessary, for 1 h at room temperature. The cover slips with cells were then mounted on a glass slide with the antifade mounting medium. Fluoromount-G, viewed with Zeiss Confocal microscope at a magnification of 60×, and pictures were captured digitally. The digital images were quantitatively analyzed for fluorescence intensities with the ImageJ software.

2.7. Transient transfection of cells

The cDNA for DN-ERK1/ERK2 (K71R) (generously provided by Dr. M. Cobb of the University of Texas Southwestern Medical Center) [40] was subcloned in the pcDNA3.1 Zeo (+) expression plasmid (Invitrogen, San Diego, CA). The DN-p38α/β mutant cDNA containing His-tag cloned in the pcDNA3 mammalian expression vector (Invitrogen, San Diego, CA) was kindly provided by Dr. Michael Ostrowski (The Ohio State University, Columbus, OH) [41]. BLMVECs (3×105 cells) cultured on sterile 35-mm dishes were transiently transfected with 2 μg of cDNA corresponding to either DN-ERK1 or ERK2 or DN-p38 MAPK or GFP using the Effectene transfection reagent (Qiagen, Valencia, CA) according to manufacturer’s instructions. GFP expression was examined 18–24 h following transfection. To obtain transfection efficiency, the average GFP-positive cells from the images taken with the Olympus X50 fluorescence microscope, were corrected for the difference in vector size of the GFP versus the pcDNA3 vectors containing the DN-ERK1 or DN-ERK2 or DN-p38 MAPK. From 5 independent transfections, the range of transfection efficiency obtained was 21–44% and the average was 32.6%.

2.8. Protein determination

Protein was determined by BCA protein assay (Pierce).

2.9. Statistical analysis

All experiments were done in triplicate. Data were expressed as mean± standard deviation (S.D.). Statistical analysis was carried out by ANOVA using SigmaStat (Jandel). The level of statistical significance was taken as P<0.05.

3. Results

3.1. Vitamin C induces PLD activation in BLMVECs in a dose- and time-dependent fashion

Vitamin C, in a dose-dependent manner (0–10 mM), at 120 min of incubation, induced significant activation of PLD in BLMVECs as compared with the vehicle-treated control cells (3-, 9-, 18-, 28-, and 37-fold at 1, 3, 5, 7, and 10 mM, respectively) (Fig. 1A). At 30 min of incubation of ECs with vitamin C (3, 5, and 7 mM), although an increase (2-fold) in the activation of PLD was observed as compared with that in the cells exposed to the same for 0 min (9.6-, 18-, and 22-
fold at 3, 5, and 7 mM, respectively) (Fig. 1B). Also, at 120 min of incubation, the other chosen analogs of vitamin C such as δ-glucuronolactone (the precursor of vitamin C with similar molecular weight), L-ascorbyl-2-sulfate and L-ascorbyl-2-phosphate, at the same concentrations (5 and 10 mM) did not induce the activation of PLD in the cells (data not shown), indicating that vitamin C-induced activation of PLD in BLMVECs was not due to the osmotic shock exerted by the vitamin at pharmacological doses.

3.2. Antioxidants and iron chelators attenuate vitamin C-induced PLD activation

Our earlier studies have revealed the antioxidant attenuation of oxidant-induced activation of PLD in ECs [32]. The role of iron in oxidative stress has been well established [5,42,43]. Antioxidants such as epigallocatechin gallate (50 μM), propyl gallate (100 μM) and N-acetyl cysteine (1 mM) and iron chelators (diethylenetriaminepentaacetic acid and desferal, 1 mM) [44] caused a marked and significant inhibition of vitamin C-induced PLD activation in BLMVECs (data not shown). Extracellular treatment of cells with bovine erythrocyte superoxide dismutase (SOD, 100 μg) caused a slight (14%) decrease in the vitamin C-induced activation of PLD in BLMVECs exposed to the vitamin for 120 min (Fig. 2). Addition of bovine liver catalase (100 μg/ml) to the medium resulted in a significant and almost a complete attenuation of PLD activation in BLMVECs exposed to vitamin C (5 mM) for 120 min (Fig. 2). These results confirmed the involvement of iron, O₂⁻ and H₂O₂ in the vitamin C-induced activation of PLD in BLMVECs.

3.3. MAPK inhibitors attenuate vitamin C-induced PLD activation

Earlier, we have established the involvement of p38 MAPK in the oxidant-mediated activation of PLD in ECs [36]. Therefore, here we investigated the role of MAPKs in the vitamin C-mediated activation of PLD in BLMVECs. In this study, the chosen concentration of vitamin C was 5 mM and designated time of incubation was 120 min. As shown in Fig. 3, pretreatment of BLMVECs for 2 h with a selective p38 MAPK inhibitor, SB203580 (20 μM) and a specific inhibitor of MEK1/2 (upstream regulator of ERK1/ERK2), PD98059 (20 μM) caused a significant attenuation of vitamin C-induced activation of PLD (55% and 44% decrease in ECs treated with SB203580...
3.4. DN-p38 MAPK and DN-ERK1/ERK2 attenuate vitamin C-induced PLD activation

Fig. 4A illustrates the typical transfection efficiency (32.6%) of DN-p38 MAPK and DN-ERK1/ERK2 in BLMVECs as evidenced by GFP fluorescence. As shown in Fig. 4B, vitamin C (5 mM)-induced PLD activation at 120 min of treatment, in DN-p38 MAPK-transfected cells, was significantly lower (41% attenuation) as compared to that in the vector-transfected control cells. On the other hand, in the DN-ERK1- and DN-ERK2-transfected cells, vitamin C-induced PLD activation was significantly attenuated by 15% and 23%, respectively (Fig. 4C). These results further indicated that p38 MAPK and ERK1/ERK2 were involved in the upstream regulation of vitamin C-induced PLD activation in BLMVECs.

3.5. Vitamin C induces phosphorylation and activation of MAPKs

We first investigated the vitamin C-induced phosphorylation of p38 MAPK and ERK1/ERK2 in BLMVECs. Cells were exposed to vitamin C (5 mM) for 0–120 min and the phosphorylation of p38 MAPK and ERK1/ERK2 in cell lysates was determined by SDS–PAGE and Western blotting. Beginning with 10 min of exposure of ECs to vitamin C, the phosphorylation of p38 MAPK was clearly evident (1.6-fold as compared with the 0 min treatment), attained a maximum at 15 min of treatment with the vitamin (7.3-fold as compared with the 0 min treatment), and from 30–120 min of treatment of cells...
with the vitamin, the extent of phosphorylation declined, but still was greater as compared with that at 0 min of treatment with the vitamin (Fig. 5). On the other hand, the vitamin C-induced phosphorylation of ERK1/ERK2 exhibited a similar pattern as that of the p38 MAPK. At 10 min of exposure of BLMVECs with vitamin C, the phosphorylation of ERK1/ERK2 was dramatically higher (2-fold as compared with the 0 min treatment), reached a maximum at 15 min of treatment with the vitamin (2.4-fold as compared with the 0 min treatment), attained a plateau between 30 and 60 min of exposure to the vitamin, and at 120 min of exposure to the vitamin, the extent of phosphorylation declined to the level that was seen at 0 min of incubation (Fig. 5). Examination of phosphorylation of MAPKs in situ in BLMVECs by fluorescence confocal microscopy also revealed a time-dependent and significant increase in the vitamin C-induced phosphorylation of both p38 MAPK and ERK1/ERK2 up to 60 min and thereafter a decrease in the same at 120 min of treatment (data not shown).

We further determined the activities of p38 MAPK and ERK1/ERK2 in their specific IPs of BLMVECs treated with vitamin C (5 mM) for 0–120 min. As shown in Fig. 6A, vitamin C induced a time-dependent and significant enhancement of activity of p38 MAPK with a maximum at 15 min and a gradual decline from there on wards to 60 min. At 120 min of treatment of cells with vitamin C, the activity of p38 MAPK reached almost the extent that was exhibited by the cells exposed to vitamin C for 0 min. Vitamin C induced a maximum and significant enhancement of ERK1/ERK2 activity in BLMVECs at 10 min of treatment as compared with that in cells exposed to the same for 0 min (Fig. 6B). From 15 to 60 min of exposure of cells to vitamin C, although a significant increase in the ERK1/ERK2 activity was noticed as compared to that in the cells exposed to the same for 0 min, it gradually decreased with time. At 120 min of treatment of cells with vitamin C, the activity of ERK1/ERK2 was even less than that was observed in the cells treated with the vitamin for 0 min (Fig. 6B). Collectively, these results established that vitamin C induced the activation of both p38 MAPK and ERK1/ERK2, in a time-dependent manner which was upstream of PLD activation.

3.6. Vitamin C induces translocation of PLD

Translocation and redistribution of PLD1 and PLD2 isoforms in BLMVECs were examined following exposure of cells to different concentrations of vitamin C (5, 7, and 10 mM) for 120 min by confocal immunofluorescence microscopy. Both PLD1 and PLD2 were visualized by their green fluorescence. PLD1 in the vehicle-treated control cells was solely perinuclear in distribution with a scanty presence in the cytosol, whereas in the cells treated with vitamin C, a dose-dependent translocation and redistribution of the isoform were evident throughout the cytosol, cell membrane, filipodia and lamellipodia (Fig. 7B). These results clearly showed that vitamin C induced translocation and redistribution of both PLD1 and PLD2 in BLMVECs.
3.7. Vitamin C induces association of p38 MAPK and ERK1/ERK2 with PLD

Here, we investigated the association of p38 MAPK and ERK1/ERK2 with PLD isoforms by confocal immunofluorescence microscopy and SDS–PAGE and Western blotting following the exposure of cells to vitamin C (5 mM) for 0–120 min. At 0 min of incubation with vitamin C, p38 MAPK was distributed around PLD1 but at 120 min of incubation of cells under identical conditions, PLD1 was relocalized in the form of a ring around the nucleus and p38 MAPK was intensely distributed around PLD1 in a punctuate manner. The association of p38 MAPK with PLD2 gradually increased from 0 min, attained maximum at 60 min, and declined at 120 min of treatment of cells with vitamin C (data not shown). With the progression of time of exposure of vitamin C, from 15 to 60 min, the association of PLD1 and ERK1/ERK2 gradually increased and thereafter declined dramatically at 120 min of treatment. The translocation and association of PLD2 and ERK1/ERK2 started from 15 min and was the greatest at 60 min of treatment of cells with vitamin C (data not shown).

Utilizing the SDS–PAGE and Western blotting, we further examined the extent of association of MAPKs with PLD isoforms in the PLD1 and PLD2 IPs of BLMVECs exposed to vitamin C (5 mM) for 120 min. The extents of association of ERK1/ERK2 with PLD1 and PLD2 in vitamin C-treated cells as compared to that in the control vehicle-treated cells were 82% and 112%, respectively, as determined by the gel intensity measurements (Fig. 8). The extents of association of
p38 MAPK with PLD₁ and PLD₂ in vitamin C-treated cells, under identical conditions, as compared to that in the control vehicle-treated cells were 52% and 94%. Collectively, these results further established that the association of ERK₁/ERK₂ and p38 MAPK was greater with the PLD₂ isoform than with the PLD₁ isoform in BLMVECs exposed to vitamin C.

3.8. Vitamin C induces threonine phosphorylation of PLD

We further hypothesized that MAPKs would phosphorylate PLD and thus regulate its activity in ECs under vitamin C exposure. Therefore, we first utilized SDS–PAGE and Western blotting to examine the extent of phosphorylation of threonine in PLD₁ and PLD₂ IPs of BLMVECs exposed to vitamin C (5 mM) for 120 min. As shown in Fig. 9A, vitamin C induced phosphorylation of threonine in PLD₁ and PLD₂ isoforms up to 75% and 101% as compared to the same in control vehicle-treated cells, suggesting that the extent of threonine phosphorylation in PLD₁ was greater than that in PLD₂ in cells upon treatment with vitamin C. Utilizing the only commercially available phosphothreonine-PLD₁ antibody to detect phosphothreonine in PLD₁, we further investigated the in situ MAPK-mediated threonine phosphorylation of PLD₁ by confocal immunofluorescence microscopy in BLMVECs following exposure to vitamin C (5 mM) for 0–120 min. As shown in Fig. 9B, vitamin C (5 mM), in a time-dependent manner, induced the threonine phosphorylation in PLD₁ in BLMVECs. Between 30 and 120 min of exposure of cells with vitamin C, threonine phosphorylation in PLD₁ increased significantly, reaching a maximum at 120 min of exposure of cells with the vitamin. Pretreatment of BLMVECs with p38 MAPK-specific inhibitor, SB203580 (25 μM), and MEK1/2-specific inhibitor, PD98059 (25 μM) for 2 h, completely and significantly attenuated the increase in phosphorylation of threonine in PLD₁ at 60 and 120 min of exposure to vitamin C, suggesting that threonine phosphorylation of PLD₁ was mediated by p38 MAPK and ERK₁/ERK₂ in BLMVECs.

Fig. 7. Vitamin C induces translocation of PLD. BLMVECs (10⁴ cells) were grown on sterile cover slips and treated with MEM or MEM containing vitamin C (5, 7 and 10 mM) for 120 min under a humidified atmosphere of 95% air–5% CO₂ at 37 °C. At the end of the incubation period, cells were processed as described in Materials and methods that included treatment with the primary rabbit anti-PLD₁ (N-terminal and Internal) (A) or anti-PLD₂ (N-terminal and Internal) (B) antibodies for 1 h at room temperature, and subsequent treatment with secondary anti-rabbit AlexaFluor 488-conjugated antibody for 1 h at room temperature. The processed cells on cover slips were viewed with Zeiss Confocal microscope at a magnification of 60×, and pictures were captured digitally. Each micrograph is a representative of at least three independent experiments.

Fig. 8. Vitamin C enhances association of p38 MAPK and ERK₁/ERK₂ with PLD₁ and PLD₂. BLMVECs (5 × 10⁵ cells/35-mm dish) were incubated in MEM or MEM containing vitamin C (5 mM) for 120 min under a humidified atmosphere of 95% air–5% CO₂ at 37 °C. At the end of incubation period, IPs of PLD₁ and PLD₂ were subjected to SDS–PAGE and Western blotting analysis for detection of PLD₁, PLD₂, p38 MAPK, and ERK₁/ERK₂ as described under Materials and methods. Results shown are representative blot of three independent experiments.
3.9. DN-p38 MAPK and DN-ERK1/ERK2 attenuate vitamin C-induced threonine phosphorylation of PLD1 and PLD2

The extent of threonine phosphorylation in the IPs of PLD1 and PLD2 isozymes of BLMVECs treated with vitamin C (5 mM) for 120 min was investigated by SDS-PAGE and Western blotting. As shown in Fig. 10A, vitamin C induced a marked phosphorylation of threonine in both PLD1 and PLD2 in the vector-transfected cells, whereas that of PLD2 in DN-p38 MAPK-transfected cells was completely attenuated under identical conditions. However, in DN-ERK1/ERK2-transfected cells, the extent of vitamin C-induced phosphorylation of threonine in PLD1 and PLD2 was diminished, wherein that of the former was strikingly lower, as compared to that in the vector-transfected control cells (Fig. 10B). These results suggested that threonine phosphorylation of PLD1 was mediated by ERK1/ERK2 whereas that of PLD2 was mediated by both p38 MAPK and ERK1/ERK2.

4. Discussion

In the present study, we demonstrated that vitamin C at mM pharmacological concentrations induced the activation of PLD in BLMVECs in a time- and dose-dependent fashion that was
significantly attenuated by antioxidants, p38 MAPK- and ERK-specific inhibitors, and transient transfection of cells with DN-p38 MAPK and DN-ERK1/ERK2. In addition, the study also revealed that vitamin C induced translocation of PLD1 and PLD2, enhanced their association with p38 MAPK and ERK1/ERK2, and induced phosphorylation of threonine in PLD1 and PLD2, which were all associated with the observed PLD activation in BLMVECs. We, for the first time, showed that vitamin C at pharmacological doses, activated PLD in the lung microvascular ECs through oxidative stress and MAPK activation.

Vitamin C infusion into circulation at pharmacological doses (mg – ~g) is emerging as a therapeutic intervention for certain types of cancers and vascular diseases [5]. The prooxidant nature of vitamin C, capable of generating ROS and causing oxidative stress-mediated cytotoxicity, is well established [5,45–56]. Others and we have shown that oxidants and ROS induce the activation of PLD in different cultured mammalian cell systems, including ECs [22–32,36]. In this study, we clearly established that vitamin C, at pharmacological doses, induced the activation of PLD in BLMVECs, involving iron-mediated oxidative stress. Earlier, others and we have reported the activation of PLD in mammalian cells including ECs by H₂O₂, fatty acid hydroperoxide, 4-hydroxynonenal, diperoxovanadate (DPV), and redox regulation [13,25–32,36,38,57,59–61]. Together, these studies support our results of the current study that vitamin C at pharmacological doses generated ROS, induced oxidative stress, and activated PLD in BLMVECs through a redox-active prooxidant mechanism.

PLD is activated by several agonists such as hormones, growth factors, neurotransmitters, cytokines, and ROS in different mammalian cell systems and tissues, involving agonist- and cell-specific complex signaling mechanisms of regulation [9,13,20,27,38]. Mechanism of oxidant (ROS)-induced PLD activation has also been shown to be oxidant-specific, which has been shown to be regulated upstream by PKC or protein tyrosine kinases or MAPKs [13,23–31,36,62,64]. In some model cell systems, oxidant-induced PLD activation has not been attenuated by PKC inhibitors or down-regulation of PKC [25,28,30,36]. However, the involvement of tyrosine kinases and PKC in oxidant-induced PLD activation in several mammalian cell systems including ECs has been documented [23,37,63–65]. The present study demonstrated the activation of p38 MAPK and ERK1/ERK2 by vitamin C in BLMVECs, upstream of PLD activation. Involvement of ERK1/ERK2 in H₂O₂-induced activation of PLD in PC12 cells has been shown [58,59]. Our earlier work
has shown the role of p38 MAPK in DPV-induced activation of PLD in BPAECs [36]. It has been observed that the tea antioxidant, epigallocatechin gallate induces activation of PLD in astrocytes that is mediated by upstream activation of p38 MAPK [60,61]. Therefore, these earlier reports support our current findings on the involvement of p38 MAPK and ERK1/ERK2 in vitamin C-induced activation of PLD in BLMVECs.

MAPKs, the stress-activated protein kinases, are broadly divided into three classes, namely (1) ERK, (2) JNK, and (3) p38 MAPK, which participate in different cell signaling cascades, following activation through phosphorylation of both threonine and tyrosine residues by distinct dual-specific serine/threonine MAPK kinases [36,66]. Our current study also showed that vitamin C induced (i) in situ translocation/relocalization of PLD isoforms but also possible protein–protein interactions among the PLD isoforms and MAPKs, thus leading to the regulation of PLD activity. Our earlier study has also established oxidant-mediated enhancement of interaction and association among p38 MAPK and PLD isoforms in ECs [36] and further supports our current findings that vitamin C induced interaction/association of PLD isoforms with p38 MAPK and ERK1/ERK2 in BLMVECs.

The mechanisms of agonist-induced PLD activation are still complex and controversial. Although a phosphorylation mechanism of PKC-mediated regulation of PLD activation has been suggested, in vitro studies on PKC-dependent stimulation of PLD activity have suggested a non-phosphorylation-dependent protein–protein interaction as a mechanism of activation of PLD [9,20,36]. Our current findings also revealed the role of p38 MAPK- and ERK1/ERK2-mediated threonine phosphorylation of PLD1 and PLD2 in vitamin C-induced activation of those isoforms in BLMVECs. This is further supported by our earlier study which has shown that oxidant-induced phosphorylation of PLD leading to its activation in intact BPAECs is attenuated by SB202190, suggesting p38 MAPK-mediated phosphorylation of PLD isoforms [36]. The exact mechanism of MAPK activation and resultant site-specific threonine phosphorylation of PLD isoforms leading to their activation needs to be clearly established.

In conclusion, we, for the first time, showed in the present study that vitamin C, at pharmacological doses, induced activation of PLD in BLMVECs through oxidative stress and upstream activation of p38 MAPK and ERK1/ERK2 (Scheme 1). Thus, the findings of this study underscore the important role of MAPK-regulated activation of PLD in ECs by vitamin C at pharmacological doses.

Acknowledgements

We acknowledge Mr. Terry Welsh for his excellent technical expertise. This work was supported by grants from the National
Heart, Lung, and Blood Institutes (HL69909, POI HL58064, HL38324, HL66108, HL67176, POI HL70294, HL63744, and HL65608), National Institutes of Health (CA102264), and the American Lung Association.

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