Mercury activates phospholipase D in vascular endothelial cells: Implications for environmental cardiovascular disease

A Senior Honors Thesis

Presented in Partial Fulfillment of the Requirements for graduation *with distinction* in Biology in the undergraduate colleges of The Ohio State University

By
Thomas Hagele

The Ohio State University
June 2006

Project Advisor: Narasimham Parinandi, Ph.D.
Division of Pulmonary, Critical Care, and Sleep Medicine
Department of Medicine
Dorothy M. Davis Heart and Lung Research Institute
College of Medicine
The Ohio State University

Acknowledgements

Slightly more than a year ago I first came to Dr. Parinandi's lab. Like many of his undergraduate researchers, I originally came to the lab as a volunteer through the University Hospital. I did not know what opportunities would await me. As a volunteer, I planned on cleaning glass ware for a couple hours a week in his lab. I quickly learned, becoming a part of Dr. Parinandi's lab involved no such thing. Dr. Parinandi told me it would no longer be necessary to wear the black volunteering smock and offered me an opportunity to work in his lab; very different from my original glass ware cleaning expectations. As an undergraduate looking for research opportunities, I agreed to join the lab. Over the past year, the lab has become a second home to me. I have built relationships within the lab and within the research community. I am thankful for the opportunities and the hospitality I have been given.

I wish to express thanks to all those who helped me during the thesis research process. I want to thank my co-workers, Bruce Kaufman, Jessie Mazerik, Joe Hild, Valorie Ciapala, Alon Peltz, Emily Steinhour and Anita Gregory for aiding me with experiments. Their time and effort have been appreciated. I also wish to thank Dr. Parinandi for mentoring and assisting me over the past year. His guidance is much appreciated and was pertinent in the development of the thesis.

Table of Contents

Table of Figures

Abstract

Currently, mercury has been identified as a risk factor of cardiovascular diseases among humans. Here, we have hypothesized 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. In order to test the hypothesis, monolayers of bovine pulmonary artery ECs (BPAECs) in culture, following labeling of membrane phospholipids with [^{32}P], were exposed to mercuric chloride (inorganic form), methylmercury chloride (environmental), and thimerosal (pharmaceutical), 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 (0-50 μM) and time-dependent (0-60 min) fashion. Metal chelators significantly attenuated mercury-induced PLD activation in BPAECs, suggesting that cellular mercury-ligand interaction(s) is required for the enzyme activation and that chelators are suitable blockers for mercury-induced PLD activation in ECs. Sulfhydryl (thiol-protective) agents and antioxidants also significantly attenuated the mercury-induced PLD activation in BPAECs, suggesting that the cellular thiols are probable targets for mercury action and that reactive oxygen species (ROS) play a role in mercury-induced PLD activation in ECs. Increases in ROS generation were observed in BPAECs following the exposure of cells to methylmercury that was attenuated by antioxidants; this confirmed the involvement of ROS/oxidative stress in the mercury-induced PLD activation in BPAECs. For the first time, the results of this study revealed that mercury induced the activation of PLD in the vascular ECs wherein cellular thiols and ROS acted as signal mediators for the enzyme activation. The results suggest further that PLD signaling may play an important role in mercury-induced endothelial dysfunctions and cardiovascular diseases.

Introduction

Mercury (Hg) is a heavy metal belonging to the transition element series of the periodic table. The element has been in commercial (industrial) and medical uses for centuries (1) which results in occupational exposures to mercury. Although mercury occurs naturally in the environment, anthropogenic activities cause the release of the element into the environment leading to the pollution of air, water, and soil (1,2). Coal and oil-fired power plants have been identified as the largest source of emission of mercury into the environment in the United States (4). Inorganic mercury is toxic to many organisms and is converted to more toxic organic forms (methylmercury) through biomethylation by microorganisms (bacteria) (3). Methylmercury reaches the organisms in the food chain and ultimately accumulates in humans, the top consumers (3). Methylmercury toxicity to the experimental animals, poisoning and environmental toxicity to humans are well documented (5). Dental amalgam fillings containing mercury have been in uncontrolled use in dental care worldwide(6). Mercury leaching from the implanted dental amalgams has been attributed to mercury-induced adverse health effects in humans (6,7). Mercury has also been recognized as a toxicant in occupational environments, contributing to work related disorders/diseases such as upper respiratory tract diseases, lung diseases, cardiovascular diseases, musculoskeletal disorders, and nervous system diseases among workers (10). 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 (11,12).

Fish are contaminated with mercury in the form of methylmercury in the aquatic environment (12) and fish eaters are exposed to methylmercury. Methylmercury has been a well-documented environmental teratogen in animals including humans (13). Fish are also good

sources of omega-3 fatty acids, which have been shown to offer a multitude of heath benefits including better cardiovascular health among humans (13,16). Consumption of fish has been shown as a major source of environmental mercury in humans that could lead to suppression of the beneficial effects of omega-3 fatty acids on the coronary artery disease (14,15).

The role of mercury toxicity as a possible risk factor in cardiovascular disease has been discussed (17). Reports have been made on the toxic effects of metals in several diseases among humans including the vascular diseases (18). The mercury levels in the toe nails of humans have been shown to be associated with the risk of myocardial infarction and high mercury levels have been attributed to the diminished cardioprotective effects of fish consumption among humans (19). Therefore, elevated body levels of mercury, due to fish consumption by humans, have been hypothesized as a risk factor in coronary heart disease (20). 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 (21). An association between the occupational exposure to mercury in mining and refining and risk of cardiovascular diseases has been shown (22).

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 lacking at the current time. 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 (23). Mercury-induced vascular endothelial damage and vasculitis in humans upon autopsy have been documented (24). 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 the structural and functional entities. Phospholipases are enzymes which specifically hydrolyze the membrane phospholipids and generate bioactive lipid second messengers, which play a vital role in cell signaling (25). 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 (26) (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 (26-28). Agonist-mediated activation of PLD plays a pivotal role in signal transduction in mammalian cells (26,29-31). Several functions, such as promotion of mitogenesis in fibroblasts, stimulation of oxidative bursts in neutrophils, increase of intracellular calcium, and activation of protein kinases and phospholipases have been attributed to the signaling actions of PA/LPA (26,29-31). Two major forms of PLD, $hPLD_1$ and $hPLD_2$, have been cloned in mammalian cells; They are selectively activated by various cofactors such as Arf, Rho, Cdc42, detergents, and phosphatidylinositol 4,5-biphosphate (PIP_2) in cell-free preparations (32-38).

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 system (BPAECs) was chosen here as the EC 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 was investigated. For the first time, this study revealed that mercury as mercuric chloride, methylmercury chloride, and thimerosal representing the inorganic, environmental, and pharmacological forms of the element respectively, induced PLD activation in vascular ECs which was attenuated by heavy metal chelator agents and drugs, sulfhydryl group protectants, and antioxidants, suggesting that mercury-induced PLD activation in vascular ECs in culture was mediated by a mechanism involving cellular sulfhydryl groups (thiols) and intracellular ROS.

Materials and Methods

Materials. Bovine pulmonary artery endothelial cells (BPAECs) (passage 2) were purchased from Cell Applications Inc. (San Diego, CA). Minimal essential medium (MEM), fetal bovine serum (FBS), trypsin, nonessential amino acids, DMEM phosphate-free medium, mercury chloride, methylmercury chloride, mercury acetate, mercury sulfate, thimerosal, propyl gallate, absorbic acid (vitamin C), catalase, ethylenediaminetetracetic acid (EDTA), pyrrollinedine dithiocarbamate (PDTC), D-penicillamine, dietyldithiocarbamic acid (DETC), dithiothreitol (DTT), N-acetyl-L-cysteine (NAC), and meso-2,3-dimercapto-succinic acid (DMSA) were obtained from Sigma Chemical Co. (St. Louis, MO). MnTBAP was obtained from Calbiochem (San Diego, CA). [^{32}P]orthophospate 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, antibiotics, and growth as described previously (39). Cells in culture were maintained at 37º 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 X 10-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.

PLD activation in ECs. BPAECs in 35-mm dishes (5 X 10^5 cells/dish) were incubated with [^{32}P]orthophosphate (5 μCi/ml) in phosphate-free DMEM containing 2% FBS for 18-25 h at 37º C in 5% CO_2 and 95% air (39). 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 mercuric sulfate or mercuric acetate or methylmercury chloride or thimerosal) at the chosen μM concentrations in the presence of 0.05% butanol for specified lengths of time (0-60 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 absence or presence of the pharmacological inhibitors and in the presence of 0.05% butanol for specified lengths of time. Incubations were terminated by the addition of 1 mL of methanol:HCl (100:1 vol/vol) and lipids were extracted in chloroform:methanol (2:1 vol/vol) (39). [^{32}P]PBt formed as a result of PLD activation and concomitant transphysphatidylation reaction (an index of *in vivo* PLD activation) (Schema-2) 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 vol) 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.

ROS measurement by *DCFDA fluorescence.* Formation of ROS in BPAECs in 35-mm dishes (5 X 10^5 cells/dish) was determined by DCFDA fluorescence in cells which were loaded with 10 μM DCDFA for 30 min in complete MEM at 37º C in a 95% air-5% CO_2 environment prior to exposure to mercury compound(s) (40). 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 cell was transferred to 1.5-ml microcentrifuge tubes and centrifuged at 8,000 g for 10 min at 4º C. The supernatant medium was aspirated, and the cell pellet was washed twice with ice-cold PBS and sonicated on ice with a probe sonicator at a setting of 5 for 15 s in 500 µl of ice-cold PBS to prepare cell lysates. 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.

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, mercuric sulfate, mercuric acetate, thimerosal, and methylmercury chloride) would activate PLD in BPAECs in a dose dependent (0-50 μM) fashion following incubation of cells for 1 and 2 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 (Fig. 1A). At 2 h of incubation, although mercuric chloride caused a significant activation of PLD at 10 and 25 μM concentrations similar to that observed at 1 h of treatment of cells with the same, the cell exhibited 25-fold increase in PLD activation upon exposure of cells to 50 μM of mercuric chloride as compared to that in the vehicle-treated cells (Fig. 1A). BPAECs also showed similar extent of activation of PLD following exposure to mercuric sulfate. Mercuric sulfate significantly caused 5-, 9-, and 12-fold increase in activation of PLD at 10, 25, and 50 μM doses, respectively upon treatment of cells for 1 h as compared to that in cells exposed to vehicle alone (Fig. 1B). On the other hand, mercuric sulfate, only at 25 μM and 50 μM doses, significantly caused 9- and 8.5-fold activation of PLD in BPAECs following exposure of cells to the compound for 2 h as compared to that in cells treated with vehicle alone (Fig. 1B). Both chloride and sulfate forms of mercury significantly caused a increase in the activation of PLD in BPAECs with increase in dose. Mercury acetate significantly resulted in 4.5-, 7.5-, and 5-fold increases in PLD activation at 10, 25, and 50 μM doses of the compound respectively, following exposure of cells to 1 h treatment with the compound as compared to the same in the cells exposed to vehicle

alone (Fig. 1C). BPAECs exhibited 2.5-, 7-, and 4-fold increases in PLD activation when exposed to 10, 25, and 50 μM mercury acetate respectively for 2 h as compared to the cells treated with vehicle alone (Fig. 1C). Although mercury acetate resulted in a significant dose-dependent increase with PLD activation in a linear fashion up to 25 μM concentrations of the agonist, at the 50 μM dose, the extent of mercury acetate-induced activation of the enzyme declined by approximately 3 orders of magnitude as compared to the same observed at 25 μM treatment of the same compound at 1 and 2 h (Fig. 1C). However, mercury acetate caused a dose-dependent activation of PLD in BPAECs. Thimerosal 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 (Fig. 1D). Thimerosal alone significantly causes 8-, 13.5-, and 10.5-fold increases in PLD activity in cells at 10, 25, and 50 μM concentrations, respectively following 1 h exposure of cells to the compound as compared to the same in the vehicle-treated cells (Fig. 1D). It was evident from this study that thimerosal resulted in significant and dose-dependent activation of PLD in BPAECs. Methylmercury chloride at 10, 25, and 50 μM doses caused a 3.5-, 2.5-, and 3- fold increase in PLD activity at 1h of treatment of cells with the compound respectively as compared to the same in cells treated with vehicle alone (Fig. 1E). On the other hand, methylmercury chloride significantly resulted in 4.5- 2.5, and 3-fold increase in PLD activity in BPAECs at 10, 25, and 50 μM doses respectively, following exposure of cells to the compounds for 2 h as compared to the same in the cells exposed to vehicle alone (Fig. 1E). The study also suggested that methylmercury chloride caused the maximum extent of activation at a concentration of 10 μM following exposure of cells for 1 and 2 h and upon further increasing the concentration of the agonist to 25 and 50 μM. Although methylmercury caused a significant increase in activation of PLD 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 (Fig. 1E). 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 four different mercury compounds (mercuric chloride, mercuric sulfate, thimerosal, and methylmercury chloride) was studied. As compared to the vehicle-treated cells, mercuric chloride (25 μM), even at 0 min of incubation caused a significant (9-fold) increase in PLD activation. At 10 min of treatment, mercuric chloride caused a significant activation of PLD (20-fold) in cells, which declined slightly (15-fold) at 20 and 30 min of treatment with the agonist as compared to the same in cells exposed to the agonist for 0 min (Fig. 2A). This study also revealed that cells exposed to mercuric chloride exhibited a plateau in the activation of PLD at 20 and 30 min of treatment. Mercuric sulfate (25 μM) significantly caused 2.5-, 2.5-, and 5.5-fold increase in PLD activation in BPAECs at 15, 30, and 60 min respectively, as compared to the same in cells treated with mercuric sulfate for 0 min (Fig. 2B). These results also revealed that mercuric sulfate induced a time-dependent and linear activation of PLD in ECs. Thimerosal (25 μM) induced 8-, 13-, and 26-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 activation of the enzyme in BPAECs (Fig. 2C). Methylmercury chloride significantly caused 3-, 6-, and 6-fold activation of PLD in BPAECs at 10, 20, and 30 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 four chosen forms of mercury caused a time-dependent activation of PLD in ECs.

Metal chelating agents attenuate mercuric chloride induced PLD activation

Chelating agents complex with transition metals and have been shown to protect against metal-mediated adverse effects and metal toxicity (41). Therefore, here, different chelating agents were included in this study in order to attenuate the mercuric chloride-induced PLD activation in BPAECs. Prior to exposure of cells to mercuric chloride (25 μM) for 30 min, cells were pretreated for 1 h with basal MEM or MEM containing the selected chelating agent and then incubated with mercuric chloride in presence of the chelating agent for 30 min. The classic trace element chelating agent, EDTA, significantly attenuated the mercury chloride-induced PLD activation in BPAECs (30% and 80% inhibition at 1 and 5 mM of EDTA treatment, respectively) (Fig. 3A). One of the thiocarbamate derivatives, a widely used heavy-metal chelating agent, DETC offered significant and dose dependent attenuation of mercury chloride-induced activation of PLD in BPAECs (20%, 40%, and 60% attenuation at 100 μM, 500 μM, and 1 mM of DETC treatment, respectively) (Fig. 3B). Another well-known thiocarbamate chelating agent, PDTC also significantly attenuated the mercuric chloride induced PLD activation in BPAECs (50%, 55%, and 60% attenuation at 100 μM, 500 μM, and 1 mM of PDTC treatment, respectively) in a dose-dependent fashion (Fig. 3C). The results revealed that EDTA was the most effective chelating agent in causing almost complete inhibition of the mercuric chloride induced activation in BPAECs followed by the thiocarbamate class of chelating agents.

Antioxidants and sulfhydryl agents attenuate mercuric chloride-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 (39,42). Also,

it has been reported that the thiols (non-protein and protein) are the targets for heavy metal cellular actions (41,42). Altered thiol redox has been shown to activate PLD in ECs (41,42). Therefore, in order to establish the role of oxidative stress (ROS) and thiol alterations in mercuric chloride-induced PLD activation in BPAECs, here the effects of well established antioxidants (vitamin C, propyl gallate, catalase, and NAC) and thiol (sulfhydryl) protective agents (DMSA, NAC, DTT, and D-penicillamine) were investigated on the mercuric chloride-induced PLD activation in BPAECs. Prior to treatment of cells with mercuric chloride (25 μM) for 30 min, cells were pretreated for 1 h with basal MEM or MEM containing the chosen antioxidants or thiol protective agents and then incubated with mercuric chloride in presence of the antioxidants or thiol protective agents for 30 min. Vitamin C, even at a dose of 100 μM, significantly attenuated the mercuric chloride-induced activation of PLD in BPAECs (50% decrease) and upon further increasing the concentration of vitamin C to 500 μM and 1 mM caused further significant attenuation (56% and 67% decrease) of the mercuric chloride-induced PLD activation in cells (Fig. 4A). Although catalase failed to inhibit the mercuric chloride-induced PLD activation in BPAECs, propyl gallate (100 μM) caused significant attenuation (67% decrease) of mercuric chloride-induced PLD activation (Fig. 4B). All the tested thiol protective agents (DMSA 1 mM, NAC 500 μM, DTT 500 μM, and D-penicillamine 500 μM) were the most effective agents in causing significant inhibition of mercuric chloride-induced PLD activation in BPAECs. DMSA, NAC, DTT, and D-Penicillamine caused 95%, 73%, 86%, and 90% inhibition of mercuric chloride induced PLD activation, respectively in ECs (Fig. 4B and 4C).

Metal chelating agents and antioxidants attenuate methylmercury chloride-induced PLD activation. The earlier experiments of this study revealed that the PLD activation in BPAECs

induced by the inorganic form of mercury, mercury chloride, was attenuated by the heavy metal chelating agents. Therefore, here, the effects of well established chelating agents including EDTA (1 mM), PDTC (1 mM), DETC (1 mM) and D-penicillamine (1-10 μM) were examined on the methylmercury chloride-induced PLD activation in BPAECs. Prior to treatment of cells with methylmercury chloride (10 μM) for 30 min, cells were pretreated for 1 h with basal MEM or MEM containing the chosen chelating agents and then exposed to methylmercury chloride in presence of the chelating agents for 30 min. Although EDTA caused a 9% inhibition of methylmercury chloride-induced PLD activation in BPAECs, the extent of enzyme inhibition was not statistically significant (Fig. 5A). However, the thiocarbamate chelating agents, PDTC and DETC, significantly caused 27% and 55% of attenuation of the methylmercury chloride-induced activation of PLD in BPAECs, respectively (Fig. 5B). The widely used heavy-metal chelator drug, D-penicillamine (1 and 5 μM), significantly caused 30% inhibition of the methylmercury chloride-induced PLD activation and upon increasing the concentration of D-penicillamine to 10 μM, the extent of inhibition of methylmercury chloride-induced PLD activation was 50% and significant (Fig. 5C). These results showed that the metal chelating agents offered up to 50% inhibition of methylmercury chloride-induced PLD activation in BPAECs, as opposed to attenuating 60-80% of the mercuric chloride-induced PLD activation in BPAECs.

Antioxidants and sulfhydryl agents attenuate methylmercury chloride-induced PLD activation. As the earlier results of this study revealed that mercuric chloride-induced PLD activation in ECs was significantly attenuated by antioxidants, here, the effects of antioxidants on the methylmercury chloride-induced PLD activation in BPAECs were investigated. Prior to challenging the cells with methylmercury chloride (10 μM) for 30 min, BPAECs were pretreated

for 1 h with antioxidants (NAC 1 mM; propyl gallate 50, 100, and 500 μM; vitamin C 1 mM; catalase 50 μg/mL; MnTBAP 20 μM, DTT 500 μM, and DMSA 1 mM), and then treated with methylmercury chloride for 30 min. NAC, a thiol antioxidant, significantly and almost completely (80% inhibition) attenuated the methylmercury chloride-induced PLD activation in BPAECs (Fig. 5A). Propyl gallate, the well-known phenolic antioxidant, significantly attenuated the methylmercury-induced PLD activation in a dose-dependent fashion in BPAECs (19%, 29%, and 30% decrease at 50, 100, and 500 μM doses of propyl gallate, respectively) (Fig. 5D). However, in comparison with the mercuric chloride-induced PLD activation in ECs that was attenuated by vitamin C, methylmercury chloride-induced PLD activation in BPAECs was not inhibited by vitamin C but was significantly enhanced by the vitamin (Fig. 6A). On the other hand, catalase, significantly attenuated methylmercury chloride-induced PLD activation in BPAECs (18% decrease) (Fig. 6A). MnTBAP, a superoxide dismutase mimetic, failed to attenuate but significantly enhanced the methylmercury-induced PLD activation in BPAECs (Fig. 6B). Sulfhydryl protective agents (thiols), DTT and DMSA, significantly and almost completely attenuated the methylmercury chloride-induced PLD activation in BPAECs (90% and 92% decrease at 500 μM DTT and 1 mM DMSA, respectively) (Fig. 6B). These results suggested that some selective antioxidants and all the tested sulfhydryl (thiol) protective agents significantly attenuated the methylmercury chloride-induced PLD activation in BPAECs.

Metal chelating agents and redox-active antioxidants attenuate thimerosal-induced PLD activation. As the earlier results of this study showed that metal chelating agents and antioxidants caused attenuation of mercuric chloride- and methylmercury chloride-induced PLD activation in BPAECs, here, the effects of metal chelating agents and redox-active antioxidants on the thimerosal-induced PLD activation in BPAECs were investigated. Prior to treatment of

cells with thimerosal (25 μM) for 30 min, BPAECs were pretreated with EDTA (1 mM), NAC (1 mM), PDTC (1 mM), D-penicillamine (1 mM), vitamin C (1 mM), DMSA (1 mM), and (DETC 1 mM) for 1 h and then treated with thimerosal for 30 min in presence of the metal chelating agents and antioxidants. EDTA caused significant attenuation (30% decrease) of the thimerosal-induced PLD activation in BPAECs (Fig. 7A). The two well established metal chelating agents, PDTC and D-penicillamine, significantly attenuated the thimerosal-induced PLD activation in BPAEC (30% and 65% decrease, respectively) (Fig. 7B). DETC, the other thiocarbamate chelating agent, also was effective in causing significant attenuation (40% decrease) of the thimerosal induced PLD activation in BPAEC (Fig. 8A). NAC, the well-established sulfhydryl (thiol) group protector and thiol antioxidant, significantly and completely (96% decrease) attenuated the thimerosal-induced PLD activation in BPAECs (Fig. 7A). Vitamin-C, the well characterized redox active antioxidant, significantly attenuated (35% decrease) the thimerosal induced PLD activation in cells. These results revealed that the metal chelating agents and redox active antioxidants caused significant inhibition of thimerosal-induced PLD activation in BPAECs.

Antioxidants and sulfhydryl agents attenuate thimerosal-induced PLD activation. The earlier results of this study showed that certain selected antioxidants and sulfhydryl (thiol) agents attenuated PLD activation induced by mercuric chloride and methylmercury chloride. Therefore, here, the effects of the antioxidants and sulfhydryl agents on the thimerosal-induced PLD activation in BPAECs were investigated. Prior to treatment of cells with thimerosal (25 μM) for 30 min, BPAECs were pretreated with NAC (1 mM), catalase (50 μg/mL), DTT (500 μM), MnTBAP (20 μM), DMSA (1 mM), and propyl gallate (100 μM) for 1 h, and then exposed to thimerosal for 30 min. Catalase failed to attenuate thimerosal-induced PLD activation (Fig. 8A)

whereas both MnTBAP and propyl gallate significantly attenuated (30% and 10% decrease at MnTBAP 20 μM and propyl gallate 100 μM, respectively) the thimerosal-induced PLD activation in BPAECs (Fig. 8B). All the three chosen sulfhydryl agents, NAC, DMSA, and DTT significantly and almost completely attenuate the thimerosal-induced PLD activation in BPAECs (96%, 83%, and 80% decrease at NAC 1 mM, DMSA 1 mM, and DTT 500 μM, respectively) (Figs. 7A, 7C, and 8A). Collectively, these results revealed that antioxidants and sulfhydryl (thiol protective) agents also significantly attenuated the thimerosal-induced PLD activation in BPAECs.

Methylmercury chloride induces ROS generation in a dose dependent fashion.

As antioxidants attenuated PLD activation in BPAECs induced by mercury (mercuric chloride, methylmercury chloride, and thimerosal), 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 (0-15 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 which 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) as compared to that in the vehicle-treated cells (Fig. 9A). 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 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 extent exhibited by the cells treated with vehicle alone (Fig. 9B). However, the superoxide dismutase mimetic, MnTBAP, was only effective to cause a partial (30% decrease) attenuation of the methylmercury chloride-induced intracellular ROS generation in BPAECs (Fig. 9C). Overall, NAC appeared to be the most efficient antioxidant in causing a marked attenuation of methylmercury chloride-induced intracellular ROS formation in BPAECs.

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 antioxidants attenuated the mercury-induced activation of PLD in ECs. The observation in this study that methylmercury chloride induced the generation of ROS in ECs suggested the involvement of ROS/oxidative stress in the methylmercury chloride-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 (Scheme-3).

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 (39). Oxidant (ROS)-induced activation of PLD in many cell systems including ECs has been shown to be regulated by PKC, mitogen-activated protein kinases, and tyrosine kinases (39,43,44). Redox regulation of PLD in ECs involving the thiol-redox system has been documented (45). 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 (42). Depletion of GSH and binding of the metal to protein thiols have been linked to heavy metal toxicity (42). Several

studies have also shown that methylmercury induces oxidative stress in cellular systems leading to cytotoxicity (46). The results of the current study are also in agreement with the earlier reports that methylmercury-induced generation of ROS in BPAECs. Together, the observations that mercury induced 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. Reports made earlier showing that oxidants and thiol redox alterations activate PLD in ECs support the findings of the current study along these lines (43). This is further supported by the observation in the current study that mercuric chloride induced the loss of cellular GSH in BPAECs (data not shown). However, the upstream regulation of mercury-induced PLD activation in BPAECs by PKC, MAPKs, and tyrosine kinases is not ruled out as oxidative stress and thiol redox alterations have been shown earlier to modulate PLD activation in different cellular systems through the kinase signaling cascades (43-45). This further warrants a detailed investigation.

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 (41). 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. PLD is an enzyme with histidine in the active site (38) 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. Nevertheless, upstream signaling kinases and other associated proteins which are rich in metal-

binding residues (-SH and histidine) can also form coordination complexes with mercury, undergo activation, and subsequently modulate the activity of PLD in ECs. Needless to say, the findings of this study on the attenuation of mercury-induced PLD activation by certain metal chelators indicated the arrest of possible complexation of mercury with the protein targets in BPAECs and thus assured the possible use of those chelators in alleviating the adverse effects of mercury at the cellular level such as the activation of membrane phospholipid hydrolyzing PLD which can also lead to changes in membrane fluidity and disruption in the bilayer.

The physiological significance of agonist-induced PLD activation in modulating EC function is emerging (43). 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 (43,48). Currently, the mechanisms by which PA regulates EC barrier function are not clearly understood and warrant further study. Based on the results of the current study that mercury induced the activation of PLD in BPACEs, it is surmised here that mercury through PLD-generated PA formation, may cause EC barrier dysfunction.

In conclusion, the results of this study showed for the first time that mercury induced the activation of PLD in ECs further 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

1. Clarkson TW, Magos L, Myers GJ (2003). The Toxicology of Mercury- Current Exposures and Clinical Manifestations. N. Engl. J. Med. 349, 1731-37.
2. Sarkar BA (2005). Mercury in the environment: effect on health and reproduction. Rev. Environ. Health. 20, 39-56.
3. Boening DW (2000). Ecological effects, transport, and fate of mercury: a general review. Chemosphere. 40, 1335-51.
4. Kuehn B (2005). Medical Groups Sue EPA Over Mercury Rule. JAMA. 294, 415-16.
5. Dopp E, Hartman LM, Florea AM, Rettenmeier AW, Hirner AV (2004). Environmental distribution, analysis, and toxicity of organometal (loid) compounds. Crit. Rev. Toxicol. 34, 301-33.
6. Pleva J (1994). Dental mercury—a public health hazard. Rev. Environ. Health. 10, 1-27.
7. Mutter J, Naumann J, Sadaghiani C, Walach H, Drasch G (2005). Int. J. Hyg. Environ. Health. 208, 435-8.
8. Clements CJ, McIntyre PB (2006). When science is not enough-a risk/benefit profile of thiomersal containing vaccines. Expert Opin. Drug Saf. 5, 17-29.
9. Mutter J, Naumann J, Schneider R, Walach H, Haley B (2005). Mercury and autism: accelerating evidence? Neuro. Endocrinol Lett. 26, 439-46.
10. Ustinaviciene R, Obelenis V, Eterminas D (2004). Occupational health problems in modern work environment. Medicina. 40, 897-904.
11. Counter SA, Buchanan LH (2004). Mercury exposure in children: a review. Toxicol. Appl. Pharmacol. 198, 209-30.

12. Clarkson TW (2002). The Three Modern Faces of Mercury. Environmental Health Perspectives. 110, 11-23.
13. Bouzan C, Cohen JT, Connor WE, Kris-Etherton PM, Gray GM, Konig A, Lawrence RS, Savitz DA, Teutsch SM (2005). Am. J. Prev. Med. 29, 347-52.
14. Landmark K, Aursnes I (2004). [Mercury, fish, fish oil and the risk of cardiovascular disease]. Tidsskr Nor. Laegeforen. 124, 198-200.
15. Chan HM, Egeland GM (2004). Fish consumption, mercury exposure, and heart disease. Nutr. Rev. 62, 68-72.
16. Diaz JH (2004). Is fish consumption safe. J. La. State Med. Soc. 156, 44-49.
17. Kostka B (1991). Toxicity of mercury compounds as a possible risk for cardiovascular diseases. Br. J. Ind. Med. 48, 845.
18. Nash RA (2005). Metals in medicine. Altern. Ther Health Med. 11, 18-25.
19. Guallar E, Sanz-Gallardo MI, Veer PV, Bode P, Aro A, Gomez-Aracena J, Kark JD, Riemersma RA, Martin-Moreno JM, Kok FJ (2002). Mercury, Fish Oils, and the Risk of Myocardial Infarction. N. Engl. J. Med. 347, 1747-54.
20. Yoshizawa K, Rimm EB, Morris JS, Spate VL, Hsieh CC, Spiegelman D, Stampfer MJ, Willett (2002). Mercury and The Risk of Coronary Heart Disease in Men. N. Engl. J. Med. 347, 1755-60.
21. Kim DS, Lee EH, Yu SD, Cha JH, Ahn SC (2005). Heavy metal as risk factor of cardiovascular disease—an analysis of blood lead and urinary mercury. J. Prev. Med Pub. Health. 38, 401-7.

22. Boffetta P, Sallsten G, Garcia-Gomez M, Pompe-Kirn V, Zaridze D, Bulbulyan M, Caballero J-D, Ceccarelli F, Kobal A B, Merler E (2001). Mortality from cardiovascular disease and exposure to inorganic mercury. Occup. Environ. Med. 58, 461-466.
23. Wakita Y (1987). Hypertension induced by methyl mercury in rats. Toxicol Appl. Pharmacol. 89, 144-7.
24. Egermayer P (2000). Epidemics of vascular toxicity and pulmonary hypertension: what can be learned? 247, 11-17.
25. N. Divecha, R.F. Irvine, Cell 80 (1995) 269-278.
26. Exton JH. Regulation of Phospholipase D. *Biochim Biophys Acta* 1439:175-186, 1999.
27. Billah MM, Lapetina EG, Cuatrecasas P. Insulinlike effect of vanadate on hepatic glycozen metabolism in nondiabetic and streptozocin-induced diabetic rats. *J Biol Chem* 256:5399-5403, 1981.
28. D.N. Brindley, D.W. Waggoner, Chem. Phys. Lipids 80 (1996) 45-57.
29. J.H. Exton, J. Biol. Chem. 272 (1977) 15579-15582.
30. V. Natarajan, J. Lab. Clin. Med. 125 (1995) 126-137.
31. W.D. Singer, H.A. Brown, X. Jiang, P.C. Sternweis, J. Biol. Chem. 271 (1996) 4504-4510.
32. W.C. Colley, T.C. Sung, R. Roll, J. Jenco, S.M. Hammond S, Y. Altshuller, D. Bar-Sagi, A.J. Morris, M.A. Frohman, Curr. Biol**.** 7 (1997) 191-201.
33. S.M. Hammond, Y.M. Altshuller, T. C. Sung, S.A. Rudge, K. Rose, J. Engebrecht, A.J. Morris, M.A. Frohman, J. Biol. Chem. 270 (1995) 29640-29643.
34. I. Lopez, R.S. Arnold, J.D. Lambeth, J. Biol. Chem. 273 (1998) 12846-12852.
35. M.G. Houle, S. Bourgoin, Biochim. Biophys. Acta 1439 (1999) 135-139.
36. M. Liscovitch, V. Chalifa-Caspi, Chem. Phys. Lipids 80 (1996) 37-44.

37. M.A. Frohman, T.C. Sung, A.J. Morris, Biochim. Biophys. Acta 1439 (1999) 175-186.

38. A.J. Morris, M.A. Frohman, J. Engebrecht, Anal. Biochem. 252 (1997) 1-9.

39. Varadharaj S, Steinhour E, Hunter MG, Watkins T, Baran CP, Magalang U, Kuppusamy P, Zweier JL, Marsh CB, Natarajan V, Parinandi NL. (2005). Vitamin C-induced activation of phospholipase D in lung microvascular endothelial cells: Regulation by MAP kinases. Cell Signal. 2005 Dec 20; [Epub ahead of print]

40. Parinandi NL, Kleinberg MA, Usatyuk PV, Cummings RJ, Pennathur A, Cardounel AJ, Zweier JL, Garcia JG, Natarajan V. (2002). Hyperoxia-induced NAD(P)H oxidase activation and regulation by MAP kinases in human lung endothelial cells. Am J Physiol Lung Cell Mol Physiol. 2003 Jan;284(1):L26-38.

41. Blanusa M, Varnai VM, Piasek M, Kostial K. (2005). Chelators as antidotes of metal toxicity: therapeutic and experimental aspects. Curr Med Chem. 2005;12(23):2771-94.

42. Valko M, Morris H, Cronin MT. (2005). Metals, toxicity and oxidative stress. Curr Med Chem. 2005;12(10):1161-208.

43. Parinandi NL, Roy S, Shi S, Cummings RJ, Morris AJ, Garcia JG, Natarajan V. (2001). Role of Src kinase in diperoxovanadate-mediated activation of phospholipase D in endothelial cells. Arch Biochem Biophys. 2001 Dec 15;396(2):231-43.

44. Natarajan V, Scribner WM, Morris AJ, Roy S, Vepa S, Yang J, Wadgaonkar R, Reddy SP, Garcia JG, Parinandi NL. (2001). Role of p38 MAP kinase in diperoxovanadate-induced phospholipase D activation in endothelial cells. Am J Physiol Lung Cell Mol Physiol. 2001 Aug;281(2):L435-49.

45. Parinandi NL, Scribner WM, Vepa S, Shi S, Natarajan V. (1998). Phospholipase D activation in endothelial cells is redox sensitive. Antioxid Redox Signal. 1999 Summer;1(2):193-210.

46. Garg TK and Chang JY. (2006). Methylmercury causes oxidative stress and cytotoxicity in microglia: attenuation by 15-deoxy-delta 12, 14-prostaglandin J2. J Neuroimmunol. 2006 Feb;171(1-2):17-28.

47. Onyido I, Norris AR, Buncel E. (2004). Biomolecule--mercury interactions: modalities of DNA base--mercury binding mechanisms. Remediation strategies. Chem Rev. 2004 Dec;104(12):5911-29.

48. English D, Cui Y, Siddiqui RA. Messenger function of phosphatidic acid . *Chem Phys Lipids* 80:117-132, 1996.

Figure Legends

Figure 1: Mercury activates PLD in a dose-dependent fashion.

BPAECs (5 X 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 (0-50 μM) of mercuric chloride (**A**), mercuric sulfate (**B**), mercuric acetate (**C**), thimerosal (**D**), and methylmercury chloride (**E**) for 1 and 2 h in MEM containing 0.05% butanol. At the end of incubation [^{32}P]PBt formed was determined as described under Materials and Methods. Data represent means $\pm$ S.D. of three independent experiments. *Significantly different at $P<0.05$ as compared to cells not treated with the mercury compound(s).

Figure 2: Mercury activates PLD in a time-dependent manner.

BPAECs (5 X 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 MEM alone or MEM containing mercuric chloride (25 μM) (**A**) or mercuric sulfate (25 μM) (**B**) or thimerosal (25 μM) (**C**) or methylmercury chloride (10 μM) (**D**) for 0-60 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 $\pm$ S.D. of three independent experiments. *Significantly different at $P<0.05$ as compared to cells not treated with the mercury compound(s).

Figure 3: Metal chelating agents attenuate mercuric chloride-induced PLD activation.

BPAECs (5 X 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 and 5 mM; **A**) or DETC (100 μM, 500 μM,

1mM; **B**) or PDTC (100 μM, 500 μM, and 1 mM; **C**) and then subjected to treatment with vehicle or mercuric chloride (25 μM) 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 ± S.D. of three independent experiments. *Significantly different at $P<0.05$ as compared to cells treated with vehicle alone. ** Significantly different at $P<0.05$ as compared to cells treated with mercuric chloride alone.

Figure 4: Antioxidants and sulfhydryl agents attenuate mercuric chloride-induced PLD activation.

BPAECs (5 X 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 (100 μM, 500 μM, and 1 mM; **A**) or antioxidants (propyl gallate 100 μM, Catalase 50 μg/mL, and DMSA 1mM; **B**) or sulfhydryl agents (NAC, DTT, and D-penicillamine 500 μM; **C**) and then treated with mercuric chloride (25 μM) 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 ± S.D. of three independent experiments. *Significantly different at $P<0.05$ as compared to cells treated with vehicle alone. ** Significantly different at $P<0.05$ as compared to cells treated with mercuric chloride alone.

Figure 5: Metal Chelating agents and antioxidants attenuate methylmercury chloride-induced PLD activation.

BPAECs (5 X 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 EDTA (1 mM) and NAC (1 mM) (**A**) or PDTC (1

mM) and DETC (1 mM) (**B**) or D-penicillamine (1, 5, and 10 μM) (**C**) or propyl gallate (50, 100, and 500 μM) (**D**) and treated with methylmercury chloride (10 μM) 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 ± S.D. of three independent experiments. *Significantly different at $P<0.05$ as compared to cells treated with vehicle alone. ** Significantly different at $P<0.05$ as compared to cells treated with methylmercury chloride alone.

Figure 6: Antioxidants and sulfhydryl agents attenuate methylmercury chloride-induced PLD activation.

BPAECs (5 X 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 (1mM) and catalase (1 μg/μL) (**A**) or MnTBAP (20 μM), DTT (500 μM), DMSA (1 mM) (**B**) and then treated with methylmercury chloride (10 μM) for 30 minutes 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 ± S.D. of three independent experiments. *Significantly different at $P<0.05$ as compared to cells treated with vehicle alone. ** Significantly different at $P<0.05$ as compared to cells treated with methylmercury chloride alone.

Figure 7: Metal chelating agents and redox-active antioxidants attenuate thimerosal-induced PLD activation.

BPAECs (5 X 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 EDTA (1 mM) and NAC (1 mM) (**A**) or PDTC (1 mM) and D-penicillamine (1 mM) (**B)** or vitamin c (1 mM) and DMSA (1 mM) (**C**) and then

treated with thimerosal (25 μM) 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 ± S.D. of three independent experiments. *Significantly different at P<0.05 as compared to cells treated with vehicle alone. ** Significantly different at P<0.05 as compared to cells treated with thimerosal alone.

Figure 8: Antioxidants and sulfhydryl agents attenuate thimerosal-induced PLD activation.

BPAECs (5 X 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 catalase (50 μg/μL), DTT (500 μM), and DETC (1 mM) (**A**) or MnTBAP (20 μM) and propyl gallate (100 μM) (**B**) and then treated with thimerosal (25 μM) 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 ± S.D. of three independent experiments. *Significantly different at P<0.05 as compared to cells treated with vehicle alone. ** Significantly different at P<0.05 as compared to cells treated with thimerosal alone.

Figure 9: Methylmercury chloride induces ROS generation in BPAECs: Attenuation by antioxidants

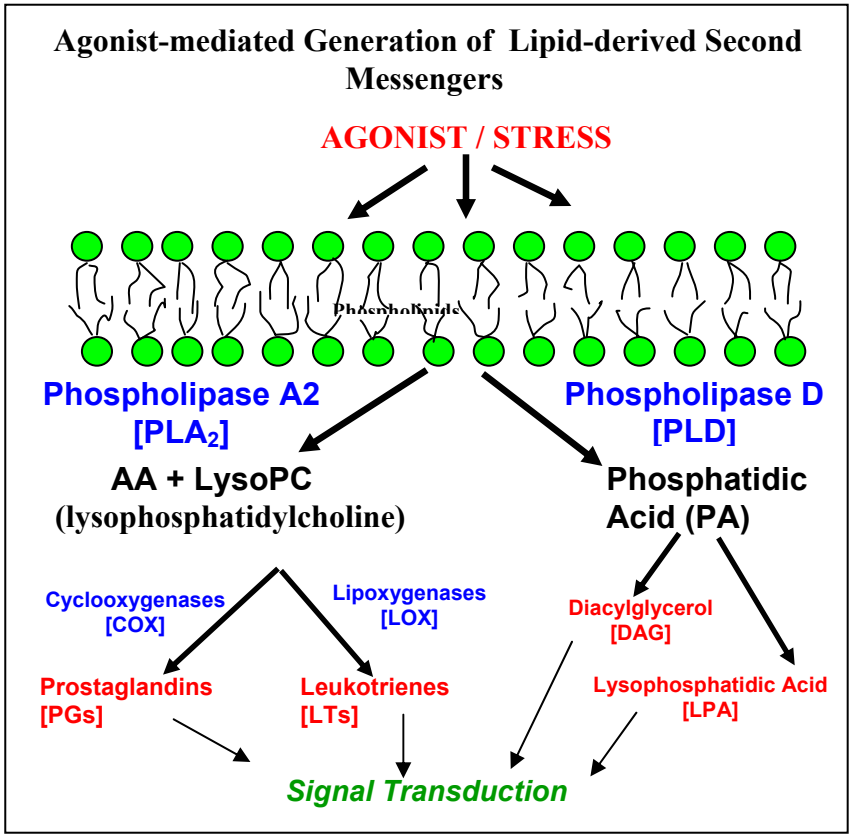
BPAECs (5 X 10^5 cells/35-mm dish) were treated with basal MEM or MEM containing increasing concentrations of methylmercury chloride (0-15 μM) and incubated for 30 min (**A**). To investigate the effects of antioxidants, BPAECs (5 X 10^5 cells/35-mm dish) were treated for 1 h with basal MEM or MEM containing 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 ± S.D. of three independent experiments.

Schema-1: Agonist-induced phospholipase A2 (PLA2) and phospholipase D (PLD) activation in cells and generation of lipid-derived second messengers and action of different phospholipases on membrane phospholipid.

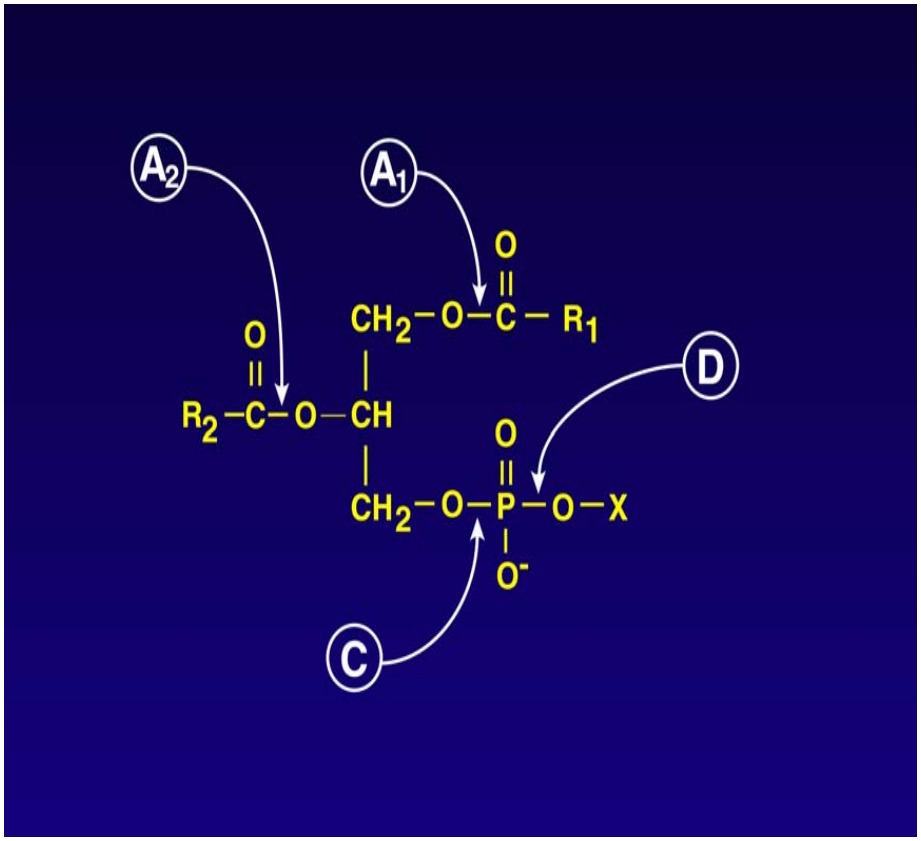
Schema-2: PLD-mediated hydrolysis of membrane phosphatidylcholine (PC) and generation of phosphatidic acid (PA), diacylglycerol (DAG), and lysophosophatidic acid (LPA). Transphoshpatidylation of the PLD-mediated PA to phosphatidylalcohol is a characteristic feature of the enzyme which is exploited for the assay of the activity of PLD in biological systems.

Schema-3: Mechanism of mercury-induced activation of PLD in ECs.

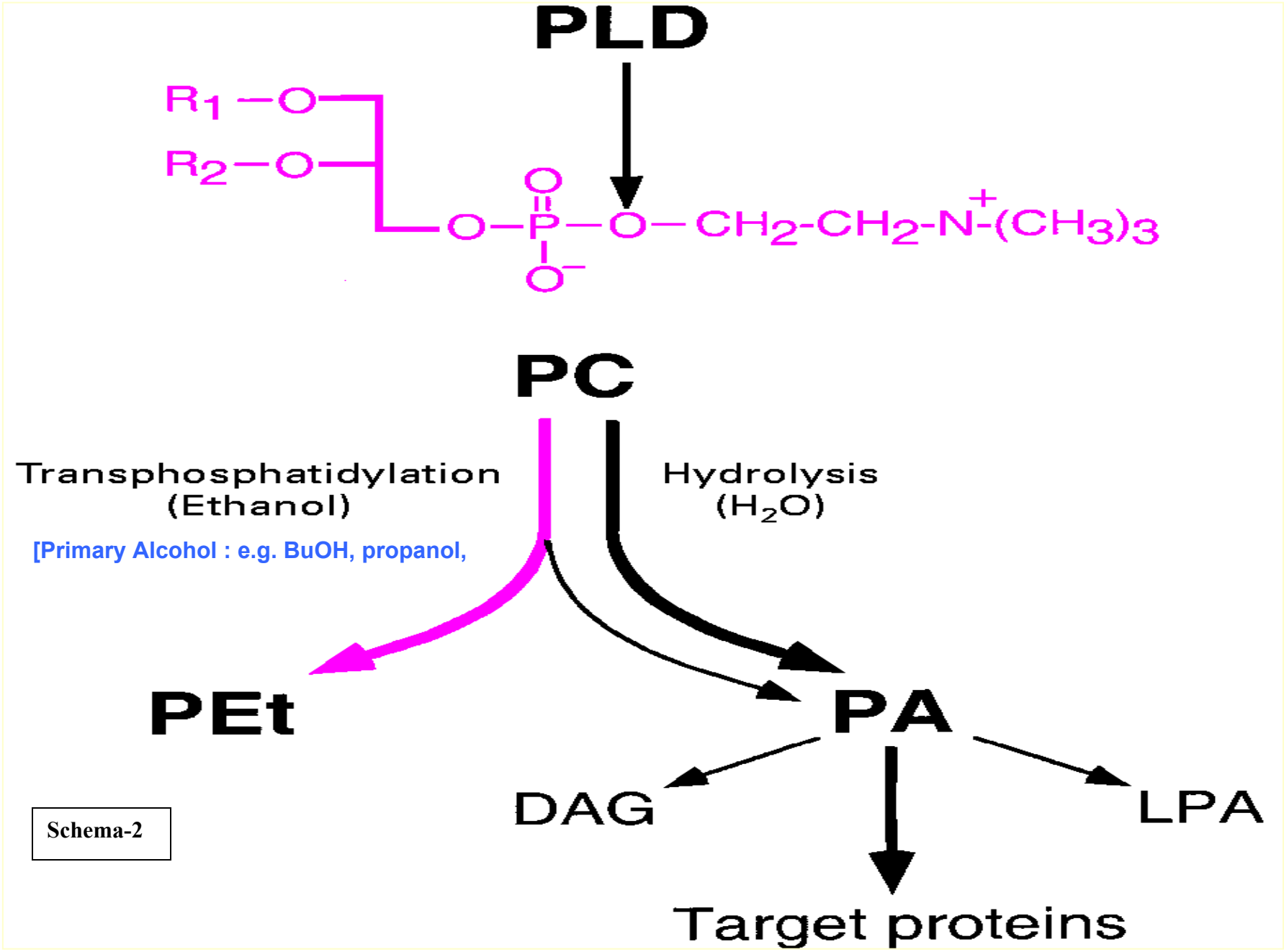


Schematic representation of agonist / stress activation of the lipid signaling enzymes which are involved in cellular signaling events by generating bioactive lipid mediators.

Schema-1



Action of different phospholipases (A_1, A_2, C, and D on membrane phospholipids.


PLD
R1 –O
R2 –O
O–P–O–CH2-CH2-N+-(CH3)3
O–
PC
Transphosphatidylation
(Ethanol)
[Primary Alcohol : e.g. BuOH, propanol,
Hydrolysis
(H2O)
PEt
PA
DAG
LPA
Target proteins

Schema-2

Fig. 1A

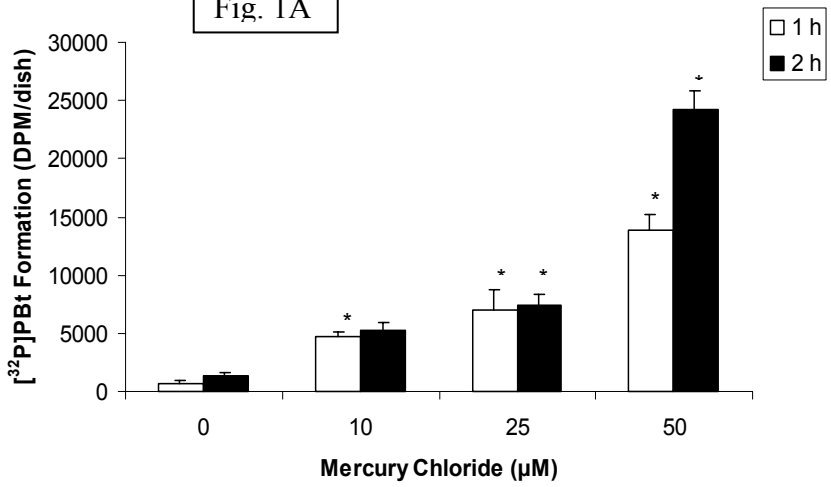


Fig. 1B

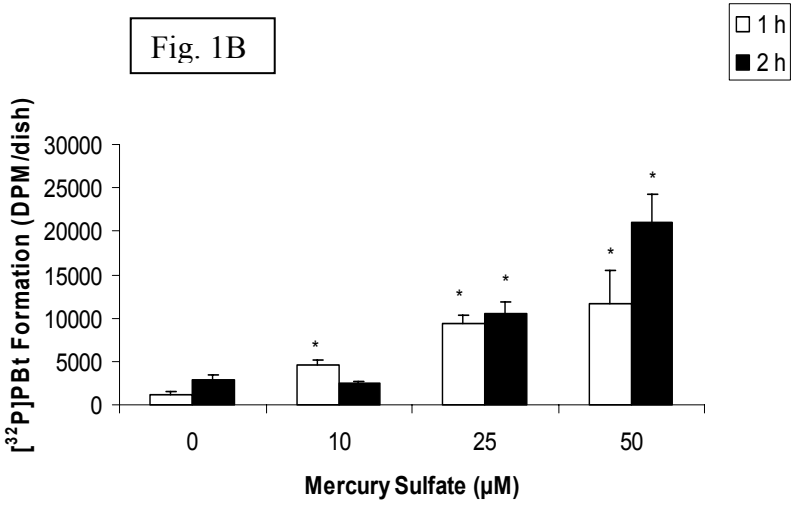


Fig. 1C

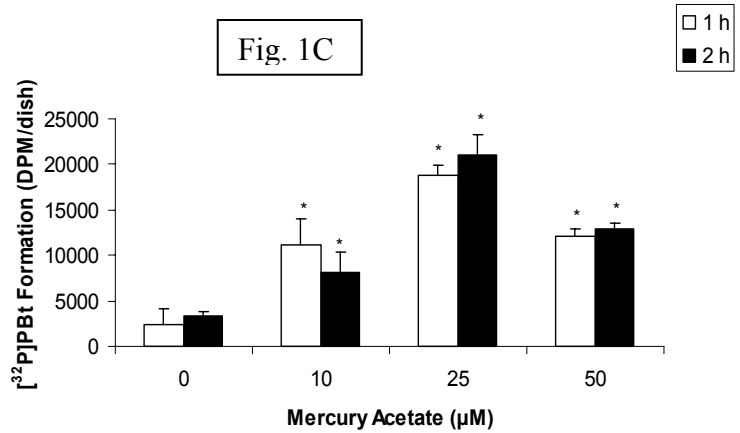


Fig. 1D

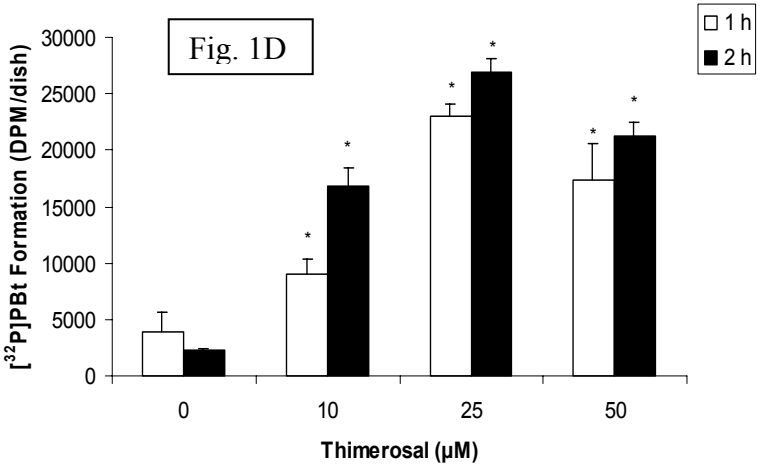


Fig. 1E

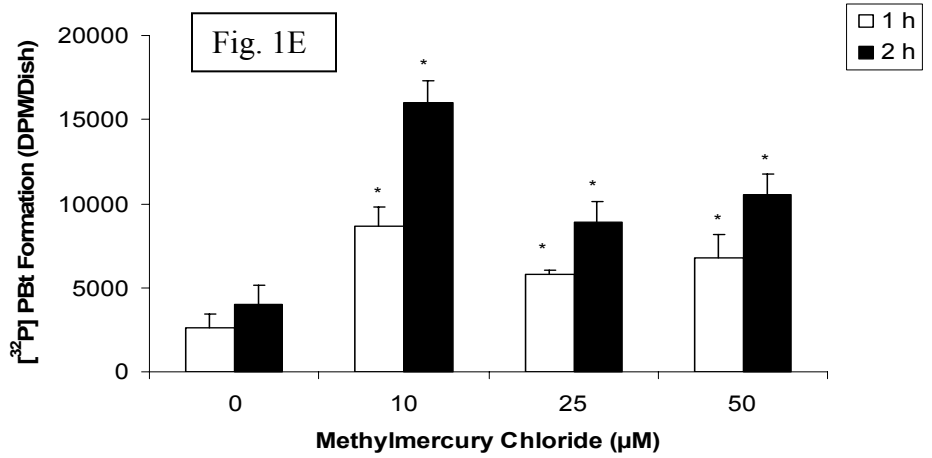


Fig. 2A

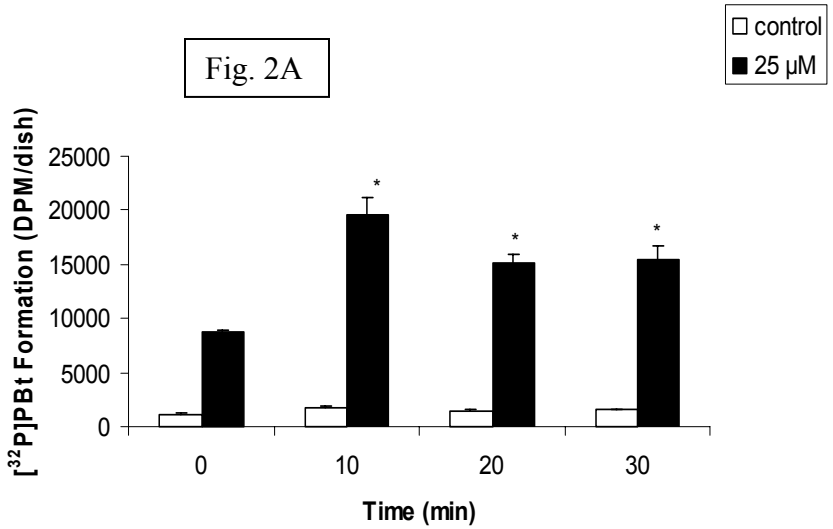


Fig. 2B

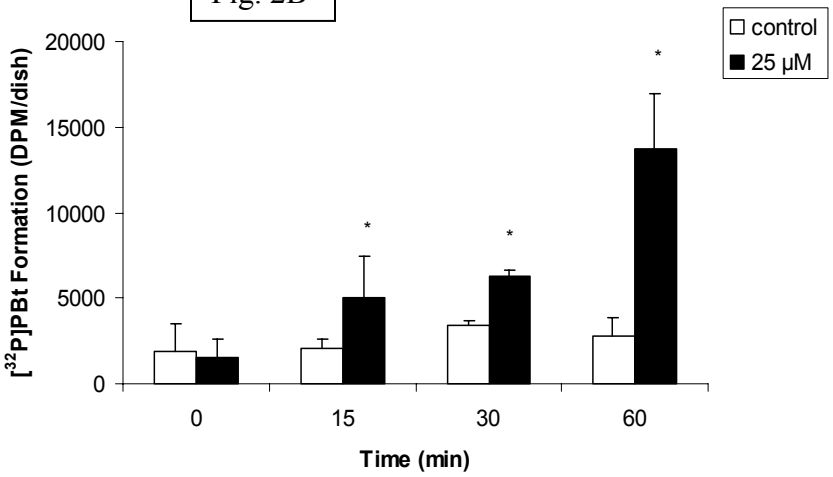


Fig. 2C

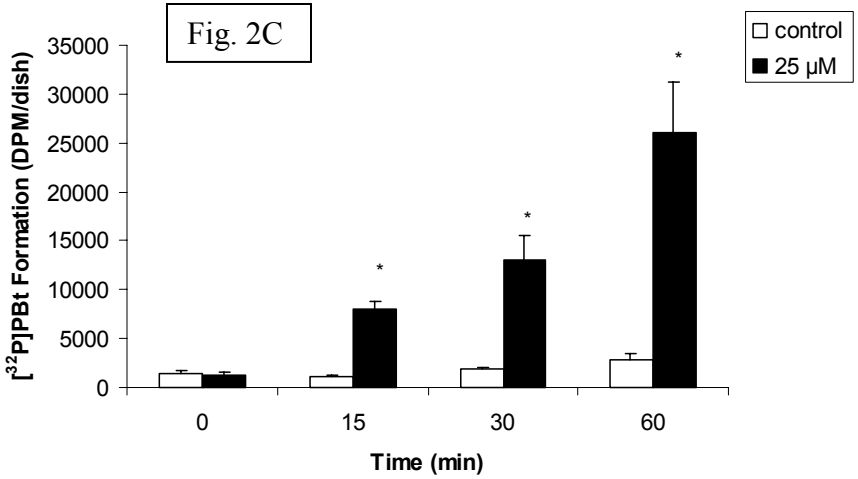


Fig. 2D

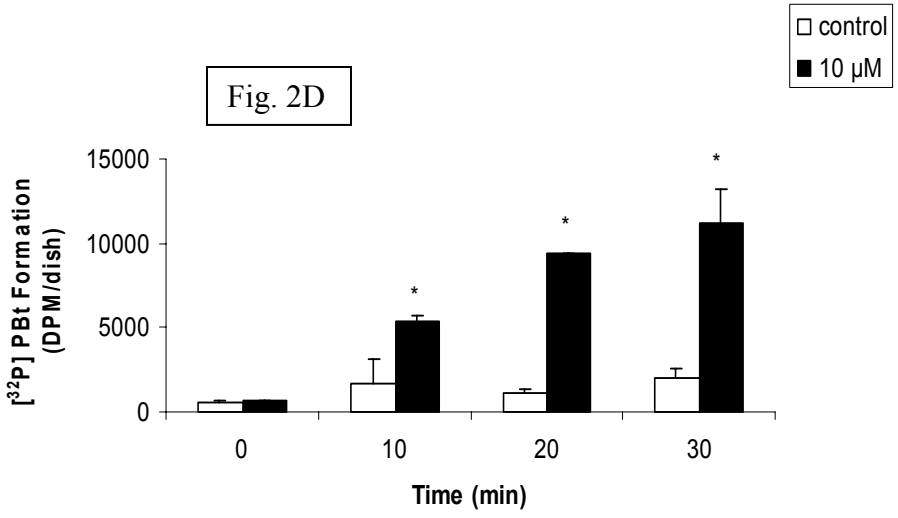


Fig. 3A

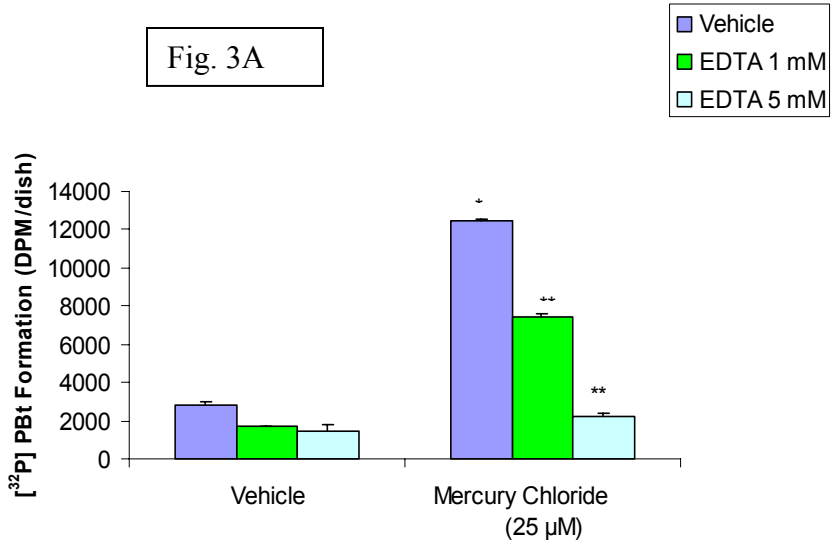


Fig. 3B

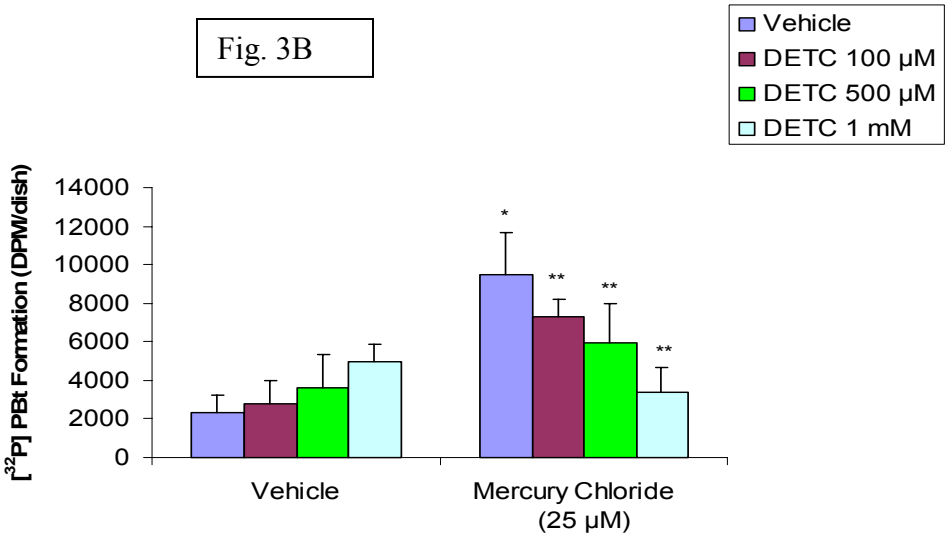


Fig. 3C

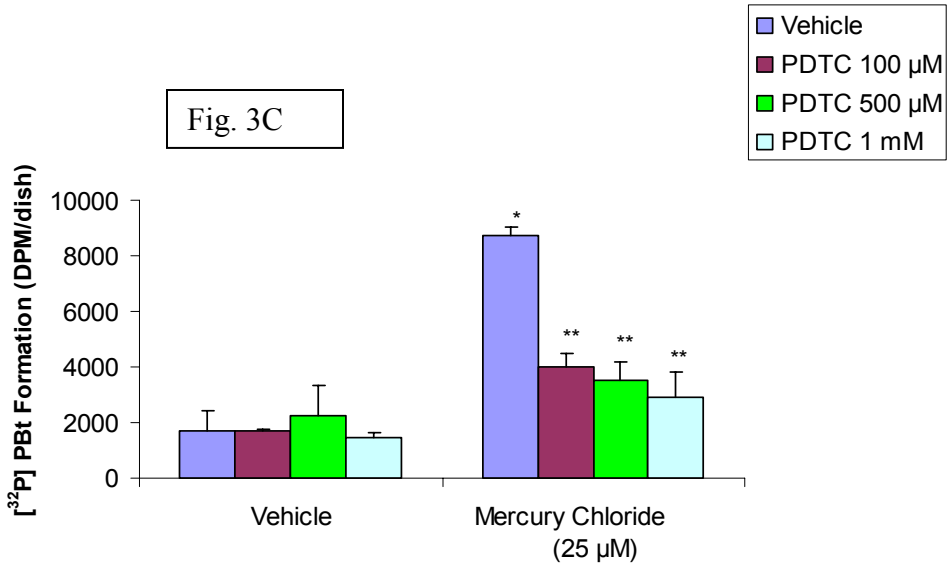


Fig. 4A

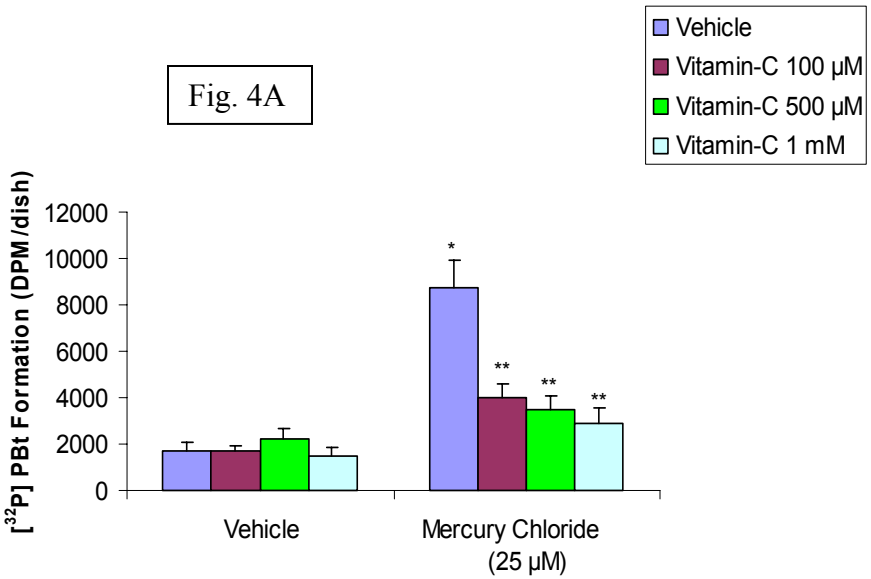


Fig. 4B

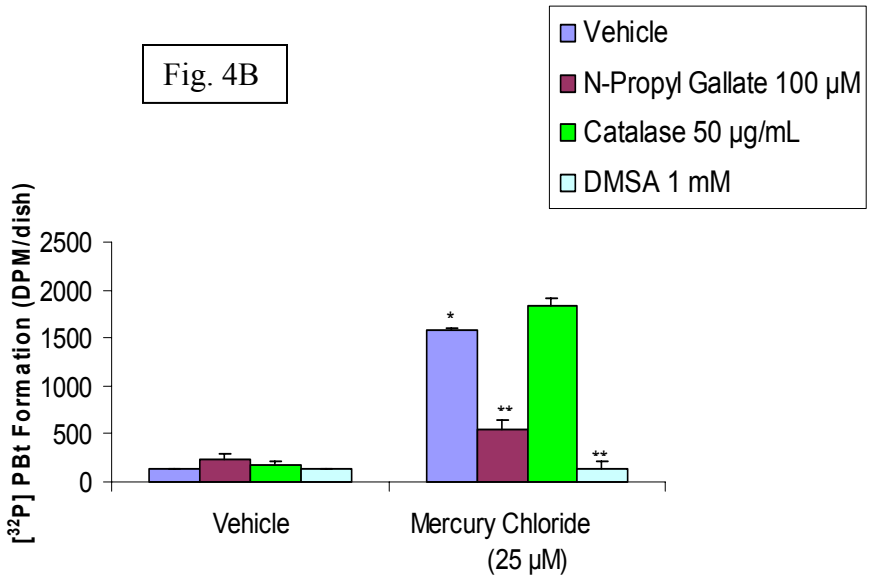


Fig. 4C

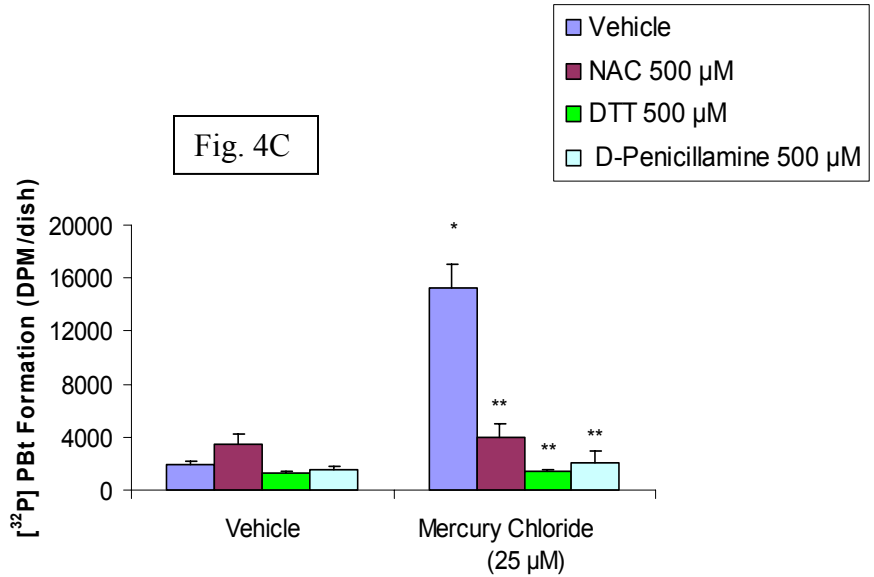


Fig. 5A

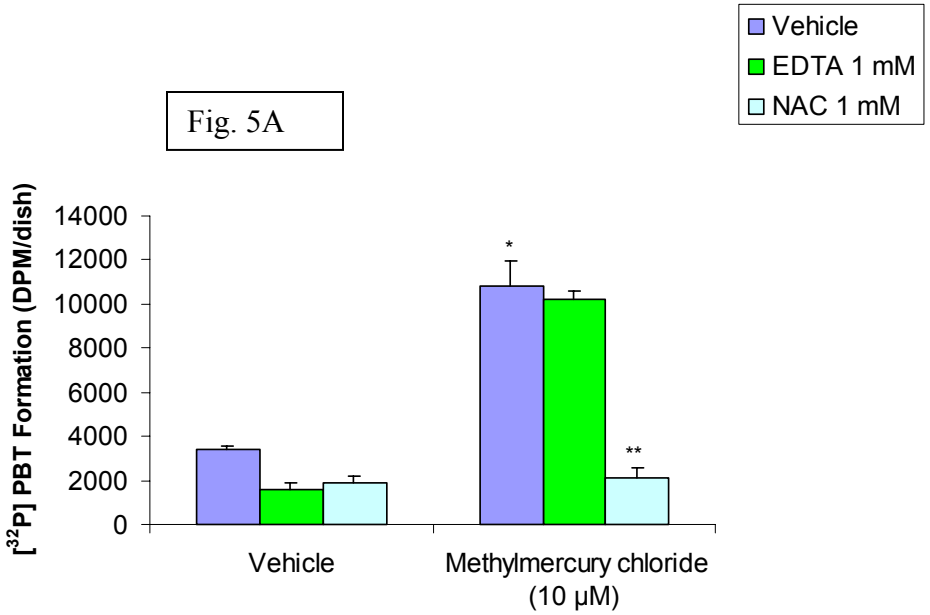


Fig. 5B

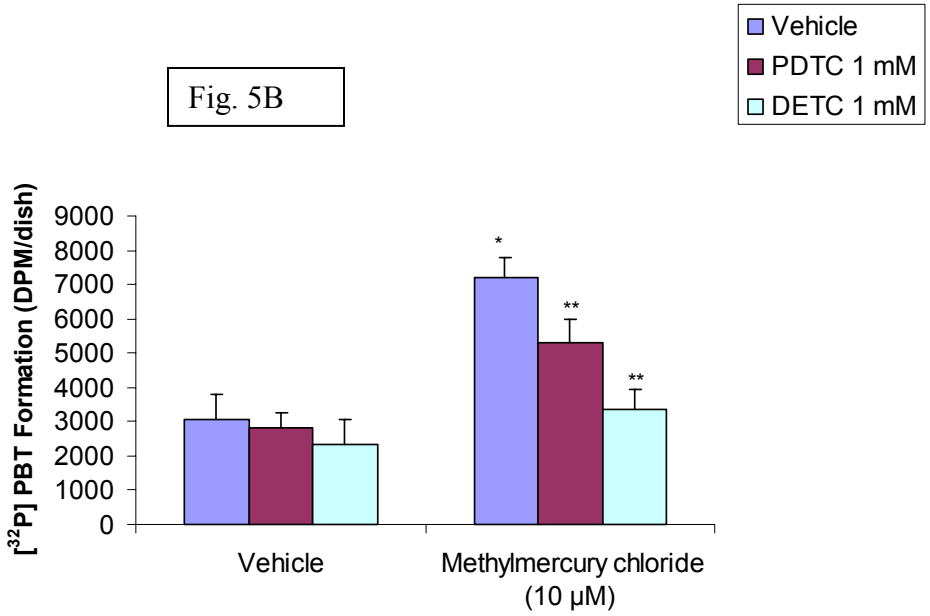


Fig. 5C

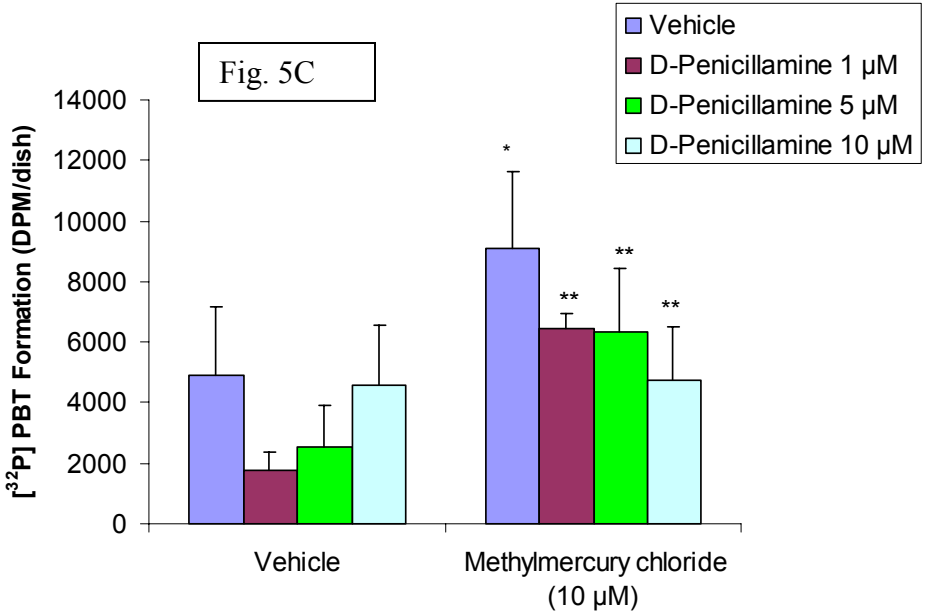


Fig. 5D

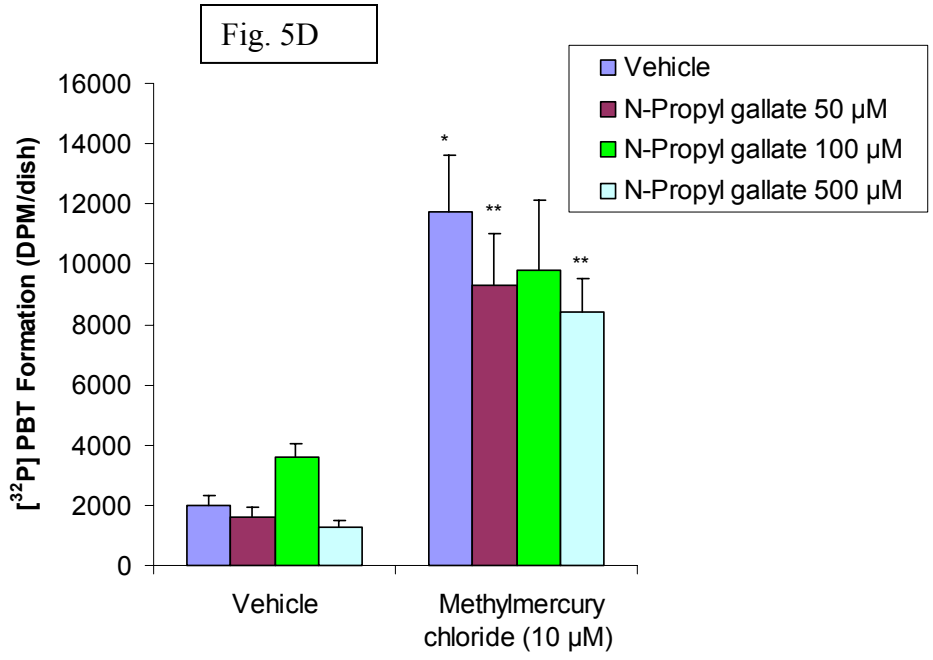


Fig. 6A

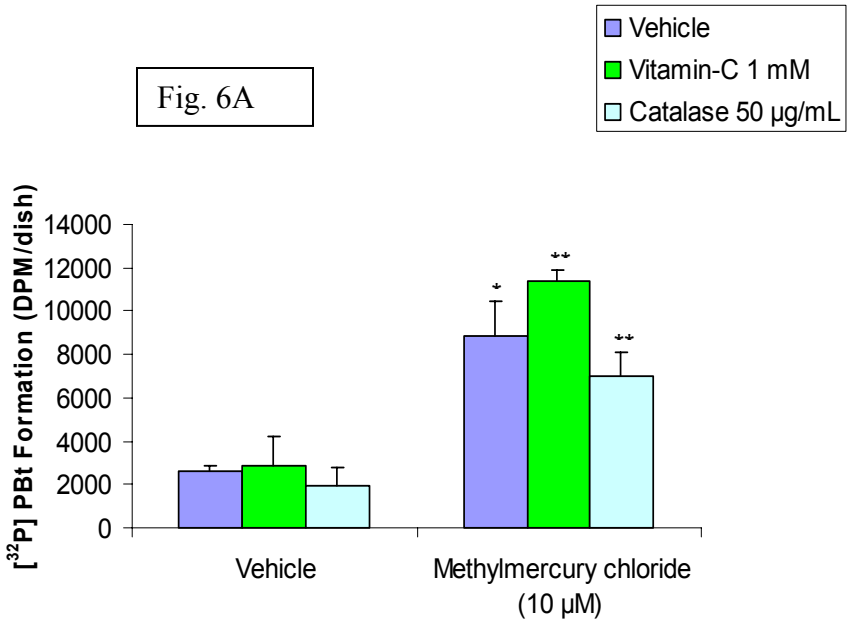

Vehicle
Vitamin-C 1 mM
Catalase 50 µg/mL
[32P] PBt Formation (DPM/dish)
14000
12000
10000
8000
6000
4000
2000
0
*
**
**
Vehicle
Methylmercury chloride (10 µM)

Fig. 6B

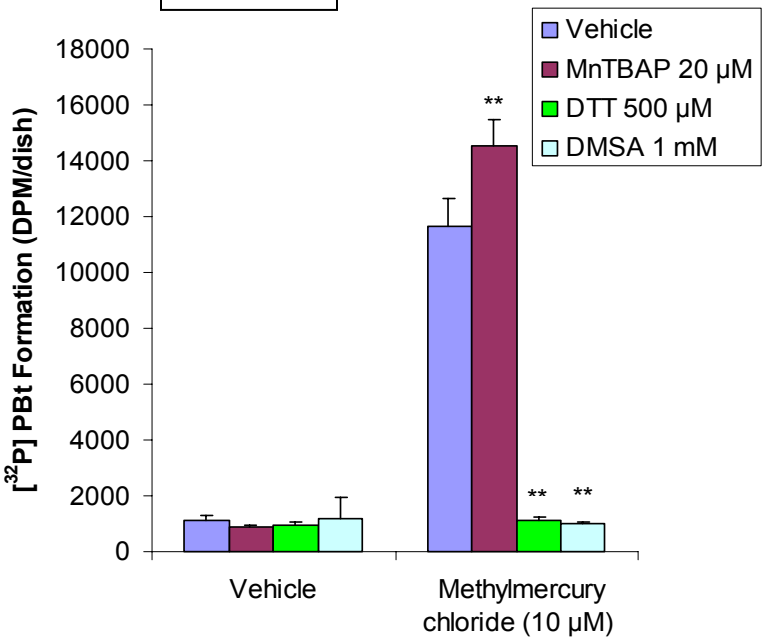

Vehicle
MnTBAP 20 µM
DTT 500 µM
DMSA 1 mM
[32P] PBt Formation (DPM/dish)
18000
16000
14000
12000
10000
8000
6000
4000
2000
0
**
**
**
Vehicle
Methylmercury chloride (10 µM)

Fig. 7A

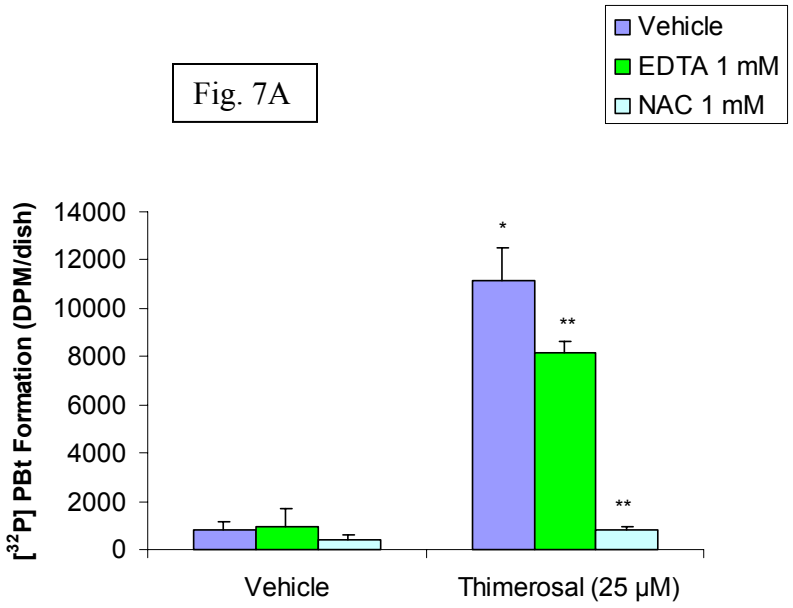


Fig. 7B

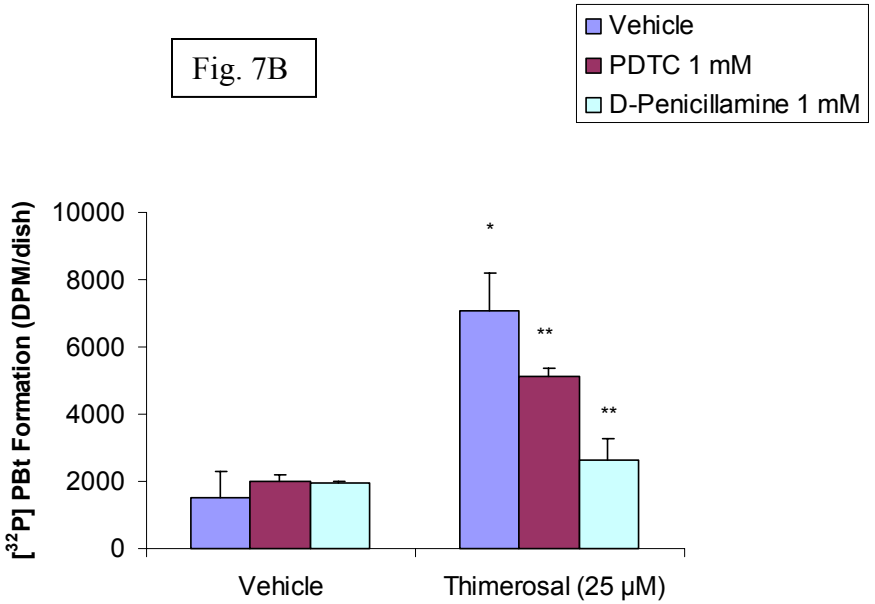


Fig. 7C

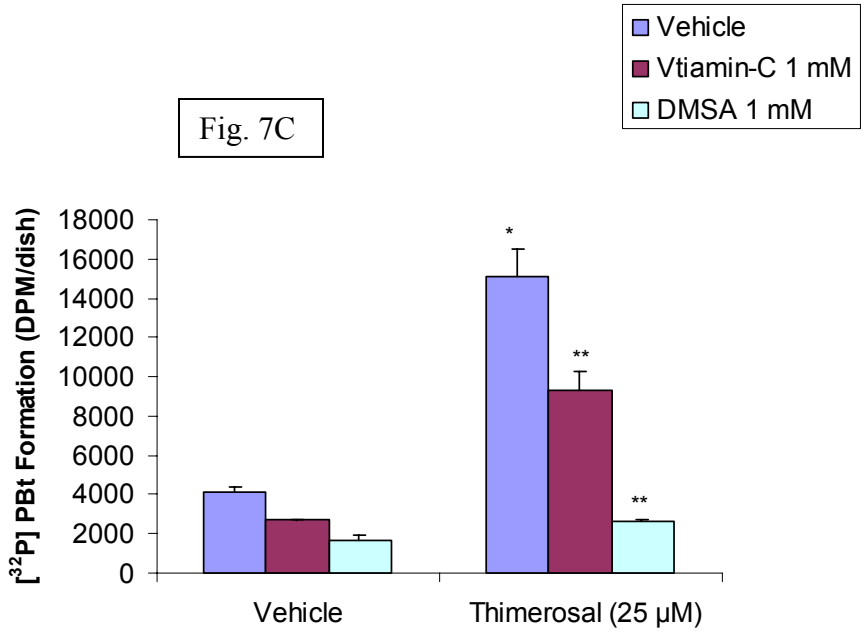


Fig. 8A

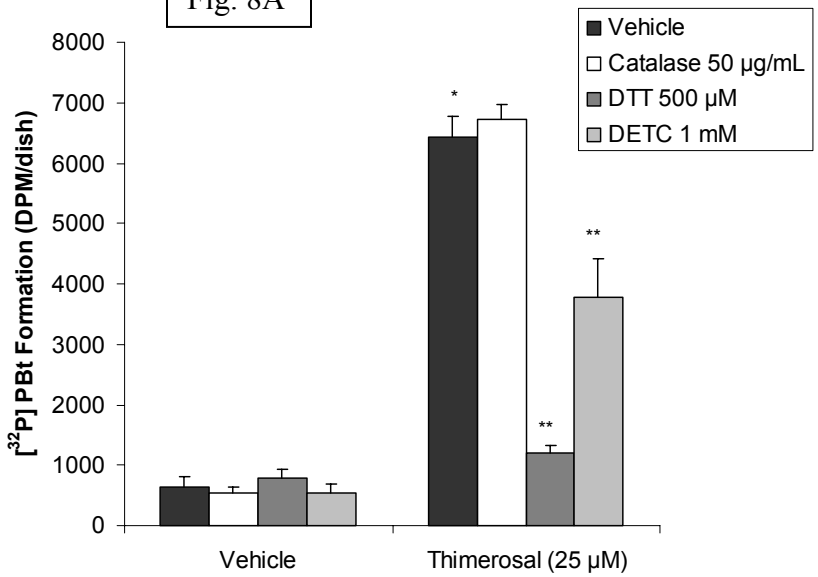

Vehicle
Catalase 50 µg/mL
DTT 500 µM
DETC 1 mM
[32P] PBt Formation (DPM/dish)
8000
7000
6000
5000
4000
3000
2000
1000
0
*
**
**
Vehicle
Thimerosal (25 µM)

Fig. 8B

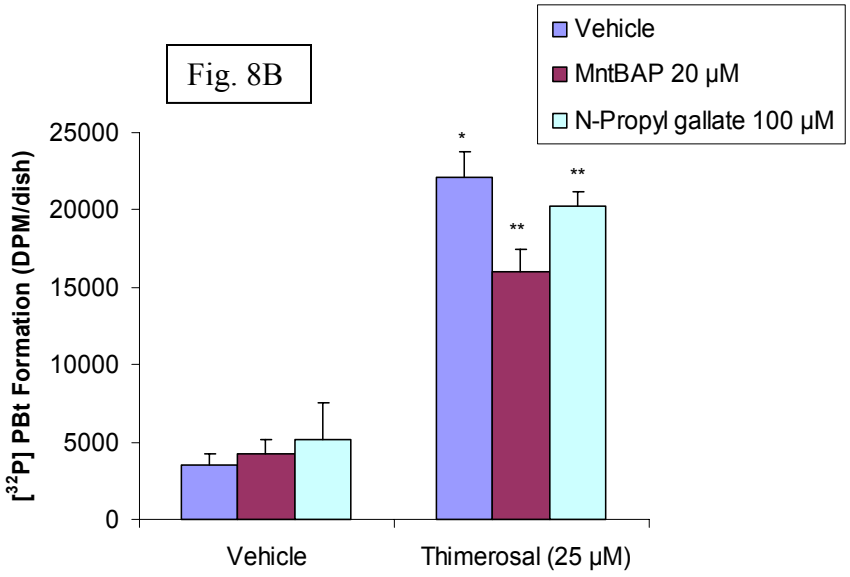

Vehicle
MntBAP 20 µM
N-Propyl gallate 100 µM
[32P] PBt Formation (DPM/dish)
25000
20000
15000
10000
5000
0
*
**
**
Vehicle
Thimerosal (25 µM)

Fig. 9A

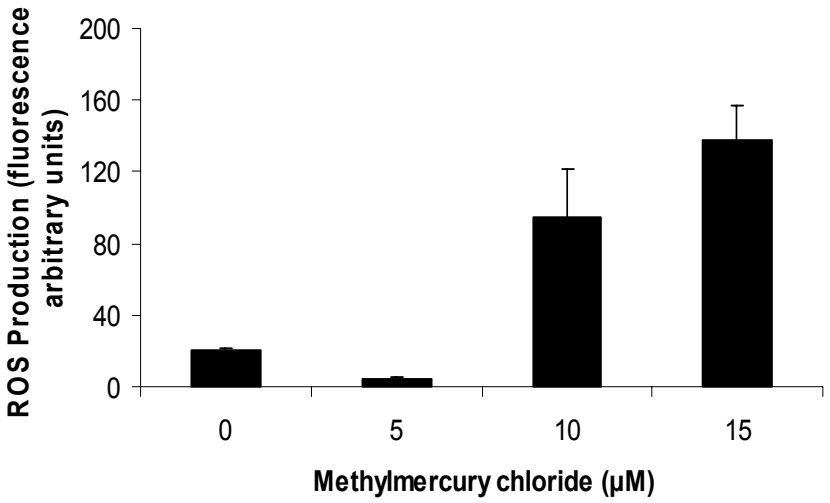


Fig. 9B

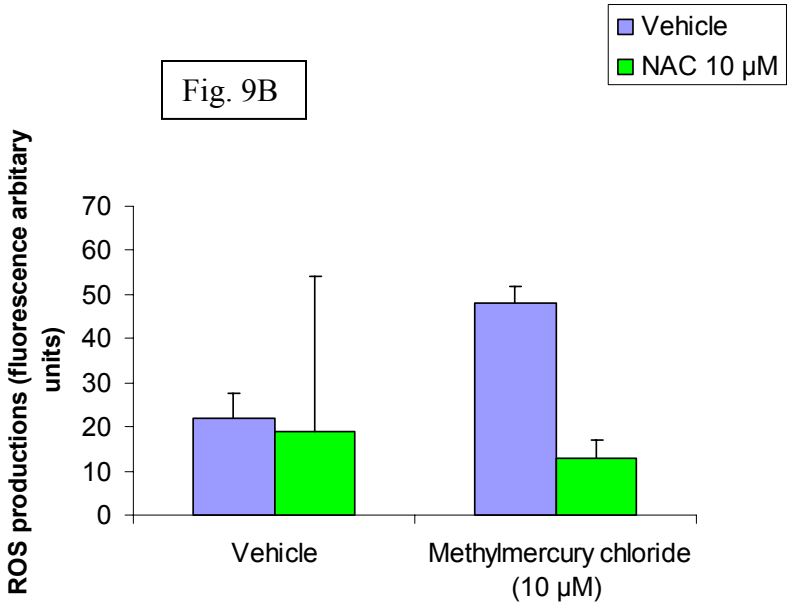
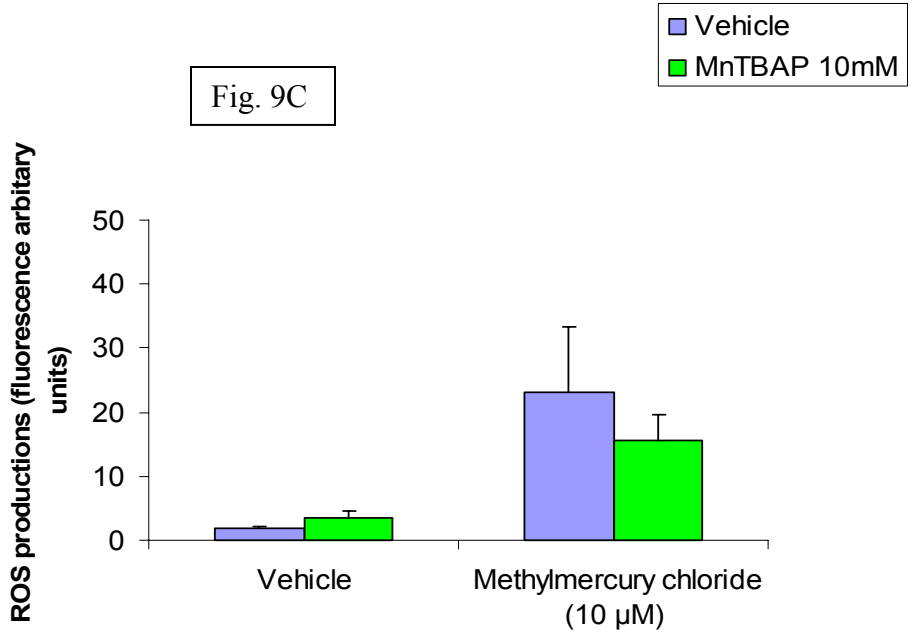
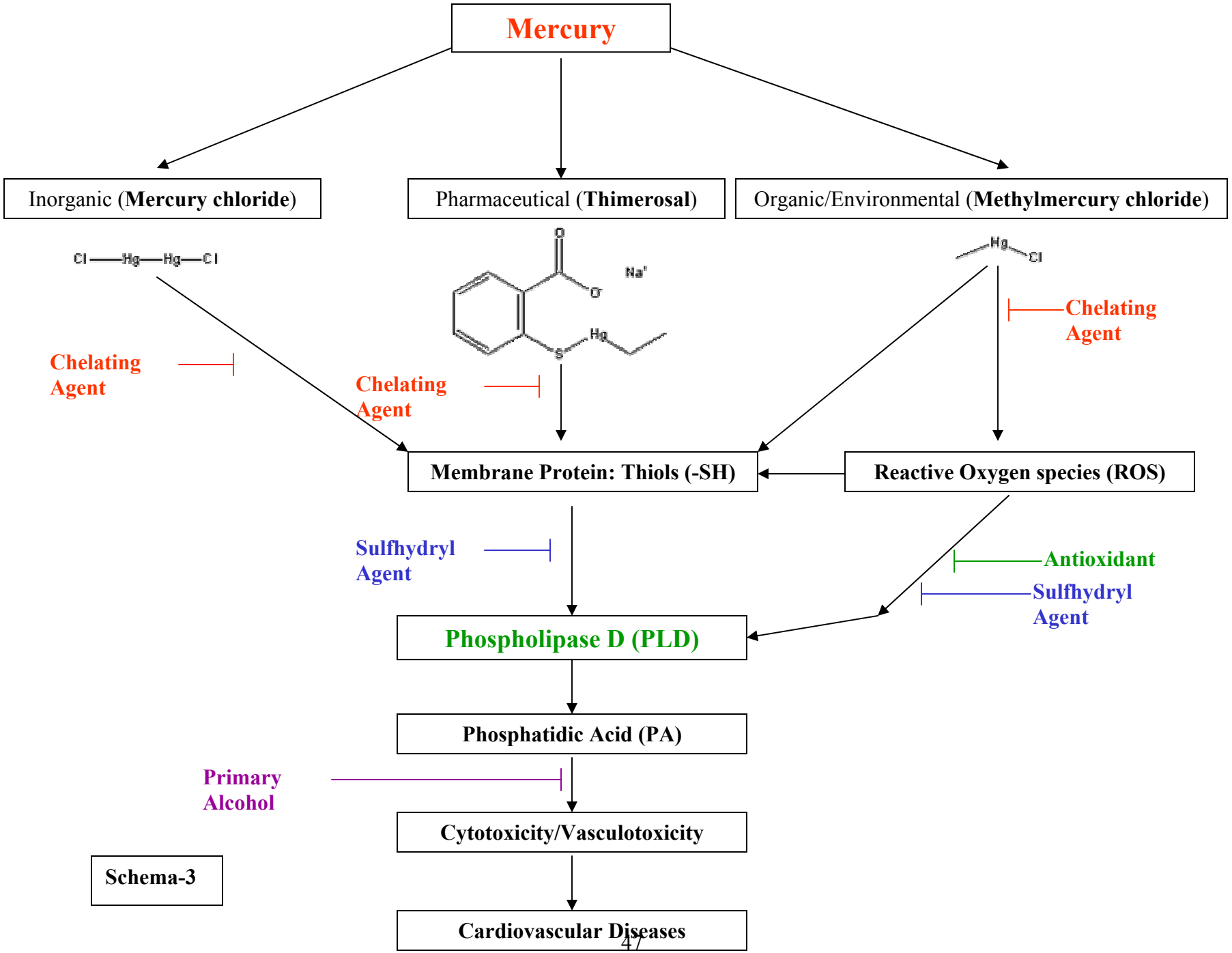


Fig. 9C




Mercury
Inorganic (Mercury chloride)
Pharmaceutical (Thimerosal)
Organic/Environmental (Methylmercury chloride)
Cl—Hg—Hg—Cl
Na+
Hg
Cl
Chelating Agent
Chelating Agent
Chelating Agent
Membrane Protein: Thiols (-SH)
Reactive Oxygen species (ROS)
Sulfhydryl Agent
Antioxidant
Sulfhydryl Agent
Phospholipase D (PLD)
Phosphatidic Acid (PA)
Primary Alcohol
Cytotoxicity/Vasculotoxicity
Cardiovascular Diseases

Schema-3