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Mercury Activates Vascular Endothelial Cell Phospholipase D through Thiols and Oxidative Stress

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Currently, mercury has been identified as a risk factor of cardiovascular diseases among humans. Here, the authors tested the hypothesis that mercury modulates the activity of the endothelial lipid signaling enzyme, phospholipase D (PLD), which is an important player in the endothelial cell (EC) barrier functions. Monolayers of bovine pulmonary artery ECs (BPAECs) in culture, following labeling of membrane phospholipids with [^{32}P]orthophosphate, were exposed to mercuric chloride (inorganic form), methylmercury chloride (environmental form), and thimerosal (pharmaceutical form), and the formation of phosphatidylbutanol as an index of PLD activity was determined by thin-layer chromatography and liquid scintillation counting. All three forms of mercury significantly activated PLD in BPAECs in a dose-dependent (0 to 50 μM) and time-dependent (0 to 60 min) fashion. Metal chelators significantly attenuated mercury-induced PLD activation, suggesting that cellular mercury-ligand interaction(s) is required for the enzyme activation and that chelators are suitable blockers for mercury-induced PLD activation. Sulfhydryl (thiol-protective) agents and antioxidants also significantly attenuated the mercury-induced PLD activation in BPAECs. Enhanced reactive oxygen species generation, as an index of oxidative stress, was observed in BPAECs treated with methylmercury that was attenuated by antioxidants. All the three different forms of mercury significantly induced the decrease of levels of total cellular thiols. For the first time, this study revealed that mercury induced the activation of PLD in the vascular ECs wherein cellular thiols and oxidative stress acted as signal mediators for the enzyme activation. The results underscore the importance of PLD signaling in mercury-induced endothelial dysfunctions ultimately leading to cardiovascular diseases.

Keywords Lipid Signaling, Mercury, Oxidative Stress, Phospholipase D, Reactive Oxygen Species, Vascular Endothelial Cells

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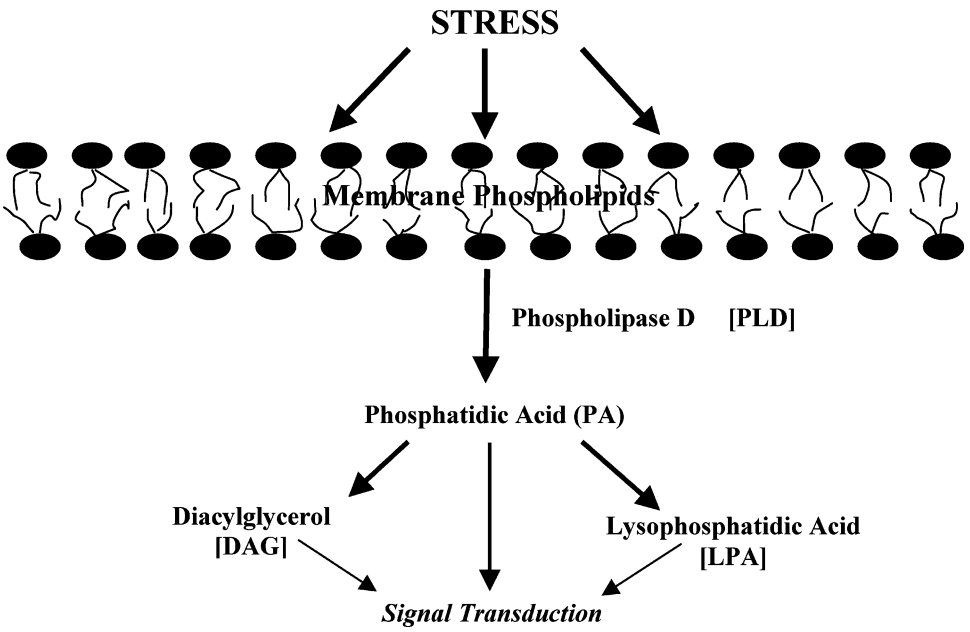
Mercury (Hg) is a heavy metal belonging to the transition element series of the periodic table. The element is used in industrial processes and medical practice (Clarkson 2002), which results in accidental and occupational exposures to mercury. Although mercury occurs naturally in the environment, anthropogenic activities cause the mercury pollution of air, water, and soil environments (Clarkson, Magos, and Meyers 2003; Sarkar 2005). Inorganic mercury is toxic to many organisms and is converted to more toxic organic forms (methylmercury) through biomethylation by microorganisms (bacteria) (Boening 2000; Dopp et al. 2004). Methylmercury reaches the organisms in the food chain and ultimately accumulates in humans, the top consumers (Boening 2000). Consumption of fish has been shown as a major source of environmental mercury (especially methylmercury) in humans that could lead to suppression of the beneficial effects of omega-3 fatty acids on the coronary artery disease (Clarkson 2002; Landmark and Aursnes 2004).

Dental amalgam fillings containing mercury have been in uncontrolled use in dental care worldwide and mercury leaching from the dental implants has been attributed to the adverse health effects in humans (Mutter et al. 2004). Thimerosal, the pharmaceutical mercury compound, has been used in vaccines as a preservative and the use of the vaccines containing this mercurial compound has been correlated with autism in children (Grether et al. 2004; Mutter et al. 2005). However, these data are subjected to debate because the analysis of mercury in hair samples does not accurately reflect the body levels of mercury, especially in children (Grether et al. 2004). Mercury has also been recognized as a toxicant in occupational environments, contributing to work related disorders/diseases such as respiratory and lung diseases, cardiovascular diseases, musculoskeletal disorders, and nervous system diseases among transport workers (Ustinaviciene, Obelenis, and Ereminas 2004). Overall, humans are exposed to all these forms of mercury through accidents, environmental pollution, food contamination, dental care, preventive medical practices, industrial and agricultural operations, and occupational operations (Clarkson 2002).



SCHEMA 1
PLD-mediated hydrolysis of membrane phosphatidylcholine (PC) and generation of phosphatidic acid (PA), diacylglycerol (DAG), and lysophosophatidic acid (LPA).

The role of mercury toxicity as a possible risk factor in cardiovascular disease has been discussed (Kostka 1991). Reports have been made on the toxic effects of metals in several diseases among humans including the vascular diseases (Nash 2005). Therefore, elevated body levels of mercury, due to fish consumption by humans, have been hypothesized as a risk factor in coronary heart disease (Yoshizawa et al. 2002). Increased levels of urinary mercury have been shown to be associated with elevated cholesterol levels in humans and mercury has been suggested as a risk factor of myocardial infarction, coronary disease, and cardiovascular disease (Kim et al. 2005).

Although mercury has been shown to be associated with cardiovascular diseases among humans, detailed studies leading to the understanding of mechanisms of mercury-induced cardiovascular diseases are currently lacking. Vascular endothelium plays a pivotal role in the structure and function of the blood vessel and maintains the homeostasis of the circulatory system and the entire body in general. Methylmercury has been shown to cause hypertension in rats (Wakita 1987), and mercury-induced vascular endothelial damage and vasculitis in humans upon autopsy have been documented (Egermayer 2000). Therefore, it is conceivable to hypothesize that mercury exerts its toxic effects on the vascular endothelium, which in turn may contribute to the mercury-induced cardiovascular diseases.

Phospholipids of cellular membranes play an important role in the cell as structural and functional entities. Phospholipases are enzymes that specifically hydrolyze the membrane phospholipids and generate bioactive lipid second messengers, which play a vital role in cell signaling (Divecha and Irvine 1995). Phospholipase D (PLD) is one such signaling enzyme, ubiquitously present in all mammalian cells, that preferentially hydrolyzes phosphatidylcholine (PC), generating phosphatidic acid (PA) and choline (Exton 1999) (Schema 1). PA is further metabolized to either 1,2-diacylglycerol (DAG) by phosphatidate phosphohydrolase or to lysophosphatidic acid (LPA) by phospholipase A_1/A_2 (Exton 1999; Varadharaj et al. 2006). Agonist-mediated activation of PLD plays a pivotal role in signal transduction in mammalian cells (Exton 1999; Natarajan 1995; Varadharaj et al. 2006). Several important roles of phospholipases have been attributed to the cellular signaling actions of PA/LPA (Exton 1999; Natarajan 1995; Varadharaj et al. 2006).

Therefore, we hypothesize that mercury activates PLD in the vascular endothelial cells (ECs), thus generating the bioactive signal lipid, PA, which may contribute to mercury-induced vascular disorders. In order to test the current hypothesis, the well-established bovine pulmonary artery EC (BPAEC) system was chosen here as the model system. Also in the present study, three different forms of mercury, mercuric chloride (inorganic form), methylmercury chloride (environmental form), and thimerosal (pharmaceutical form) were chosen and the activation of PLD in BPAECs in culture was studied following the treatment of cells with the mercury compounds. In addition, the efficacy of chelator drugs in attenuating the mercury-induced PLD activation and the role of thiols and reactive oxygen species (ROS) in the mercury-induced PLD activation were investigated.

MATERIALS AND METHODS

Materials

Bovine pulmonary artery endothelial cells (BPAECs) (passage 2) were purchased from Cell Applications (San Diego, CA). Fetal bovine serum (FBS), trypsin, and nonessential amino acids were obtained from Gibco Invitrogen (Grand Island, NY). Dulbecco's modified Eagle's (DME) phosphate-free medium, minimum essential medium (MEM), mercuric chloride, methylmercury chloride, thimerosal, propyl gallate, absorbic acid (vitamin C), catalase, ethylenediaminetetracetic acid (EDTA), pyrrollinedine dithiocarbamate (PDTC), D-pencillamine, dietyldithiocarbamic acid (DETC), dithiothreitol (DTT), *N*-acetyl-l-cysteine (NAC), meso-2,3-dimercapto-succinic acid (DMSA), 5,5′-dithio-bis (DTNB), glutathione (GSH), and Triton X-100 (t-octylphenoxypolyethoxyethanol) were obtained from Sigma Chemical (St. Louis, MO). MnTBAP was obtained from Calbiochem (San Diego, CA). [^{32}P]orthophosphate was obtained from New England Nuclear (Wilmington, DE). Phosphatidylbutanol (PBt) was purchased from Avanti Polar Lipids (Alabaster, AL). DCFDA (6-carboxy-2′,7′-dichlorodihydroxyfluorescein diacetate dicarboxy methyl ester) was purchased from Molecular Probes (Eugene, OR).

Cell Culture

BPAECs were cultured in MEM supplemented with 10% FBS, nonessential amino acids, antibiotics, and growth as described previously (Varadharaj et al. 2006). Cells in culture were maintained at 37°C in a humidified environment of 5% CO_2–95% air and grown to contact-inhibited monolayers with typical cobblestone morphology. When confluence was reached, cells were trypsinized and subcultured in T 75-cm^2 flasks or 35-mm or 100-mm tissue culture dishes. Confluent cells showed cobblestone morphology under light microscope and stained positive for factor VIII. All experiments were conducted between 8 and 20 passages (75% to 80% confluence).

PLD Activation in ECs

BPAECs in 35-mm dishes (5×10^5 cells/dish) were incubated with [^{32}P]orthophosphate (5 μCi/ml) in phosphate-free DMEM containing 2% FBS for 12 to 24 h at 37°C in 5% CO_2–95% air (Varadharaj et al. 2006). The radioactive medium was removed by aspiration and cells were incubated in serum-free MEM or MEM containing the selected forms of mercury (mercuric chloride or methylmercury chloride or thimerosal) at the chosen μM concentrations in the presence of 0.05% butanol for specified lengths of time (0 to 60 min). When required, cells prelabelled with [^{32}P]orthophosphate were pretreated with selected pharmacological agents/inhibitors for 1 h and then exposed to mercury compound(s) in the absence or presence of the pharmacological inhibitors and in the presence of 0.05% butanol for specified lengths of time. These pharmacological agents included metal chelators, thiol protectants, and antioxidants. Incubations were terminated by the addition of 1 ml of methanol:HCl (100:1 v/v) and lipids were extracted in chloroform:methanol (2:1 v/v) (Varadharaj et al. 2006). [^{32}P]PBt formed as a result of PLD activation and concomitant transphosphatidylation reaction (an index of in vivo PLD activation) was separated by thin-layer chromatography (TLC) with the upper phase of ethyl acetate: 2,2,4-trimethyl pentane: glacial acetic acid: water (65:10:15:50 by volume) as the developing solvent system. Unlabeled PBt was added as a carrier during TLC separation of lipids and was visualized upon exposure to iodine vapors. PBt spots were marked and scraped, and radioactivity-associated PBt was determined by liquid scintillation counting. All values were normalized to 1 million dpm in total lipid extract and [^{32}P]PBt formed was expressed as dpm/dish or percentage control.

Reactive Oxygen Species (ROS) Measurement by DCFDA Fluorescence

Formation of ROS in BPAECs in 35-mm dishes (5×10^5 cells/dish) was determined by DCFDA fluorescence in cells preloaded with 10 μM DCDFA for 30 min in complete MEM at 37°C in a 95% air–5% CO_2 environment prior to exposure to mercury compound(s) (Parinandi et al. 2003). When required, cells preloaded with DCFDA were pretreated with the selected pharmacological agents/inhibitors for 1 h and then exposed to mercury compound(s) for specified lengths of time. At the end of exposure to mercury compound(s) in the absence or the presence of pharmacological agents/inhibitors, the dishes containing cells were placed on ice, cells were detached with a Teflon cell scraper, and the medium containing cells was transferred to 1.5-ml microcentrifuge tubes and centrifuged at $8000 \times g$ for 10 min at 4°C. The supernatant medium was aspirated, and the cell pellet was washed twice with ice-cold phosphate-buffered saline (PBS). To prepare cell lysates, pellets were sonicated on ice with a probe sonicator at a setting of 5 for 15 s in 500 μl of ice-cold PBS. Fluorescence of oxidized DCFDA in cell lysates, an index of formation of ROS, was measured on a Gemini fluorescence plate reader with excitation and emission set at 490 and 530 nm, respectively, using appropriate blanks. The extent of ROS formation was expressed as the arbitrary fluorescence units.

Cellular Total Thiol Determination

Total cellular thiol content was measured by the DTNB-complexed spectrophotometric assay according to Parinandi et al. (1999). BPAECs were grown to 100% confluence in 100-mm dishes, then treated with MEM or MEM containing mercuric chloride (25 μM), methylmercury chloride (10 μM), or thimerosal (25 μM) for 60 min. After incubation, cells were detached by gentle scrapping and centrifuged at $5000 \times g$ for 10 min at 4°C. The cell pellets were then lysed using Triton X100. Cell lysates were then treated with 5,5′-dithiobis (DTNB) and the absorbance was determined at 412 nm on a Spectromax plate reader. Total thiol values were obtained from a standard curve prepared with GSH and normalized to 10^6 cells.

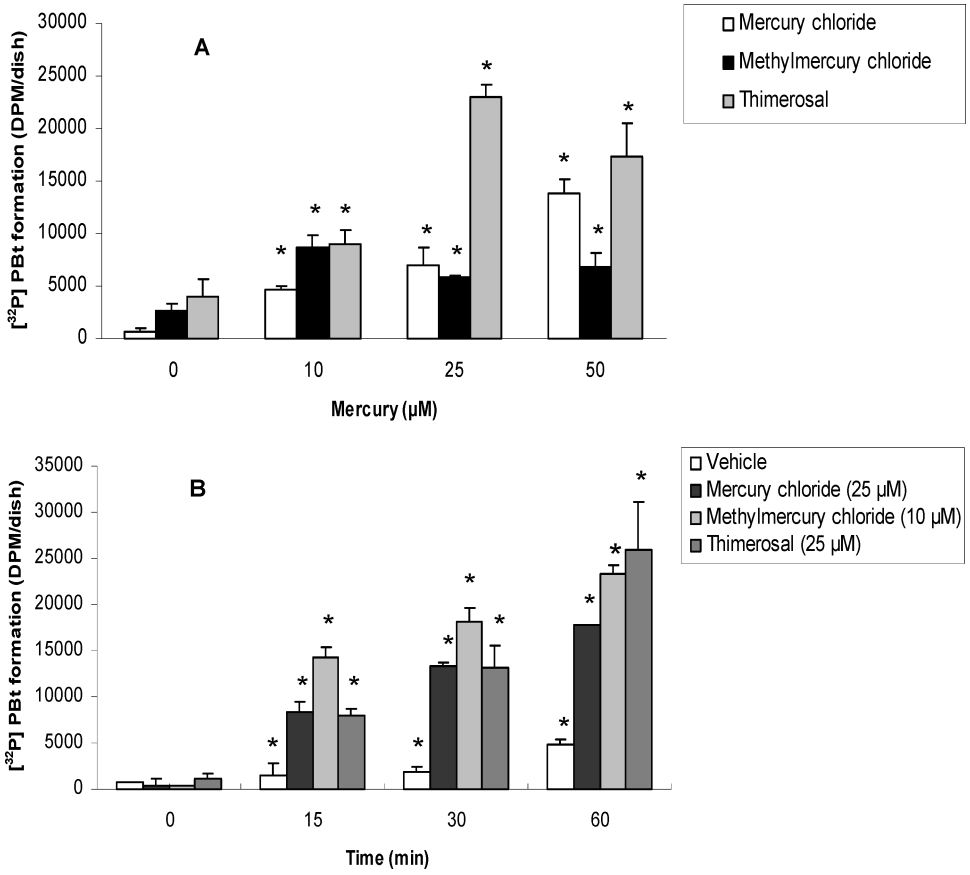


FIGURE 1

PLD activation by mercury in a dose- and time-dependent fashion. BPAECs (5×10^5 cells/35-mm dish) were labeled with [^{32}P]orthophosphate in DMEM phosphate-free medium for 12 h. Following [^{32}P]orthophosphate labeling, cells were treated with different concentrations (0 to 50 μM) of mercury (mercuric chloride or methylmercury chloride or thimerosal) (*A*) for 1 h in MEM containing 0.05% butanol. Cells were also treated with MEM alone or MEM containing mercuric chloride (25 μM) or methylmercury chloride (10 μM) or thimerosal (25 μM) (*B*) for 0 to 60 min in presence of 0.05% butanol. At the end of incubation, [^{32}P]PBt formed was determined as described under Materials and Methods. Data represent means $\pm$ *SD* of three independent experiments. *Significantly different at $p < .05$ as compared to cells treated with the vehicle alone (*A*) and cells treated for 0 min (*B*).

Statistical Analysis of Data

Standard deviation (SD) for each data point was calculated from triplicate samples. Data were subjected to one-way analysis of variance and pair wise multiple comparisons was done by Dunnett's method with $P < 0.05$ indicating significance.

RESULTS

Mercury Activates PLD in a Dose-Dependent Fashion

As no reports have been made so far on mercury-induced activation of PLD in animal cell systems including the vascular ECs, here we investigated whether different forms of mercury (mercuric chloride, methylmercury chloride, and thimerosal) would activate PLD in BPAECs in a dose-dependent (0 to 50 μM) fashion following incubation of cells for 1 h with the chosen mercury compounds. Mercuric chloride significantly caused 10-, 13-, and 20-fold activation of PLD at 10, 25, and 50 μM concentrations upon treatment of cells for 1 h, respectively, as compared to that in the cells treated with vehicle alone (Figure 1*A*). Methylmercury chloride at 10, 25, and 50 μM doses significantly caused a 3.5-, 2.5-, and 3-fold increase in PLD activity at 1 h of treatment of cells with the compound, respectively, as compared to the same in cells treated with vehicle alone (Figure 1*A*). Although methylmercury caused a significant increase in activation of PLD at 25 and 50 μM as compared to the same in vehicle-treated cells, the extent of enzyme activation at those doses as compared to the same at 10 μM dose of methylmercury chloride was significantly lower and maintained a plateau (Figure 1*A*). Thimerosal significantly induced 2-, 6-, and 4-fold activation of PLD at 10, 25, and 50 μM doses, respectively, in BPAECs at 1 h of treatment of cells as compared to that in the vehicle-treated cells (Figure 1*A*). It was evident from this study that thimerosal resulted in significant and dose-dependent activation of PLD in BPAECs. Overall, these results revealed that the tested mercury compounds were effective in causing significant and dose-dependent activation of PLD in BPAECs.

Mercury Activates PLD in a Time-Dependent Manner

Here, the time-dependent activation of PLD in BPAECs upon treatment with three different mercury compounds (mercuric chloride, methylmercury chloride, and thimerosal) was studied. At 15 min of treatment mercuric chloride caused a significant activation of PLD (27-fold) in cells, which increased at 30 and 60 min of treatment (43- and 57-fold) with the agonist as compared to the same in cells exposed to the agonist for 0 min (Figure 1*B*). Thimerosal (25 μM) induced 7-, 11-, and 22-fold increase in PLD activation at 15, 30, and 60 min of treatment of cells, respectively, as compared to the same in cells exposed to the agonist for 0 min, thus revealing a clear time-dependent and linear activation of the enzyme in BPAECs (Figure 1*B*). Methylmercury chloride significantly caused 44-, 56-, and 73-fold activation of PLD in BPAECs at 15, 30, and 60 min of treatment of cells as compared to the same in cells exposed to the agonist for 0 min. Collectively, these results revealed that all the three chosen forms of mercury caused a time-dependent activation of PLD in ECs.

Metal Chelating Agents Attenuate Mercury-Induced PLD Activation

Chelating agents complex with transition metals and have been shown to protect against metal-mediated adverse effects and metal toxicity (Blanusa et al. 2005). However, the modulatory effects of chelating agents on mercury-induced activation of PLD have not been reported so far. Therefore, here, the effects of well established chelating agents including EDTA (1 mM), PDTC (1 mM), and DETC (1 mM) were examined on the PLD activation induced by the three different chosen forms of mercury (25 μM mercuric chloride, 10 μM methylmercury, and 25 μM thimerosal) in BPAECs. Prior to the treatment of cells with the mercury compound(s), cells were pretreated for 1 h with basal MEM or MEM containing the chosen chelating agent(s) and then exposed to the mercury compound(s) in presence of the chelating agent(s) for 30 min. The classic trace element chelating agent, EDTA, significantly attenuated the mercuric chloride- and thimerosal-induced PLD activation (40% and 27% inhibition, respectively), but did not cause significant inhibition of methylmercury chloride-induced activation of PLD in BPAECs (Figure 2*A*). Another well-known thiocarbamate chelating agent, PDTC, significantly attenuated the PLD activation induced by all the three chosen mercury compounds wherein the mercuric chloride–induced enzyme activation was inhibited to the greatest extent (77% inhibition) (Figure 2*B*). One of the thiocarbamate derivatives, DETC, offered significant inhibition of mercuric chloride–, methylmercury chloride–, and thimerosal-induced PLD activation in BPAECs (64%, 54%, and 43% inhibition, respectively) (Figure 2*C*). Overall, these results showed that EDTA and thiocarbamates were the most effective chelating agents in causing significant attenuation of PLD activation in BPAECs induced by mercuric chloride and thimerosal and all the three chosen mercury compounds, respectively.

Sulfhydryl Agents Attenuate Mercury-Induced PLD Activation

Reports have been made that the thiols (nonprotein and protein) are the targets for heavy metal cellular actions (Blanusa et al. 2005; Valko, Morris, and Cronin 2005). Altered thiol redox has been shown to activate PLD in ECs (Parinandi et al. 1999). Therefore, in order to establish the role of thiols in mercury-induced PLD activation in BPAECs, here, the effects of well-established thiol (sulfhydryl) protective agents (NAC, DTT, DMSA, and D-penicillamine) were investigated on PLD activation induced by mercuric chloride, methylmercury chloride, and thimerosal. Prior to exposure of cells to mercury compound(s) (25 μM mercuric chloride, 10 μM methylmercury chloride, and 25 μM thimerosal), cells were pretreated for 1 h with MEM or MEM containing the chosen thiol protective agent(s) (1 mM) and then treated with the mercury compound(s) in the presence of the thiol protective agent(s) for 30 min. NAC, a widely used thiol protector and antioxidant, caused effective and significant attenuation of PLD activation in ECs induced by all three forms of mercury (74%, 81%, and 93% of inhibition of enzyme activation induced by mercuric chloride, methylmercury chloride, and thimerosal, respectively) (Figure 3*A*). DTT, a sulfhydryl protective agent, offered effective and significant inhibition of mercuric chloride–, methylmercury chloride–, and thimerosal-induced PLD activation in BPAECs (90%, 90%, and 82%, respectively) (Figure 3*B*). DMSA, a widely used and established sulfhydryl-containing metal chelating agent, effectively and significantly attenuated mercuric chloride–, methylmercury chloride–, and thimerosal-induced PLD activation BPAECs (90%, 91%, and 83%, respectively) (Figure 3*C*). Similarly, D-penicillamine, an amino acid analog of cysteine and metal-chelating drug, caused effective and significant inhibition of mercuric chloride–, methylmercury chloride–, and thimerosal-induced PLD activation in BPAECs (87%, 81%, and 62%, respectively) (Figure 3*D*). Collectively, these results revealed that thiol-protective agents effectively attenuated PLD activation induced by mercury compound(s), further suggesting the involvement of cellular thiol status in mercury-induced activation of PLD.

Antioxidants Attenuate Mercury-Induced PLD Activation

Heavy metals including mercury have been shown to cause oxidative stress, which in turn has been shown to activate PLD in different cellular systems including vascular ECs (Varadharaj et al. 2006; Valko, Morris, and Cronin 2005). Oxidatively modified cellular thiol redox status has been shown to activate PLD in ECs (Parinandi et al. 1999). Earlier results of this study also revealed that the thiol-protective antioxidant chelators (NAC, PDTC, and DMSA) significantly attenuated mercury-induced PLD activation in BPAECs, further suggesting the role of ROS and oxidative stress there in. Therefore, in order to establish the role of oxidative stress in mercury-induced PLD activation, the effects of well-established antioxidants (vitamin C, propyl

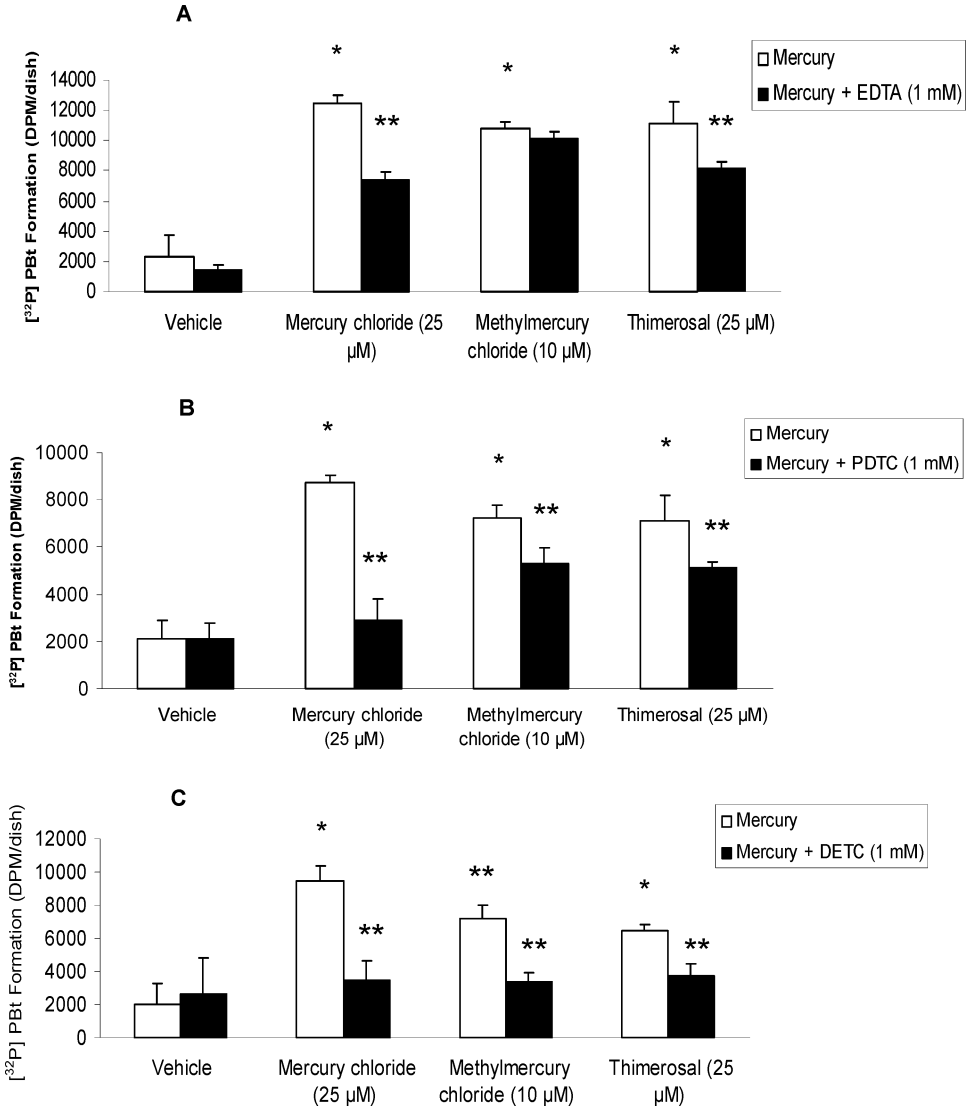


FIGURE 2

Mercury-induced PLD activation in the presence of metal chelating agents. BPAECs (5×10^5 cells/35-mm dish) were labeled with [^{32}P]orthophosphate in DMEM phosphate-free medium for 12 h. Following [^{32}P]orthophosphate labeling, cells were pretreated for 1 h with MEM or MEM containing EDTA (1 mM; *A*), PDTC (1 mM; *B*), or DETC (1 mM; *C*) and then subjected to treatment with vehicle or mercuric chloride or methylmercury chloride or thimerosal (25, 10, and 25 μM, respectively) for 30 min in presence of 0.05% butanol. At the end of incubation, [^{32}P]PBt formed was determined as described under Materials and Methods. Data represent means $\pm$ *SD* of three independent experiments. *Significantly different at $p < .05$ as compared to cells treated with vehicle alone. **Significantly different at $p < .05$ as compared to cells treated with mercury compound(s) alone.

gallate, and catalase) were investigated on mercuric chloride–, methylmercury chloride–, and thimerosal-induced enzyme activation in BPAECs. Prior to treatment with the chosen mercury compound(s) (25 μM mercuric chloride, 10 μM methylmercury chloride, 25 μM thimerosal), cells were pretreated for 1 h with MEM or MEM containing the selected antioxidant(s) (1 mM vitamin C, 100 μM propyl gallate, 50 μg/ml catalase) and then exposed to the chosen mercury compound(s) for 30 min in presence of the antioxidant(s). As shown in Figure 4*A*, vitamin C, a well-known redox-active antioxidant, caused significant inhibition of mercuric chloride– and thimerosal-induced PLD activation in ECs (67% and 38%, respectively), whereas the same did not cause significant inhibition of methylmercury chloride–induced activation of the enzyme in cells. Propyl gallate, a well-established phenolic antioxidant, effectively and significantly attenuated mercuric chloride– and methylmercury chloride–induced PLD activation in BPAECs (94% and 16%, respectively), whereas the same did not significantly attenuate thimerosal-induced PLD activation in ECs (Figure 4*B*). On the other hand, catalase, an enzymatic antioxidant that removes H_2O_2, slightly attenuated the PLD activation induced by only methylmercury chloride in BPAECs (Figure 4*C*). These results

revealed that (a) certain antioxidants were only effective in attenuating mercury induced PLD activation in BPAECs, and (b) the effectiveness of antioxidants in causing the attenuation of mercury-induced PLD activation was antioxidant-specific or mercury compound–specific or type of radical generated by the selected mercury compound. Collectively, antioxidant-mediated inhibition of activation of PLD in BPAECs suggested the role of oxidative stress in mercury-induced enzyme activation.

Methylmercury Chloride Induces ROS Generation in a Dose-Dependent Fashion

As antioxidants attenuated PLD activation in BPAECs induced by mercury, it was hypothesized here that all three chosen forms of mercury in this study would induce ROS generation in ECs. In order to test the hypothesis, BPAECs were treated with different concentrations (0 to 15 μM) of mercuric chloride, methylmercury chloride, and thimerosal for 30 min and the subsequent formation of intracellular ROS was determined. Of all the three mercury compounds, methylmercury chloride was the only mercury species that induced detectable levels of ROS in BPAECs as analyzed by the DCFDA fluorescence ROS assay. Methylmercury chloride markedly induced intracellular ROS formation (4.5-fold and 6.6-fold at 10 and 15 μM concentrations, respectively) as compared to that in the vehicle-treated cells (Figure 5*A*). These results showed that methylmercury chloride was effective in inducing generation of ROS in BPAECs.

Antioxidants Attenuate Methylmercury Chloride-Induced ROS Generation

Our previous results showed that methylmercury chloride induced intracellular ROS generation in BPAECs in a dose-dependent fashion. Therefore, here we investigated the effects of antioxidants on the methylmercury chloride-induced intracellular ROS generation in ECs. Prior to challenging the BPAEC with methylmercury chloride (10 μM) for 30 min, cells were

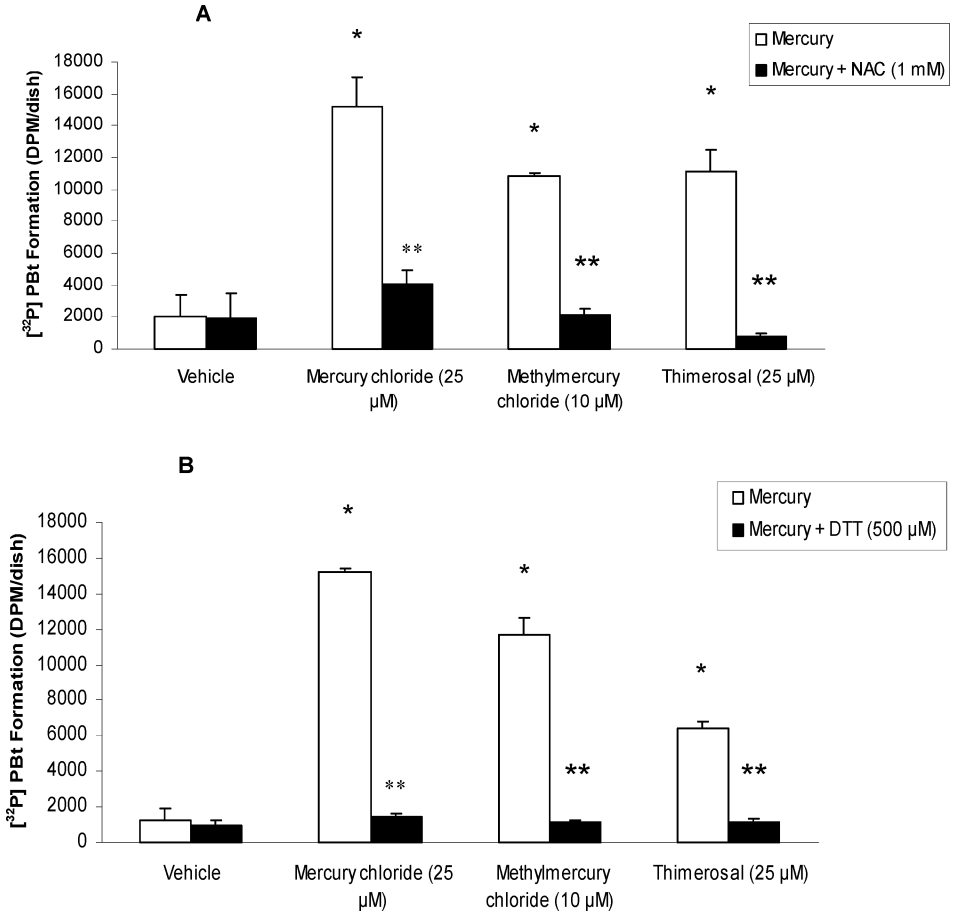


FIGURE 3

Mercury-induced PLD activation in the presence of sulfhydryl agents. BPAECs (5×10^5 cells/35-mm dish) were labeled with [^{32}P]orthophosphate in DMEM phosphate-free medium for 12 h. Following [^{32}P]orthophosphate labeling, cells were pretreated for 1 h with MEM alone or MEM containing NAC (1 mM; *A*), DTT (500 μM; *B*), DMSA (1 mM; *C*), or D-penicillamine (1 mM; *D*) and then subjected to treatment with vehicle or mercuric chloride or methylmercury chloride or thimerosal (25, 10, and 25 μM, respectively) for 30 min in presence of 0.05% butanol. At the end of incubation, [^{32}P]PBt formed was determined as described under Materials and Methods. Data represent means $\pm$ *SD* of three independent experiments. *Significantly different at $p < .05$ as compared to cells treated with vehicle alone. **Significantly different at $p < .05$ as compared to cells treated with mercury compound(s) alone. *(Continued)*

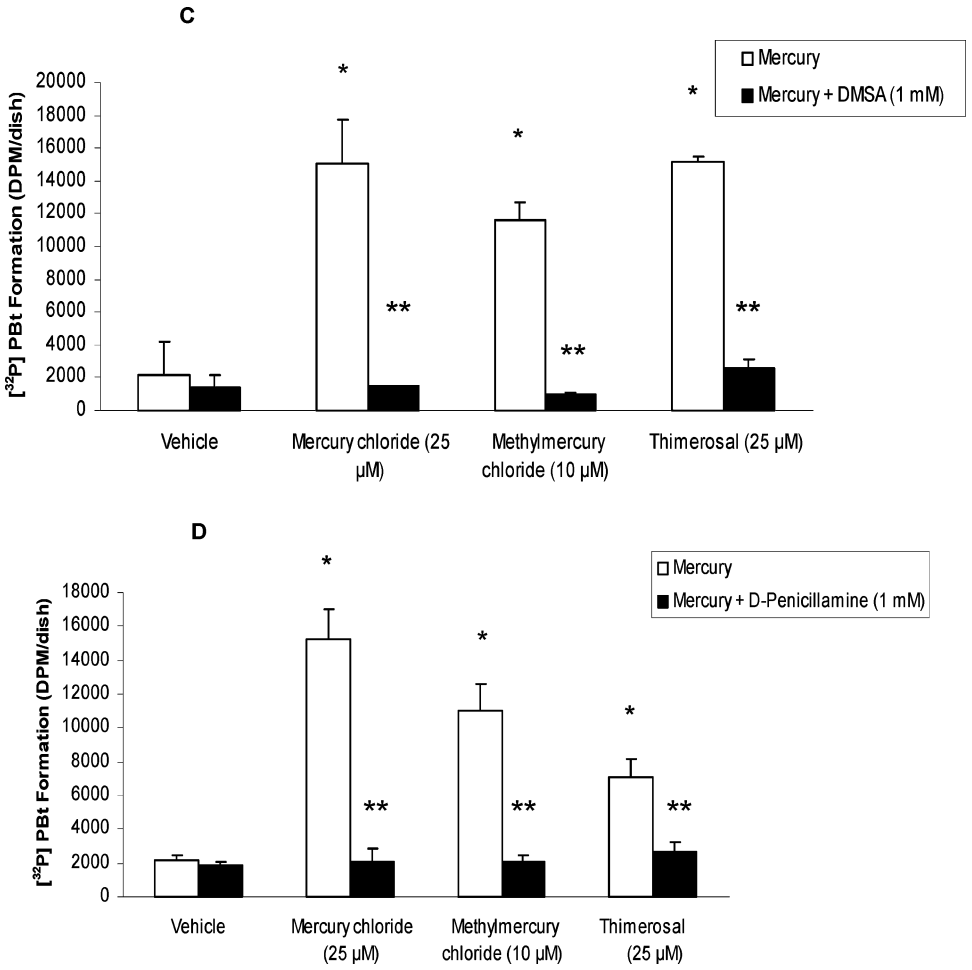


FIGURE 3
(Continued)

pretreated with two well-established antioxidants (NAC 10 mM and MnTBAP 10 μM) for 2 h and then treated with methylmercury chloride (10 μM) for 30 min. NAC markedly attenuated the methylmercury chloride-induced intracellular ROS generation (70%) to the similar extent exhibited by the cells treated with vehicle alone (Figure 5*B*). However, the superoxide dismutase mimetic, MnTBAP, was only effective to cause a lower (30% decrease) attenuation of the methylmercury chloride-induced intracellular ROS generation in BPAECs (Figure 5*C*). Overall, NAC appeared to be the most efficient antioxidant in causing a marked attenuation of methylmercury chloride-induced intracellular ROS formation in BPAECs.

Mercury Induces the Loss of Levels of Total Cellular Thiols

As it is established that mercury complexes with cellular thiols and also the results of the present study showed that sulfhydryl (thiol) protectants attenuated mercury-induced PLD activation in BPAECs, here we investigated whether the three different forms of mercury would alter the levels of total cellular thiols in the ECs. Following exposure of BPAECs to mercuric chloride (25 μM), thimerosal (25 μM), and methylmercury chloride (10 μM) for 60 min, it was observed that the levels of total cellular thiols significantly declined (Figure 6). Among all the tested mercury compounds, it was noticed that methylmercury chloride was the most effective form in decreasing the levels of total cellular thiols. Taken together, these results revealed that the inorganic and organic forms of mercury chosen in this study significantly lowered the levels of total thiols in ECs.

DISCUSSION

The results of the present study revealed that mercury as mercuric chloride (inorganic form), methylmercury chloride (environmental organic form), and thimerosal (pharmaceutical organic form) induced the activation of PLD in vascular ECs in culture. This is the first observation showing the heavy metal–induced activation of PLD in a biological system. The study also showed that metal chelators, sulfhydryl protective agents, and

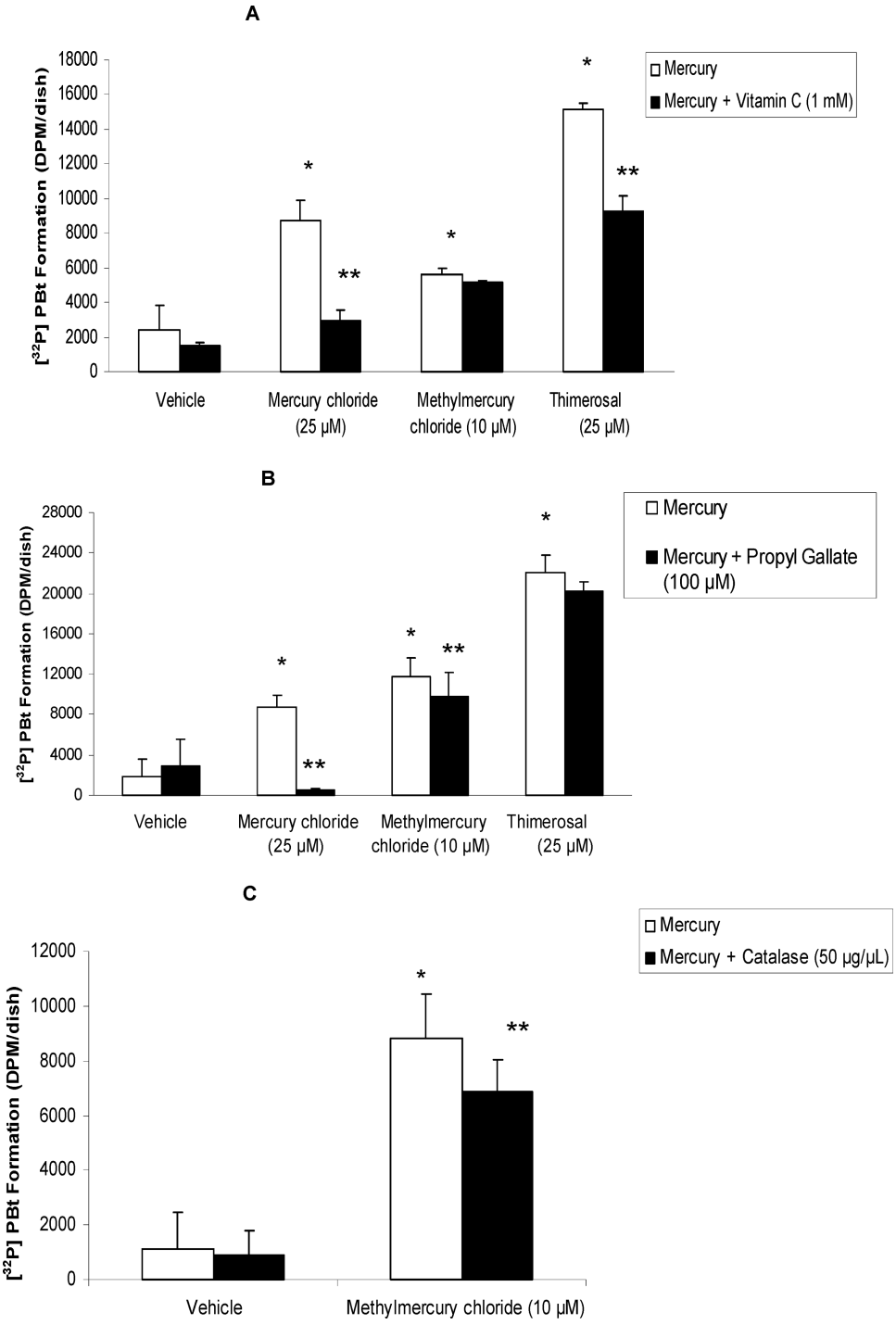


FIGURE 4

Mercury-induced PLD activation in the presence of antioxidants. BPAECs (5×10^5 cells/35-mm dish) were labeled with [^{32}P]orthophosphate in DMEM phosphate-free medium for 12 h. Following [^{32}P]orthophosphate labeling, cells were pretreated for 1 h with MEM alone or MEM containing vitamin C (1 mM; *A*), propyl gallate (100 μM; *B*), or catalase (50 μg/μl; *C*) and then subjected to treatment with vehicle or mercuric chloride or methylmercury chloride or thimerosal (25, 10, and 25 μM, respectively) for 30 min in presence of 0.05% butanol for 30 min. At the end of incubation, [^{32}P]PBt formed was determined as described under Materials and Methods. Data represent means ± *SD* of three independent experiments. *Significantly different at $p < .05$ as compared to cells treated with vehicle alone. **Significantly different at $p < .05$ as compared to cells treated with mercury compound(s) alone.

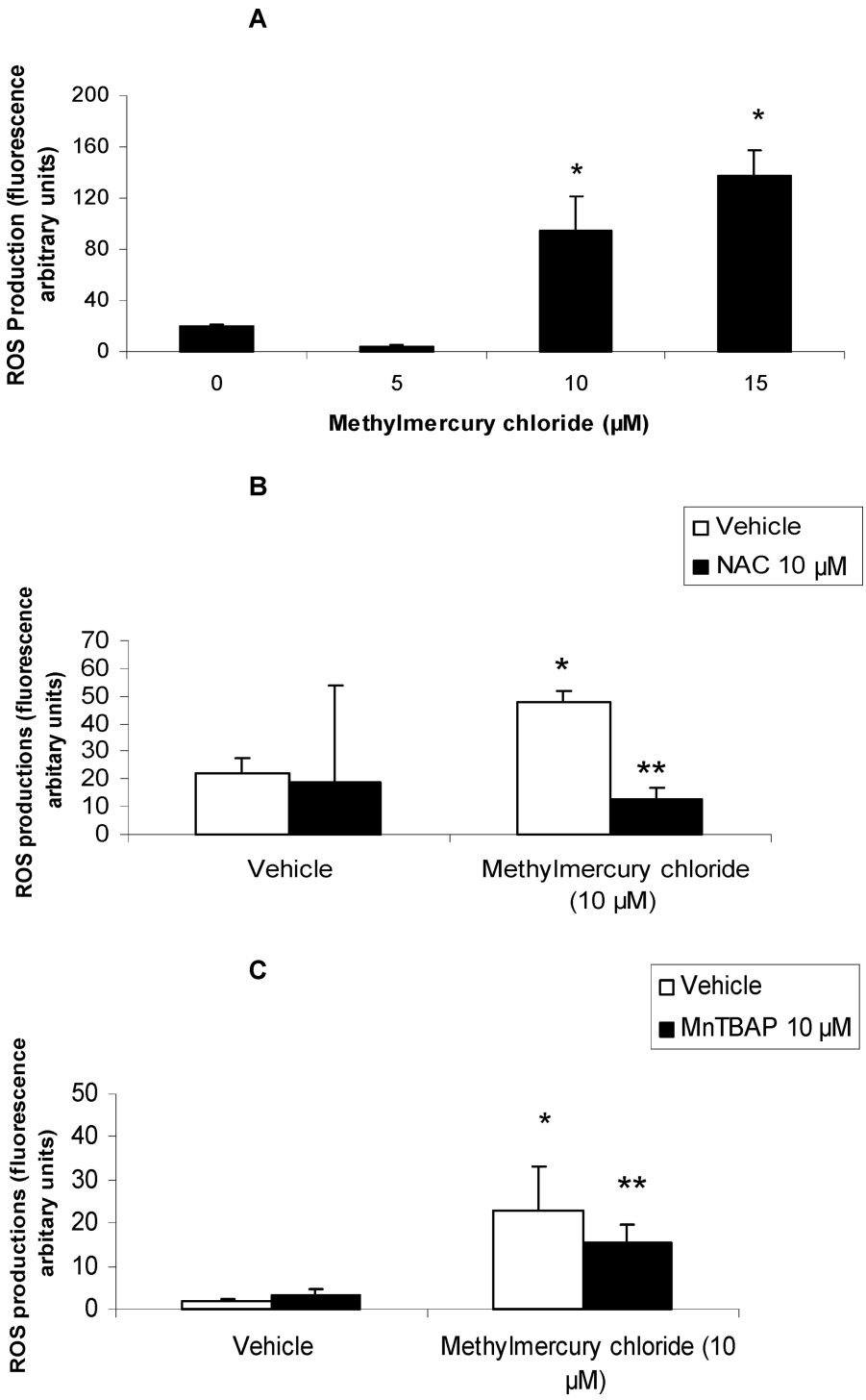


FIGURE 5

Induction of ROS generation by methylmercury chloride in a dose-dependent fashion. BPAECs (5×10^5 cells/35-mm dish) were treated with basal MEM or MEM containing increasing concentrations of methylmercury chloride (0 to 15 μM) and incubated for 30 min (*A*). To investigate the effects of antioxidants, BPAECs (5×10^5 cells/35-mm dish) were treated for 1 h with basal MEM or MEM containing NAC (10 mM) (*B*) or MnTBAP (10 μM) (*C*) and then treated with methylmercury chloride for 30 min in presence of antioxidants. At the end of incubation, intracellular formation of ROS was determined by measuring DCFDA fluorescence as described in Materials and Methods. Data represent means $\pm$ *SD* of three independent experiments. *Significantly different at $p < .05$ as compared to the cells treated with the vehicle alone. **Significantly different at $p < .05$ as compared to the cells treated with methylmercury chloride alone.

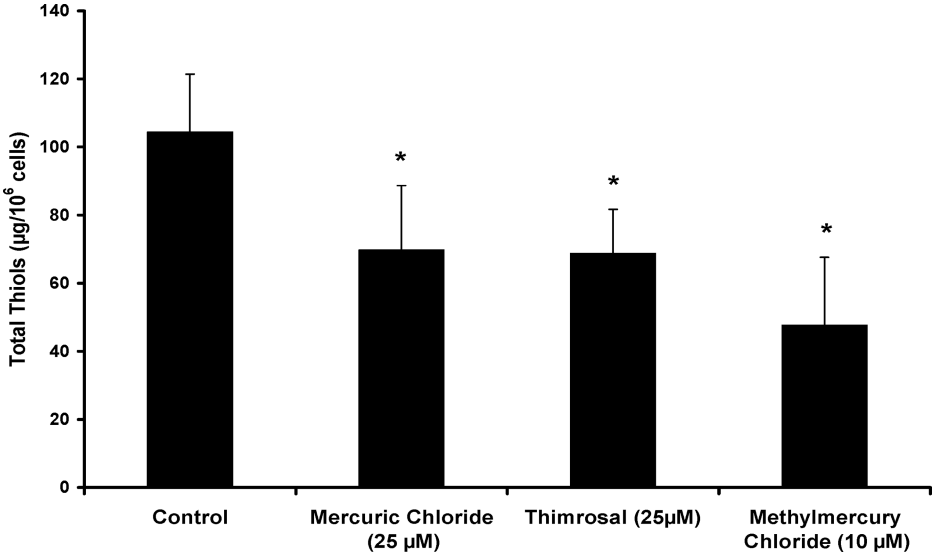
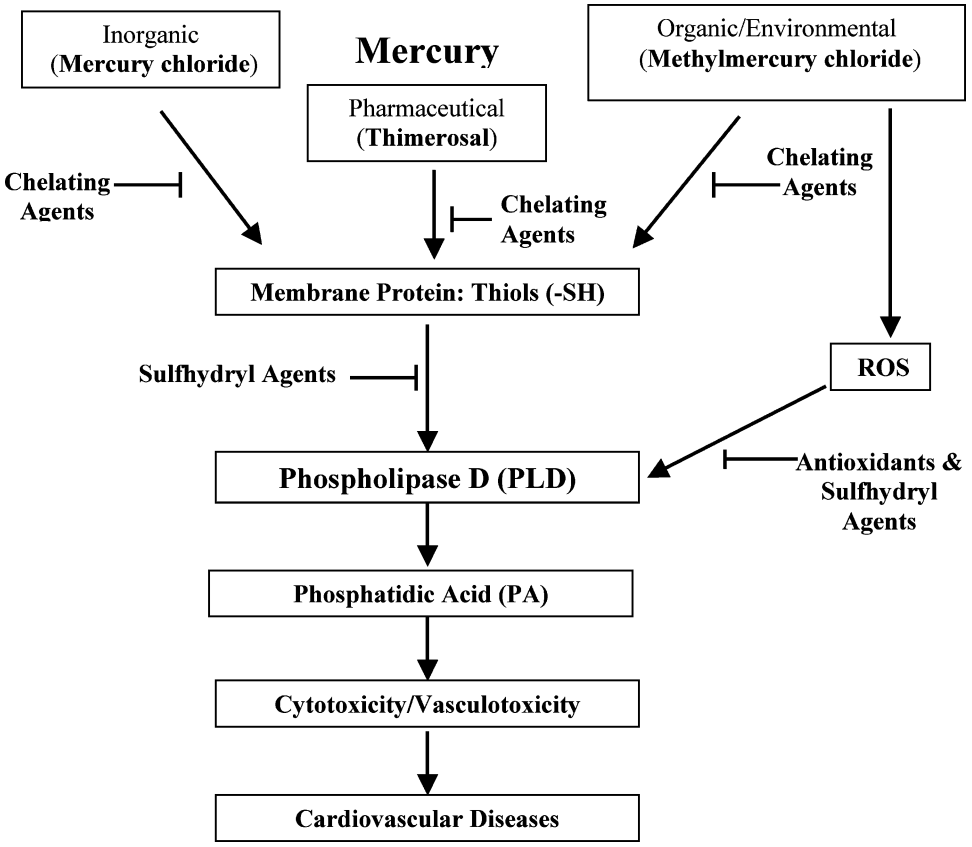


FIGURE 6

Mercury-induced decrease in the levels of total thiols. BPAECs in (2.5×10^6cells/100-mm dish) were treated with basal MEM or MEM containing mercuric chloride (25 μM), methylmercury chloride (10 μM), or thimerosal (25 μM) and incubated for 60 min. After incubation, total thiols were determined spectrophotometrically as described in Materials and Methods. Data represent means $\pm$ *SD* of three independent experiments. *Significantly different at $p < .05$ as compared to cells treated with vehicle alone.

antioxidants attenuated the mercury-induced activation of PLD in ECs. The observation in this study that methylmercury chloride induced the generation of ROS and a decrease in the levels of total thiols in ECs suggested the involvement of thiols and ROS/oxidative stress in the methylmercury chloride-induced PLD activation in ECs. Although ROS formation was induced by methylmercury, it appeared to contribute to PLD activation via feedback up to some extent as indicated by specific antioxidant effects (catalase and MnTBAP; Figure 5*C*). If PLD activation were solely induced by methylmercury through primarily ROS generation, the specific antioxidants would have completely or to the major extent inhibited the methylmercury-induced PLD activation in ECs. However, ROS formation was apparently an effect that also fed back to the activation of PLD. It also appeared that mercury-induced activation of PLD operated on a different mechanism from that induced by ROS as observed in our earlier studies with hydrogen peroxide and fatty acid hydroperoxides. It is conceivable that methylmercury would have activated membrane-bound oxidases that might have also generated extracellular ROS and extracellular addition of catalase would inhibit that ROS production. That could be the reason why as shown in the Figure 4*C*, catalase was not that effective to markedly inhibit the methylmercury-induced PLD activation in ECs. Collectively, the results of the current study showed for the first time that mercury induced the activation of PLD in BPAECs involving the cellular thiols and ROS upstream of activation of the enzyme (Schema 2).

Several agonists such as hormones, growth factors, neurotransmitters, cytokines, and ROS have been shown to activate PLD in different mammalian cells and tissues through agonist-specific and cell-specific signaling mechanisms of regulation (Varadharaj et al. 2006). Oxidant (ROS)-induced activation of PLD in many cell systems including ECs has been shown to be regulated by protein kinase C (PKC), mitogen-activated protein kinases, and tyrosine kinases (Varadharaj et al. 2006; Parinandi et al. 2001; Natarajan et al. 2001). Redox regulation of PLD in ECs involving the thiol-redox system has been documented (Parinandi et al. 1999). The results of the current study clearly showed that sulfhydryl protectants and antioxidants attenuated the mercury-induced PLD activation in BPAECs, further suggesting the oxidant and thiol regulation of mercury-induced PLD activation in ECs. Mercury toxicity has been shown to be associated with metal-induced oxidative stress involving lipid peroxidation and protein oxidation (Valko, Morris, and Cronin 2005). Depletion of GSH and binding of the metal to protein thiols have been linked to heavy metal toxicity (Valko, Morris, and Cronin 2005). From our results, we surmise that vitamin C, in addition to acting as an antioxidant, would also have complexed with mercury (mercuric chloride and mercury in thimerosal) and reduced the reactivity of the element. Similarly, the natural product antioxidant, propyl gallate may have possibly complexed with mercury (e.g., more with inorganic mercury). Several studies have also shown that methylmercury induces oxidative stress in cellular systems leading to cytotoxicity (Garg and Chang 2006). Together, the observations that mercury caused the generation of ROS and antioxidants and sulfhydryl agents attenuated the mercury-induced activation of PLD in BPAECs confirmed that oxidative stress and alterations in cellular thiols played a significant role in the mercury-induced activation of PLD in ECs. This is further supported by the observation in the current study that



SCHEMA 2
Mechanism of mercury-induced activation of PLD in ECs.

all the three chosen mercury compounds decreased the levels of total thiols in the ECs.

Metal chelators have been used widely as drugs in treating heavy metal poisoning and toxicity in cellular systems, laboratory animal models, and humans in clinical settings (Blanusa et al. 2005). Complexation of heavy metals by proteins at the histidine and cysteine (–SH) residues has been recognized as one of the primary mechanisms of heavy-metal toxicity (Onyido, Norris, and Buncel 2004). PLD is an enzyme with histidine in the active site (Morris, Frohman, and Engebrecht 1997) and it is conceivable that mercury may directly bind to this residue thus leading to its activation. Alternatively, sulfhydryl groups (GSH and protein thiols) can be good candidates to react with mercury in the cell for metal-ligand complex formation and may cause conformational changes in PLD leading to the activation of the enzyme. Thus, the possible use of those chelators in alleviating the adverse effects of mercury such as the activation of PLD may also be associated with changes in membrane fluidity and disruption in the bilayer.

The physiological significance of agonist-induced PLD activation in modulating EC function is emerging (Varadharaj et al. 2006). Exogenous administration of PA (the bioactive lipid signal mediator generated by PLD) has been shown to increase albumin paracellular flux across EC monolayers in culture (English, Cui, and Siddiqui 1996). Based on the results of the current study that mercury induced the activation of PLD in BPAECs, it is surmised here that mercury through PLD-generated PA formation may cause EC barrier dysfunction. Although it appears from the published reports that there is compelling evidence in favor of the association of mercury levels in human body and prevalence of cardiovascular disease, more experimental evidences are warranted to establish the role of mercury in cardiovascular disease. In conclusion, the results of this study showed for the first time that mercury induced the activation of PLD in Ecs, suggesting the role of PLD-generated bioactive lipid signal mediators in the toxicity of mercury to vascular endothelium and blood vessel and also mercury-associated cardiovascular diseases.

REFERENCES

Blanusa, M., V. M. Varnai, M. Piasek, and K. Kostial. 2005. Chelators as antidotes of metal toxicity: Therapeutic and experimental aspects. *Curr. Med. Chem.* 12:2771–2794.

Boening, D. W. 2000. Ecological effects, transport, and fate of mercury: A general review. *Chemosphere* 40:1335–1351.

Clarkson, T. W. 2002. The three modern faces of mercury. *Environ. Health Perspect.* 110:11–23.

Clarkson, T. W., L. Magos, and G. J. Myers. 2003. The toxicology of mercury-current exposures and clinical manifestations. *N. Engl. J. Med.* 349:1731–1737.

Divecha, N., and R. F. Irvine. 1995. Phospholipid signaling. *Cell* 80:269–278.

Dopp, E., L. M. Hartmann, A. M. Florea, A. W. Rettenmeier, and A. V. Hirner. 2004. Environmental distribution, analysis, and toxicity of organometal (loid) compounds. *Crit. Rev. Toxicol.* 34:301–333.

Egermayer, P. 2000. Epidemics of vascular toxicity and pulmonary hypertension: What can be learned? *J. Intern. Med.* 247:11–17.

English, D., Y. Cui, and R. A. Siddiqui. 1996. Messenger function of phosphatidic acid. *Chem. Phys. Lipids* 80:117–132.

Exton, J. H. 1999. Regulation of phospholipase D. *Biochim. Biophys. Acta* 1439:121–133.

Garg, T. K., and J. Y. Chang. 2006. Methylmercury causes oxidative stress and cytotoxicity in microglia: attenuation by 15-deoxy-delta 12, 14-prostaglandin J2. *J. Neuroimmunol.* 171:17–28.

Grether, J. K, L. A. Croen, C. A. Theis, M. F. Blaxill, B. E.Haley, and A. S. Holmes. 2004. Babyhair, mercury toxicity and autism. *Int. J. Toxicol.* 23:275–276.

Kim, D. S., E. H. Lee, S. D. Yu, J. H. Cha, and S. C Ahn. 2005. Heavy metal as risk factor of cardiovascular disease—an analysis of blood lead and urinary mercury. *J. Prev. Med Pub. Health* 38:401–407.

Kostka, B. 1991. Toxicity of mercury compounds as a possible risk for cardiovascular diseases. *Br. J. Ind. Med.* 48:845.

Landmark, K., and I. Aursnes. 2004. Mercury, fish, fish oil and the risk of cardiovascular disease. *Tidsskr. Nor. Laegeforen.* 124:198–200.

Morris, A. J., M. A. Frohman, and J. Engebrecht. 1997. Measurement of phospholipase D activity. *Anal. Biochem.* 252:1–9.

Mutter, J., J. Naumann, C. Sadaghiani, H. Walach, and G. Drasch. 2004. Amalgam studies disregarding basic principles of mercury toxicity. *Int. J. Hyg. Environ. Health* 207:391–397.

Mutter, J., J. Naumann, R. Schneider, H. Walach, and B. Haley. 2005. Mercury and autism: Accelerating evidence? *Neurol. Endocrinol. Lett.* 26:439–446.

Nash, R. A. 2005. Metals in medicine. *Altern. Ther. Health Med.* 11:18–25.

Natarajan, V. 1995. Oxidants and signal transduction in vascular endothelium. *J. Lab. Clin. Med.* 125:26–37.

Natarajan, V., W. M. Scribner, A. J. Morris, S. Roy, S. Vepa, J. Yang, R. Wadgaonkar, S. P. Reddy, J. G. Garcia, and N. L. Parinandi. 2001. Role of p38 MAP kinase in diperoxovanadate-induced phospholipase D activation in endothelial cells. *Am. J. Physiol. Lung Cell Mol. Physiol.* 281:L435–L449.

Onyido, I., A. R. Norris, and E. Buncel. 2004. Biomolecule–mercury interactions: Modalities of DNA base–mercury binding mechanisms. Remediation strategies. *Chem. Rev.* 104:5911–5929.

Parinandi, N. L., M. A. Kleinberg, P. V. Usatyuk, R. J. Cummings, A. Pennathur, A. J. Cardounel, J. L. Zweier, J. G. Garcia, and V. Natarajan. 2003. Hyperoxia-induced NAD(P)H oxidase activation and regulation by MAP kinases in human lung endothelial cells. *Am. J. Physiol. Lung Cell Mol. Physiol.* 284:L26–L38.

Parinandi, N. L., S. Roy, S. Shi, R. J. Cummings, A. J. Morris, J. G. Garcia, and V. Natarajan. 2001. Role of Src kinase in diperoxovanadate-mediated activation of phospholipase D in endothelial cells. *Arch. Biochem. Biophys.* 396:231–243.

Parinandi, N. L., W. M. Scribner, S. Vepa, S. Shi, and V. Natarajan. 1999. Phospholipase D activation in endothelial cells is redox sensitive. *Antioxid. Redox Signal.* 1:193–210.

Sarkar, B. A. 2005. Mercury in the environment: Effect on health and reproduction. *Rev. Environ. Health* 20:39–56.

Ustinaviciene, R., V. Obelenis, and D. Ereminas. 2004. Occupational health problems in modern work environment. *Medicina* 40:897–904.

Valko, M., H. Morris, and M. T. Cronin. 2005. Metals, toxicity and oxidative stress. *Curr. Med. Chem.* 12:1161–208.

Varadharaj, S., E. Steinhour, M. G. Hunter, T. Watkins, C. P. Baran, U. Magalang, P. Kuppusamy, J. L. Zweier, C. B. Marsh, V. Natarajan, and N. L. Parinandi. 2006. Vitamin C-induced activation of phospholipase D in lung microvascular endothelial cells: Regulation by MAP kinases. *Cell Signal* 18:1396–1407.

Wakita, Y. 1987. Hypertension induced by methyl mercury in rats. *Toxicol. Appl. Pharmacol.* 89:144–147.

Yoshizawa, K., E. B. Rimm, J. S. Morris, V. L. Spate, C. C Hsieh, D. Spiegelman, M. J. Stampfer, and W. C. Willett. 2002. Mercury and the risk of coronary heart disease in men. *N. Engl. J. Med.* 347:1755–1760.