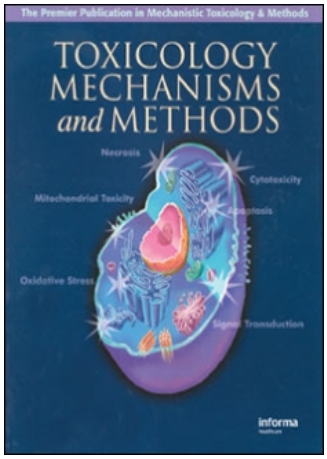


Toxicology Mechanisms and Methods
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713396575

Mercury Activates Phospholipase A_2 and Induces Formation of Arachidonic Acid Metabolites in Vascular Endothelial Cells

Jessica N. Mazerik [a]; Himabindu Mikkilineni [a]; Vivek A. Kuppusamy [a]; Emily Steinhour [a]; Alon Peltz [a]; Clay B. Marsh [a]; Periannan Kuppusamy [a]; Narasimham L. Parinandi [a]
[a] Lipid Signaling and Lipidomics Laboratory, Division of Pulmonary, Allergy, Critical Care, and Sleep Medicine, Department of Internal Medicine, The Ohio State University College of Medicine, Columbus, OH, United States

Online Publication Date: 01 November 2007
To cite this Article: Mazerik, Jessica N., Mikkilineni, Himabindu, Kuppusamy, Vivek A., Steinhour, Emily, Peltz, Alon, Marsh, Clay B., Kuppusamy, Periannan and Parinandi, Narasimham L. (2007) 'Mercury Activates Phospholipase A_2 and Induces Formation of Arachidonic Acid Metabolites in Vascular Endothelial Cells', Toxicology Mechanisms and Methods, 17:9, 541 - 557
To link to this article: DOI: 10.1080/15376510701380505
URL: http://dx.doi.org/10.1080/15376510701380505

Toxicology Mechanisms and Methods, 17:541–557, 2007

ISSN: 1537-6516 print; 1537-6524 online
DOI: 10.1080/15376510701380505

informa
healthcare

Mercury Activates Phospholipase A_2 and Induces Formation of Arachidonic Acid Metabolites in Vascular Endothelial Cells

Jessica N. Mazerik, Himabindu Mikkilineni, Vivek A. Kuppusamy, Emily Steinhour, Alon Peltz, Clay B. Marsh, Periannan Kuppusamy, and Narasimham L. Parinandi

Lipid Signaling and Lipidomics Laboratory, Division of Pulmonary, Allergy, Critical Care, and Sleep Medicine, Department of Internal Medicine, The Ohio State University College of Medicine, Columbus, OH, United States

ABSTRACT Currently, mercury has been identified as a risk factor in cardiovascular diseases among humans. Here, we tested our hypothesis that mercury modulates the activity of the vascular endothelial cell (EC) lipid signaling enzyme phospholipase A_2 (PLA_2), which is an important player in the EC barrier functions. Monolayers of bovine pulmonary artery ECs (BPAECs) in culture, following labeling of membrane phospholipids with [^{3}H]arachidonic acid (AA), were exposed to the inorganic form of mercury, mercury chloride, and the release of free AA (index of PLA_2 activity) and formation of AA metabolites were determined by liquid scintillation counting and enzyme immunoassay, respectively. Mercury chloride significantly activated PLA_2 in BPAECs in a dose-dependent (0 to 50 μM) and time-dependent (0 to 120 min) fashion. Metal chelators significantly attenuated mercury-induced PLA_2 activation, suggesting that cellular mercury–ligand interaction is required for the enzyme activation and that chelators are suitable blockers for mercury-induced PLA_2 activation in ECs. Sulfhydryl (thiol-protective) agents, calcium chelating agents, and $cPLA_2$-specific inhibitor also significantly attenuated the mercury-induced PLA_2, suggesting the role of thiol and calcium in the activation of $cPLA_2$ in BPAECs. Significant formation of AA metabolites, including the release of total prostaglandins, thromboxane B_2, and 8-isoprostane, were observed in BPAECs following their exposure to mercury chloride. Mercury chloride induced cytotoxicity as observed by the altered cell morphology and enhanced trypan blue uptake, which was attenuated by the $cPLA_2$ inhibitor $AACOCF_3$. The results of this study revealed that inorganic mercury-induced PLA_2 activation through the thiol and calcium signaling and the formation of bioactive AA metabolites further demonstrated the association of PLA_2 with the cytotoxicity of mercury in ECs. Overall, the results of the current study underscore the importance of PLA_2 signaling in mercury-induced endothelial dysfunctions.

KEYWORDS Arachidonic Acid; Isoprostane; Lipid Signaling; Mercury; Phospholipase A_2; PLA_2; Prostaglandin; Thromboxane B_2; Vascular Endothelial Cells

Received 17 January 2007; accepted 3 April 2007.

This work was supported by the National Institutes of Health grant HL 067176-05, EB 004031, and Dorothy M. Davis Heart and Lung Research Institute funds.

Address correspondence to Narasimham L. Parinandi, Ph.D., Room 611-A, Division of Pulmonary, Critical Care, and Sleep Medicine, Dorothy M. Davis Heart and Lung Research Institute, The Ohio State University, 473 W. 12th Avenue, Columbus, OH 43210. E-mail: narasimham.parinandi@osumc.edu

INTRODUCTION

Mercury (Hg), a heavy metal belonging to the transition element series of the periodic table, has been used in industrial processes and medical practice (Clarkson et al. 2003; Pleva 1994; Mutter et al. 2004) resulting in accidental and occupational exposures to mercury. Anthropogenic activities cause the increased release of the element into the environment leading to the pollution of air, water, and soil (Clarkson et al. 2003; Sarkar 2005; Kuehn 2005). Inorganic mercury is toxic to many organisms including humans and is converted to more toxic organic forms (methylmercury) through biomethylation by microorganisms (bacteria) (Boening 2000; Dopp et al. 2004). Consumption of contaminated 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 coronary artery disease (Landmark and Aursnes 2004; Chan and Egeland 2004; Clarkson 2002).

The role of mercury toxicity as a possible risk factor in cardiovascular disease has been emphasized (Kosta 1991). 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). An association between the occupational exposure to mercury in mining and refining and risk of cardiovascular diseases has been shown (Boffetta et al. 2001).

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). 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 (Dennis et al. 1991; Divecha and Irvine 1995).

Phospholipase A_2 (PLA_2) is an important membrane phospholipid hydrolyzing enzyme that catalyzes the hydrolysis of the membrane phospholipids at the *sn*-2 position generating free unsaturated fatty acid and lysophospholipid (Dennis et al. 1991). Thus, the unsaturated fatty acid released from the membrane phospholipids upon the action of PLA_2, usually arachidonic acid, is a substrate for cyclooxygenases (COXs) and lipoxygenases (LOXs), which mediate the formation of potentially bioactive arachidonic acid metabolites such as prostaglandins and leukotrienes (Chakraborti 2003). These arachidonic metabolites of COXs and LOXs have been identified to play crucial roles in inflammatory cascades and are tightly regulated by the activity of PLA_2 (Dennis et al. 1991). PLA_2 is also a very important housekeeping enzyme involved in membrane formation and repair (Balsinde et al. 2000). PLA_2 has been shown to be activated by several different agonists in different systems both in vitro and in vivo (Chakraborti 2003). Roles of PLA_2 and arachidonic acid metabolites in cardiovascular diseases have been emerging (Lambert et al. 2006). Therefore, unregulated PLA_2 activation mediated by agonists such as environmental toxicants can jeopardize the endothelial function and eventually the vessel function. As environmental mercury has been implicated in cardiovascular disease and earlier we have shown that mercury activates PLD in vascular endothelial cells (ECs) in vitro, here we have hypothesized that inorganic mercury activates PLA_2, induces the release of arachidonic acid from the membrane phospholipids, and mediates the formation of arachidonic acid metabolites, and that the activation of PLA_2 regulates the mercury-induced cytotoxicity in ECs. To test our hypothesis, we investigated whether inorganic mercury, in the form of mercury chloride, could induce PLA_2-mediated release of arachidonic acid, formation of the arachidonic acid metabolites, and regulation of mercury-induced cytotoxicity by PLA_2 in our well-established bovine pulmonary artery ECs (BPAECs) in vitro. For the first time, our current study revealed that inorganic mercury induced the activation of PLA_2, release of arachidonic acid, formation of arachidonic

acid metabolites, and regulation of cytotoxicity of mercury by PLA_2 in BPAECs.

MATERIALS AND METHODS

Materials

Bovine pulmonary artery endothelial cells (BPAECs) (passage 2) were obtained from Cell Applications Inc. (San Diego, CA). Fetal bovine serum (FBS), trypsin, and nonessential amino acids were obtained from Gibco Invitrogen Corp. (Grand Island, NY). Minimum essential medium (MEM), mercury chloride, ethylenediaminetetracetic acid (EDTA), D-pencillamine, dithiothreitol (DTT), N-acetyl-L-cysteine (NAC), meso-2,3-dimercapto-succinic acid (DMSA), ethylene glycol-bis (β-aminoethyl ether)-N,N,N′N′-tetraacetic acid (EGTA), trypan blue (0.4%), and BAPTA-AM (BAPTA) were obtained from Sigma Chemical Co. (St. Louis, MO). [^{3}H]arachidonic acid was obtained from American Radiolabeled Chemicals, Inc. (St. Louis, MO). Arachidonly trifluoromethyl ketone ($AACOCF_3$) and enzyme immunoassay kits for total prostaglandins, thromboxane B_2, and 8-isoprostane were obtained from Cayman Chemical Co. (Ann Arbor, MI).

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 × 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 (75% to 80% confluence).

Assay of Release of Arachidonic Acid and PLA_2 Activation

Release of arachidonic acid from cellular membrane phospholipids is widely assayed as an index of PLA_2 activity (Balsinde et al. 2000). BPAECs in 35-mm dishes (5×10^5 cells/dish) were labeled with carrier-free [^{3}H]arachidonic acid (5 μCi/ml) in complete EC media containing 10% FBS, nonessential amino acids, antibiotic, and growth factor for 12 h at 37°C in 5% CO_2-95% air. The radioactive medium was removed by aspiration and cells were incubated in serum-free MEM or MEM containing mercury chloride ($HgCl_2$) at the chosen concentrations (1 to 50 μM) for specified lengths of time (0 to 120 min). When required, cells prelabeled with [^{3}H]arachidonic acid were pretreated with selected pharmacological agents/inhibitors for 1 h and then exposed to mercury chloride in the absence or presence of the pharmacological inhibitors for specified lengths of time. At the end of the incubation period the amount/extent of arachidonic acid released into the medium, as an index of PLA_2 activity, was determined by liquid scintillation counting. The extent of arachidonic acid released was expressed as DPM of [^{3}H]/dish.

Determination of Cyclooxygenase-Mediated Formation of Arachidonic Acid Metabolites

The COX-mediated formation of arachidonic acid metabolites in BPAECs following their exposure to mercury chloride was determined by utilizing the commercially available EIA kits (Cayman Chemical Co., Ann Arbor, MI). Release of total prostaglandins, thromboxane B_2, and 8-isoprostane by cells was determined according to the manufacturer's recommendations. The extent of arachidonic acid metabolites released by cells was expressed as pg/mL medium.

Cellular Total Thiol Determination

Total cellular thiol content was measured by DTNB-coupled spectrophotometric assay according to Parinandi et al. (1999) and Hagele et al. (2006). BPAECs were grown to 100% confluence in 100-mm dishes and then treated with MEM or MEM containing mercury chloride (25 μM) for 60 min. After incubation, cells were detached by gentle scraping and centrifuged at 5,000 × g for 10 min at 4°C. The cell pellets were then lysed using Triton × 100. Cell lysates were 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 expressed as μg thiols/mg protein.

Assay of Cytotoxicity

Morphological changes in BPAECs grown in 35-mm dishes up to 70% confluence, following their exposure to different concentrations of mercury chloride (0, 5, 10, and 25 μM) in MEM for designated lengths of time (5, 15, 30, and 60 min) at 37°C in a humidified environment of 5% CO_2-95% air, were examined as an index of cytotoxicity. The role of $cPLA_2$ in the mercury-induced morphological changes was investigated in cells pretreated with the $cPLA_2$-specific inhibitor $AACOCF_3$ (1 μM) for 1 h in MEM and then subjecting the cells to mercury chloride (10 μM) treatment in MEM for 60 min at 37°C in a humidified environment of 5% CO_2-95% air. Simultaneous controls were established with MEM alone or MEM containing the inhibitor alone under identical conditions. Images of cell morphology were digitally captured with the Nikon Eclipse TE2000-S at either 10× or 100× magnification. Additionally, the trypan blue exclusion assay was also performed to assess the $cPLA_2$ inhibitor ($AACOCF_3$) effect on mercury-induced cytotoxicity in BPACEs according to Verity et al. (1994). Following pretreatment of cells with $AACOCF_3$ (1, 5, 10 μM) in MEM for 60 min, the cells were treated with mercury chloride (10 μM) in MEM for 60 min at 37°C in a humidified environment of 5% CO_2-95% air. Simultaneous controls were established with MEM alone or MEM containing $AACOCF_3$ alone. At the end of incubation, treatment media were removed and cells were treated with 1.0 mL trypan blue solution (1:10 dilution of stock 0.4% trypan blue solution in 0.85% saline) for 3 min at 37°C in a humidified environment of 5% CO_2-95% air. Following this, the solution was removed, cells were gently washed three times with PBS containing 0.5% glucose, and the total number of cells and the number of cells that did not exclude trypan blue were determined under Nikon Eclipse TE2000-S microscope at 20× magnification in an observed field. Mercury-induced cytotoxicity was determined from the total number of cells and the number of cells that had taken up trypan blue and expressed as % cells that had taken up trypan blue in an observed field.

Protein Determination

Cellular protein levels were determined by BCA protein assay (Pierce, Rockford, IL).

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 were done by Dunnett's method with $P < 0.05$ indicating significance.

RESULTS

Mercury Activates PLA_2 in a Dose- and Time-Dependent Fashion

As no reports have been made so far on mercury-induced activation of PLA_2 in the vascular ECs and earlier we have shown that mercury activates PLD in ECs (Hagele et al. 2006), here we investigated whether inorganic mercury (mercury chloride) would induce the release of arachidonic acid as an index of PLA_2 activation in BPAECs in a dose-dependent (0 to 50 μM) fashion following incubation of cells for 30 min with mercury chloride. Mercury chloride significantly caused the activation of PLA_2 at 10, 15, 25, and 50 μM concentrations upon treatment of cells for 30 min, as compared to that in the cells treated with vehicle alone (Fig. 1A). The time-dependant activation of PLA_2 in BPAECs upon their treatment with mercury chloride was also evident. At 15 min of treatment, mercury chloride caused a significant activation of PLA_2, which further increased at 30, 45, 60, 90, and 120 min of treatment with the metal as compared to the cells treated with the vehicle alone (Fig. 1B). Overall, these results revealed that inorganic mercury (mercury chloride) was effective in causing a significant and dose- and time-dependent activation of PLA_2 in BPAECs.

Metal Chelating Agents Attenuate Mercury-Induced PLA_2 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 PLA_2 have not been reported so far. Therefore, here, the effects of well-established chelating agents including EDTA and D-pencillamine (0.1, 0.5, and 1 mM) were examined on the PLA_2 activation induced by mercury chloride (25 μM) in BPAECs. Prior to the treatment of cells with

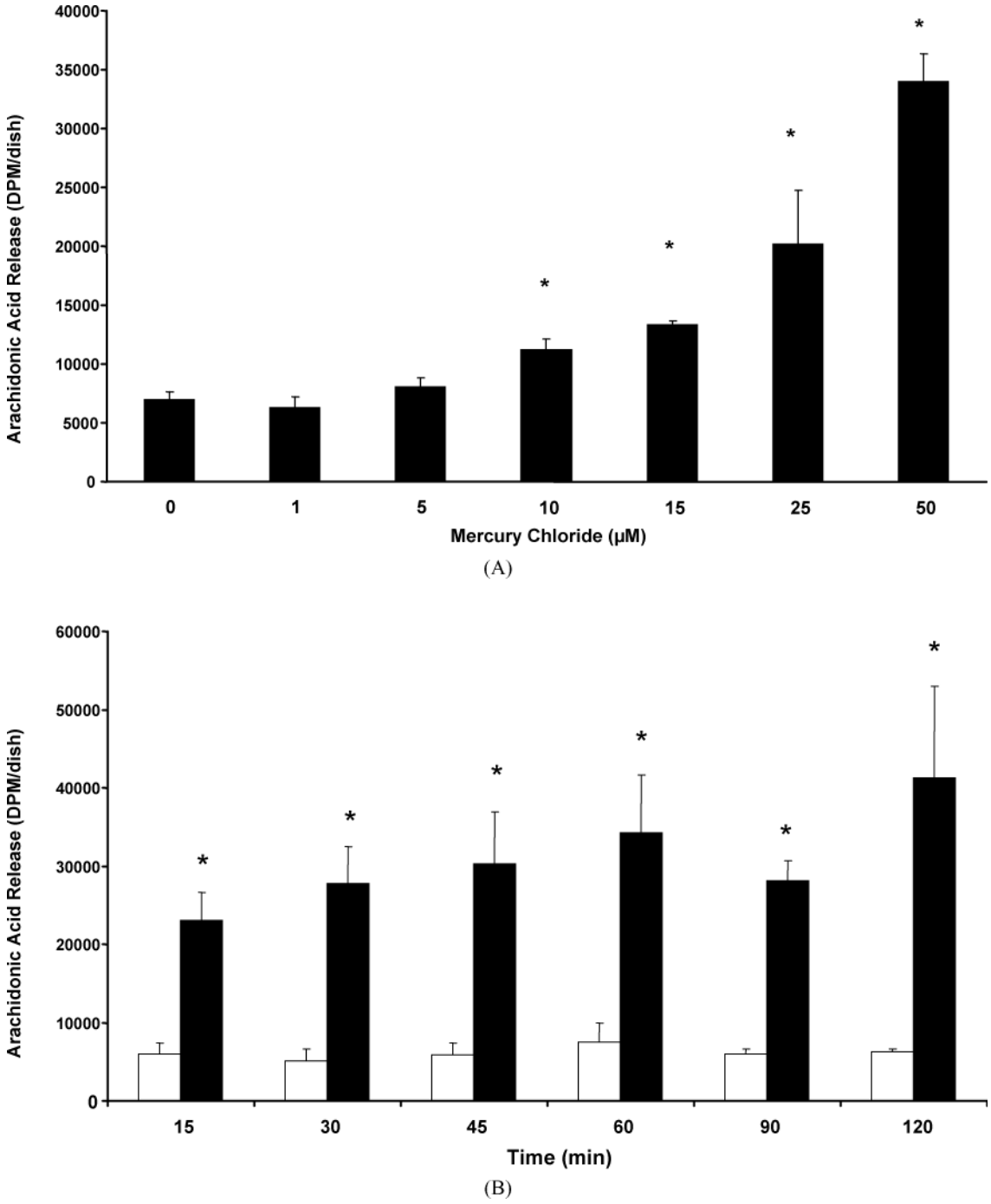


FIGURE 1 **Mercury activates PLA_2 in a dose- and time- dependent fashion. BPAECs (5×10^5 cells/35-mm dish) were labeled with [^{3}H]arachidonic acid (5 μCi, carrier-free) in complete EC medium for 12 h and then the cells were treated with different concentrations (0 to 50 μM) of mercury chloride (A) for 30 min and with mercury chloride (25 μM) (B) for 0 to 120 min. Appropriate controls were established with cells treated with MEM alone. At the end of incubation, [^{3}H]arachidonic acid released into the medium 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 the vehicle-treated control cells.**

the mercury chloride, cells were pretreated for 1 h with basal MEM or MEM containing the chosen chelating agent(s) and then exposed to the mercury compound in the presence of the chelating agent(s) for 30 min. The classic trace element chelating agent, EDTA, at all the three tested doses significantly attenuated the mercury-induced PLA_2 activation (Fig. 2A). D-penicillamine, an amino acid analog of cysteine and a metal chelating drug, caused effective and significant inhibition of the mercury-induced PLA_2 activation in BPAECs at

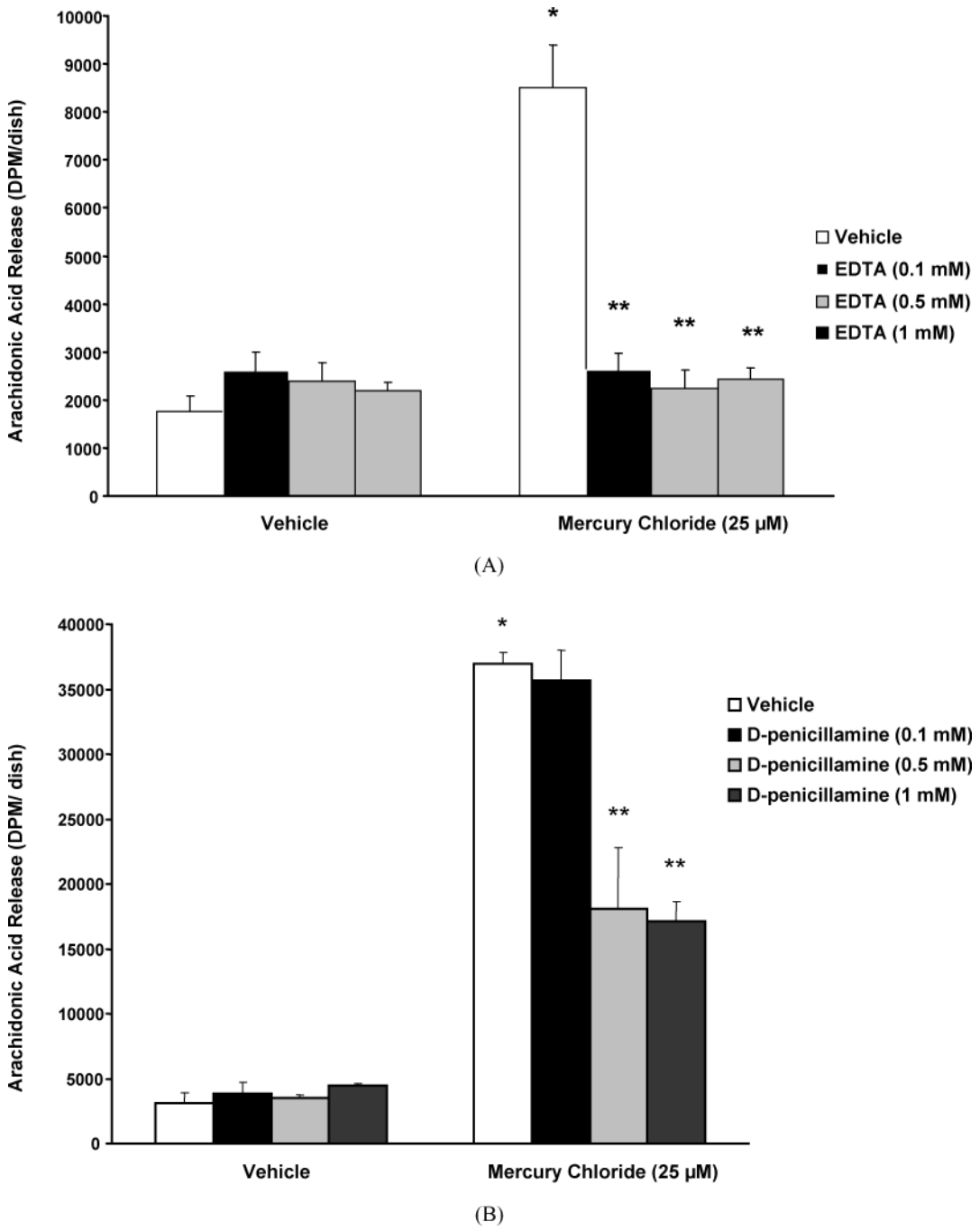


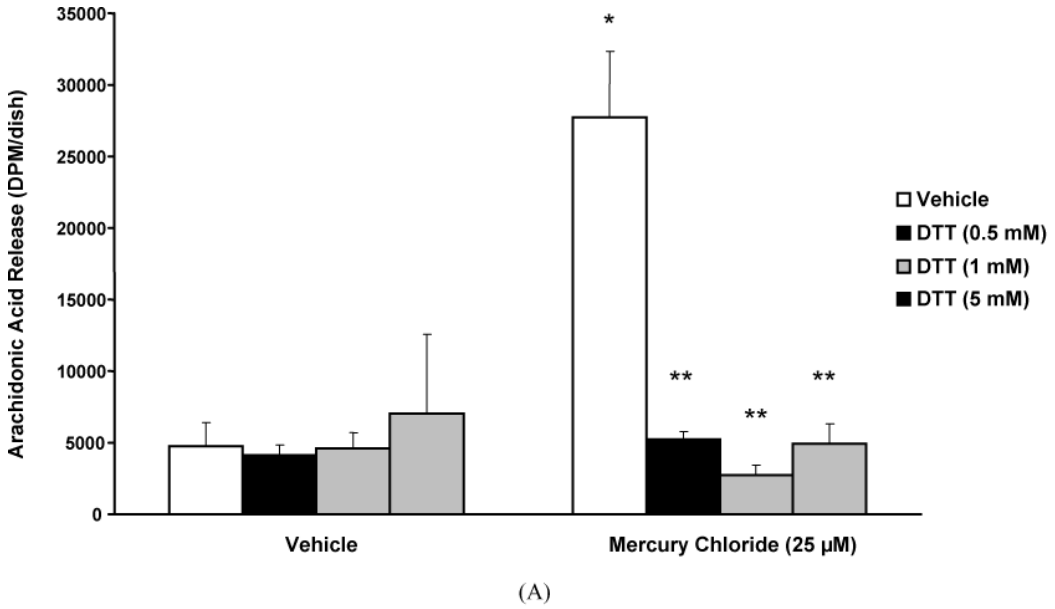
FIGURE 2 **Metal chelating agents attenuate mercury-induced PLA_2 activation. BPAECs (5×10^5 cells/35-mm dish) were labeled with [^{3}H]arachidonic acid (5 μCi, carrier-free) in complete EC medium for 12 h following which the cells were pretreated for 1 h with MEM alone or MEM containing EDTA (0.1, 0.5, and 1 mM) (A) or D- penniclillamine (0.1, 0.5, and 1 mM) (B) and then subjected to treatment with vehicle alone or mercury chloride (25 μM) for 30 min. At the end of incubation, [^{3}H]arachidonic acid released into the medium 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 mercury chloride alone.**

two chosen doses, 0.5 and 1 mM (Fig. 2B). Overall, these results showed that EDTA and D-penicillamine were effective chelating agents in causing significant attenuation of PLA_2 activation in BPAECs induced by mercury chloride.

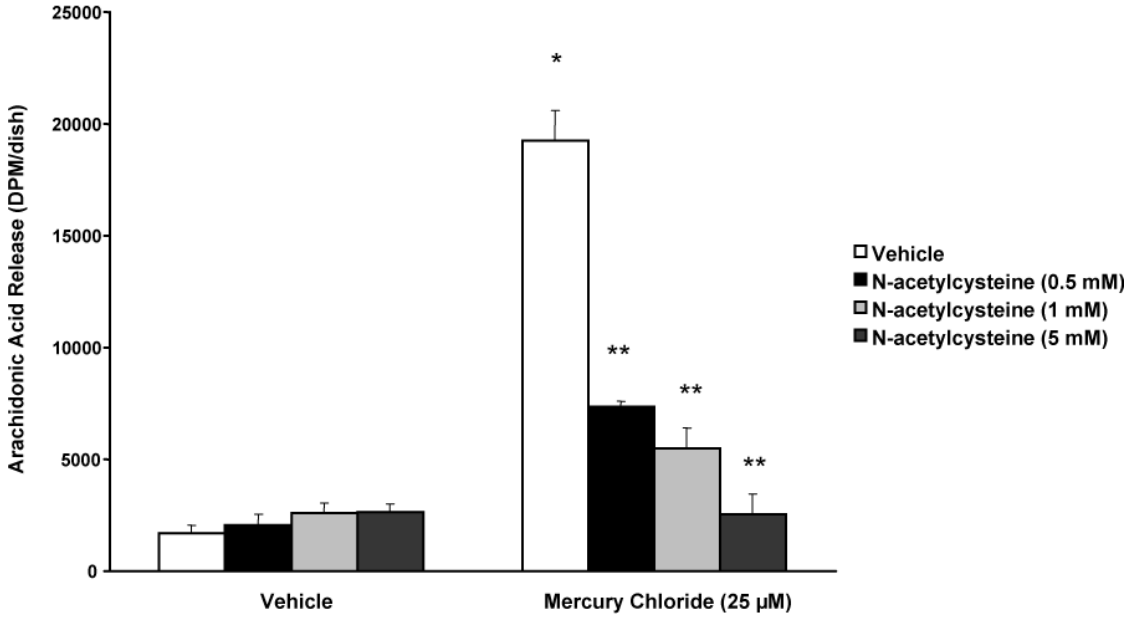
Sulfhydryl Agents Attenuate Mercury-Induced PLA_2 Activation

Reports have been made that the thiols (nonprotein and protein) are the targets for heavy metal cellular

actions (Blanusa et al. 2005; Valko et al. 2005). Therefore, in order to establish the role of thiols in mercury chloride-induced PLA_2 activation in BPAECs, here the effects of well-established thiol (sulfhydryl) protective agents (DTT and NAC) were investigated. Prior to exposure of cells to 25 μM mercury chloride, cells were pretreated for 1 h with MEM or MEM containing the chosen thiol protective agent(s) (0.5, 1, and 5 mM) and then treated with mercury chloride in the presence of the thiol protective agent(s) for 30 min. DTT, a sulfhydryl protective agent, at all the three concentrations tested, offered effective and significant inhibition of the mercury chloride-induced PLA_2 activation in BPAECs (Fig. 3A). NAC, a widely



(A)



(B)

FIGURE 3 **Sulfhydryl agents attenuate mercury-induced PLA_2 activation. BPAECs (5×10^5 cells/35-mm dish) were labeled with [^{3}H]arachidonic acid (5 μCi, carrier-free) in complete EC medium for 12 h, following which the cells were pretreated for 1 h with MEM alone or MEM containing DTT (0.5, 1, and 5 mM) (A) and NAC (0.5, 1, and 5 mM) (B) and then subjected to treatment with vehicle alone or mercury chloride (25 μM) for 30 min. At the end of incubation, [^{3}H]arachidonic acid released into the medium 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 mercury chloride alone.**

used thiol protector and antioxidant, caused effective and significant attenuation of PLA_2 activation in ECs induced by mercury (Fig. 3B). Collectively, these results revealed that thiol-protective agents effectively attenuated PLA_2 activation induced by mercury chloride, suggesting the involvement of cellular thiols.

Calcium Chelators Attenuate Mercury-Induced PLA_2 Activation

As it has been established that mammalian cells contain a calcium-dependent form of PLA_2 ($cPLA_2$), here we investigated the role of calcium in the mercury-induced activation of PLA_2 by using the widely accepted calcium quenchers EGTA and BAPTA. Prior to exposure of cells to mercury chloride (25 μM), cells were pretreated for 1 h with MEM or MEM containing the chosen calcium chelating agent (EGTA 1 mM and BAPTA 1 μM) and then treated with the mercury compound in the presence of the chelating agent(s) for 30 min. EGTA, an extracellular calcium chelator, effectively and significantly attenuated mercury-induced PLA_2 activation (Fig. 4A). A well-known intracellular calcium chelator, BAPTA, also protected against mercury-induced PLA_2 activation in BPAECs (Fig. 4B). These results revealed that calcium chelators effectively blocked mercury-induced PLA_2 activation, further suggesting the role of calcium in the enzyme activation in ECs and also the involvement of $cPLA_2$.

$cPLA_2$-Specific Inhibitor Attenuates Mercury-Induced PLA_2 Activation

As the calcium chelators caused a significant attenuation of the mercury chloride-induced PLA_2 activation in the BPAECs, here we investigated the contribution of $cPLA_2$ in the mercury-induced release of arachidonic acid. In order to demonstrate this, we utilized the $cPLA_2$-specific inhibitor $AACOCF_3$ (Riendeau et al. 1994) to examine its inhibitory effect on the mercury chloride-induced release of arachidonic acid by the ECs. Cells were pretreated in MEM or MEM containing $AACOCF_3$ (5 μM) for 1 h and then treated with mercury chloride (25 μM) in the presence of the inhibitor for 30 min. $AACOCF_3$ slightly but significantly attenuated the mercury-induced arachidonic acid release by BPAECs (Fig. 5). The results of this study confirmed that the mercury chloride activated $cPLA_2$ and thus also contributed to the release of arachidonic acid by the ECs.

Mercury Induces the Release of COX-Mediated Formation of Arachidonic Acid Metabolites

As the PLA_2-catalyzed release of arachidonic acid is known as the suitable substrate for COX activity in mammalian cells, here we investigated the release of mercury-induced COX-generated arachidonic acid metabolites by BPAECs. In order to demonstrate this, we exposed BPAECs to mercury chloride (25 μM) and the release of the COX-generated arachidonic acid metabolites (total prostaglandins, thromboxane-B_2, and 8-isoprostane) was determined at different times of incubation (15 to 90 min). As shown in Figures 6A–C, mercury chloride significantly induced the release of prostaglandins, thromboxane-B_2, and 8-isoprostane by the cells in a time-dependant manner. At 90 min of exposure of cells to mercury chloride, the release of all analyzed COX-generated arachidonic acid metabolites was at a maximum. These results demonstrated that mercury chloride induced the release of COX-generated arachidonic acid metabolites in parallel to PLA_2 activation and arachidonic acid release.

Mercury Causes the Decrease of Cellular Thiols

As it has been established that cellular thiols are targets for mercury and the current study revealed that thiol protectants attenuated the mercury-induced PLA_2 activation in BPAECs, here we examined the effect of mercury chloride (25 μM) on the levels of total cellular thiols in BPAECs. As shown in Figure 7, mercury chloride caused a significant decrease (46%) in the total thiol content following treatment of cells for 1 h as compared to the same in vehicle-treated control cells.

$cPLA_2$ Inhibitor Protects Against Mercury-Induced Cytotoxicity

As observed in the current study that the $cPLA_2$ inhibitor ($AACOCF_3$) significantly inhibited the mercury-induced activation of the arachidonic acid release from BPAECs, here we investigated whether PLA_2 activation had a role in the mercury-induced cytotoxicity. First, we observed that mercury chloride

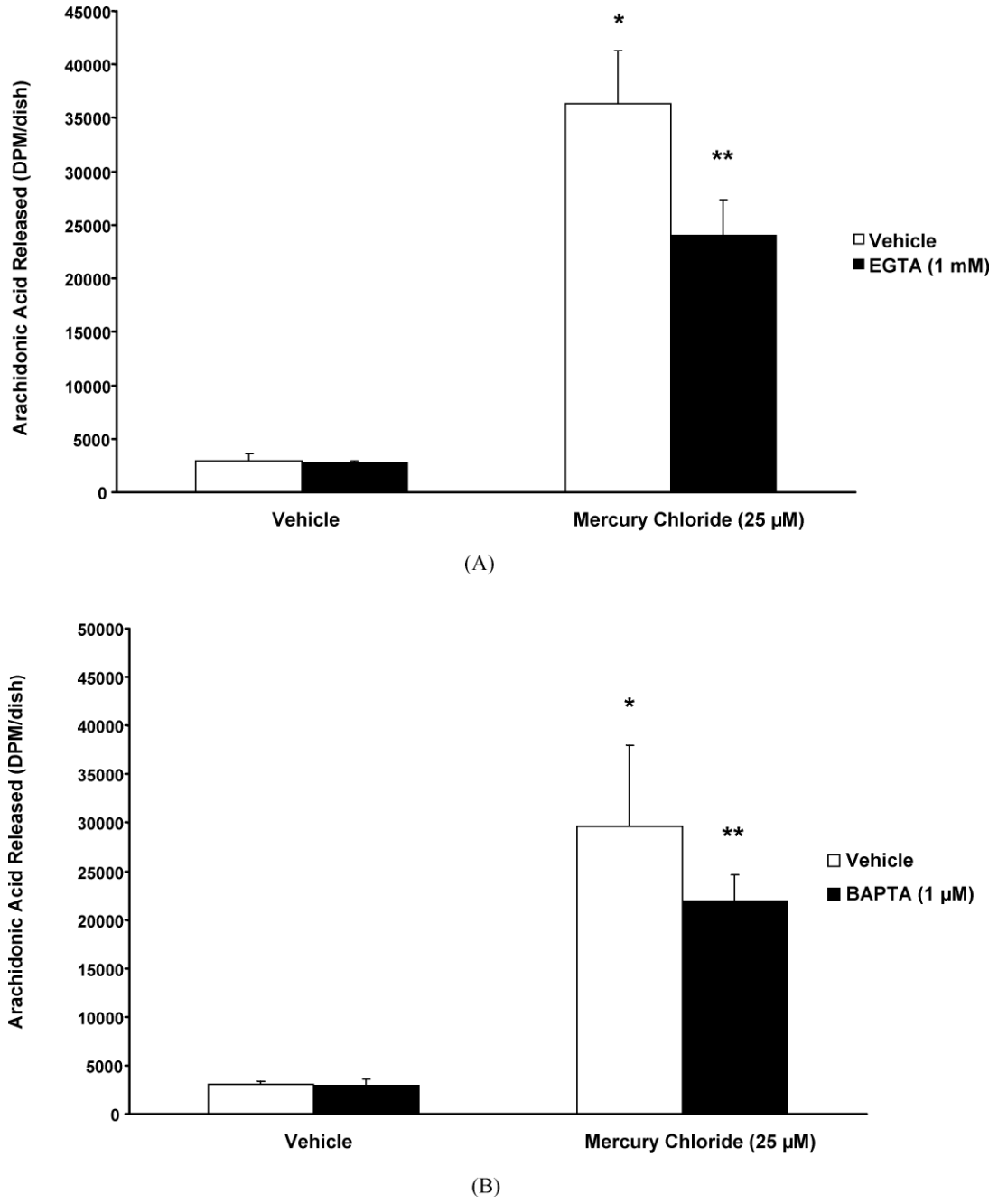


FIGURE 4 **Calcium chelators attenuate mercury-induced PLA_2 activation. BPAECs (5×10^5 cells/35-mm dish) were labeled with [^{3}H]arachidonic acid (5 μCi, carrier-free) in complete EC medium for 12 h, following which the cells were pretreated for 1 h with MEM alone or MEM containing EGTA (1 mM) (A) or BAPTA (1 μM) (B) and then subjected to treatment with vehicle alone or mercury chloride (25 μM) for 30 min. At the end of incubation, [^{3}H]arachidonic acid released into the medium 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 mercury chloride alone.**

induced cytotoxicity in BPAECs in both a dose- and time-dependent manner as observed from the alterations in cell morphology (Fig. 8). With both the increase in time of exposure and dose, mercury chloride induced the loss of cell morphology leading to the formation of circular cellular shape. $AACOCF_3$ (1 μM) restored the mercury chloride (10 μM)-induced loss of cell morphology (Fig. 9). As seen in Figure 10, mercury chloride (10 μM) also significantly enhanced the uptake of trypan blue, which was significantly attenuated by the $cPLA_2$ inhibitor $AACOCF_3$ (5 and 10 μM) in a dose-dependent manner. Collectively,

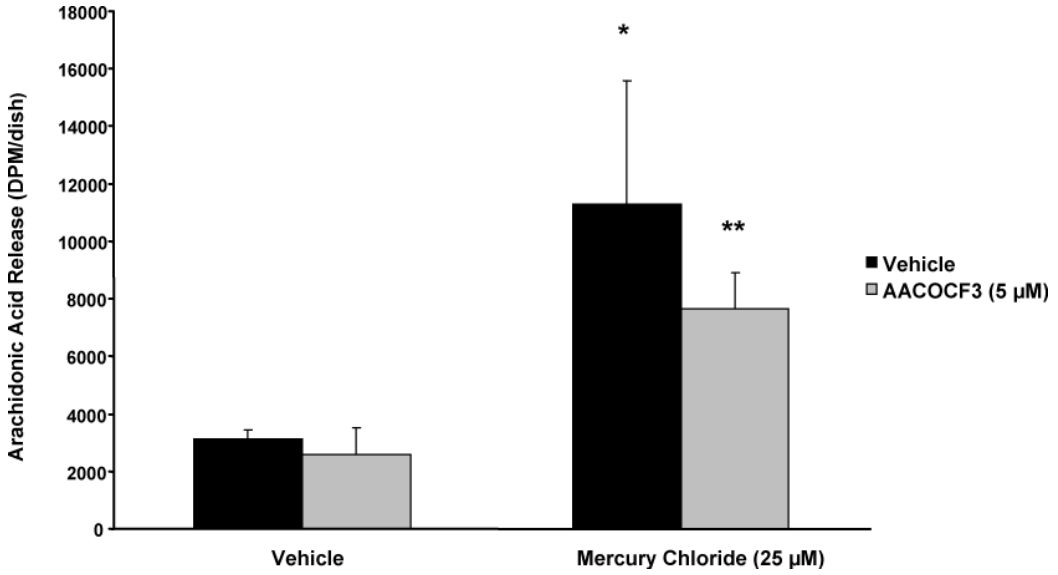


FIGURE 5 **cPLA$_2$-specific inhibitor attenuates mercury-induced PLA$_2$ activation. BPAECs (5 × 10^5 cells/35-mm dish) were labeled with [^{3}H]arachidonic acid (5 μCi, carrier-free) in complete EC medium for 12 h, following which the cells were pretreated for 1 h with MEM alone or MEM containing the cPLA$_2$-specific inhibitor AACOCF$_3$ (5 μM) and then subjected to treatment with vehicle alone or mercury chloride (25 μM) for 30 min. At the end of incubation, [^{3}H]arachidonic acid released into the medium 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 mercury chloride alone.**

these results revealed that mercury chloride induced cytotoxicity in BPAECs, which was attenuated by the cPLA$_2$ inhibitor suggesting the role of cPLA$_2$ in the inorganic mercury-induced cytotoxicity in ECs.

DISCUSSION

The current study revealed that inorganic mercury (mercury chloride) activated PLA$_2$ (release of arachidonic acid) from the membrane phospholipids of

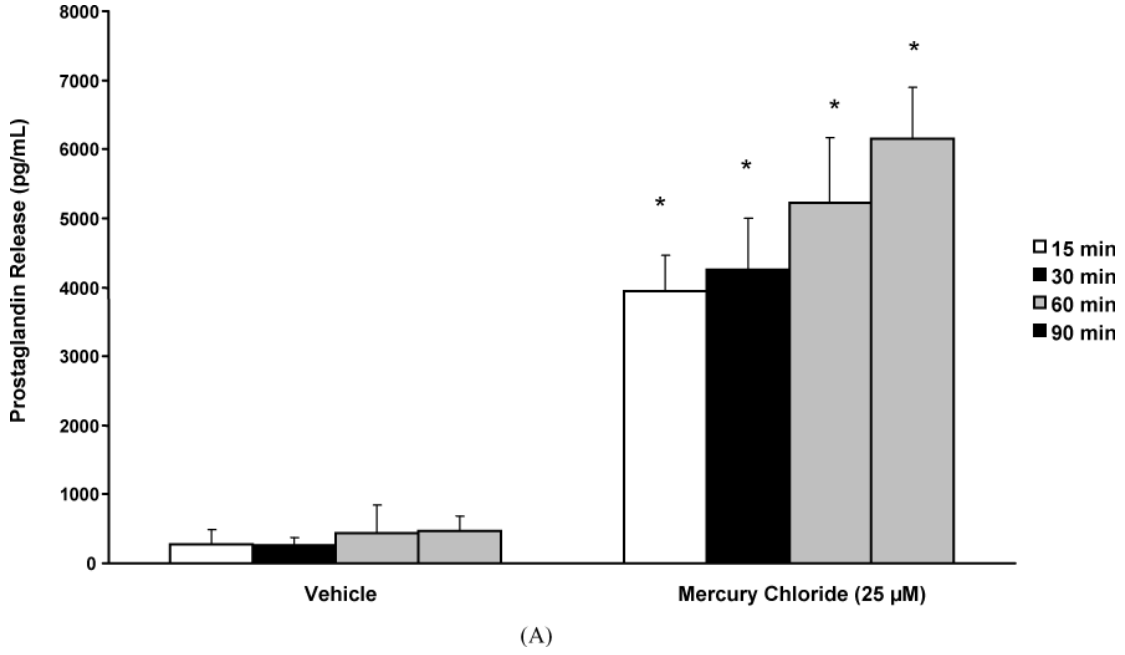
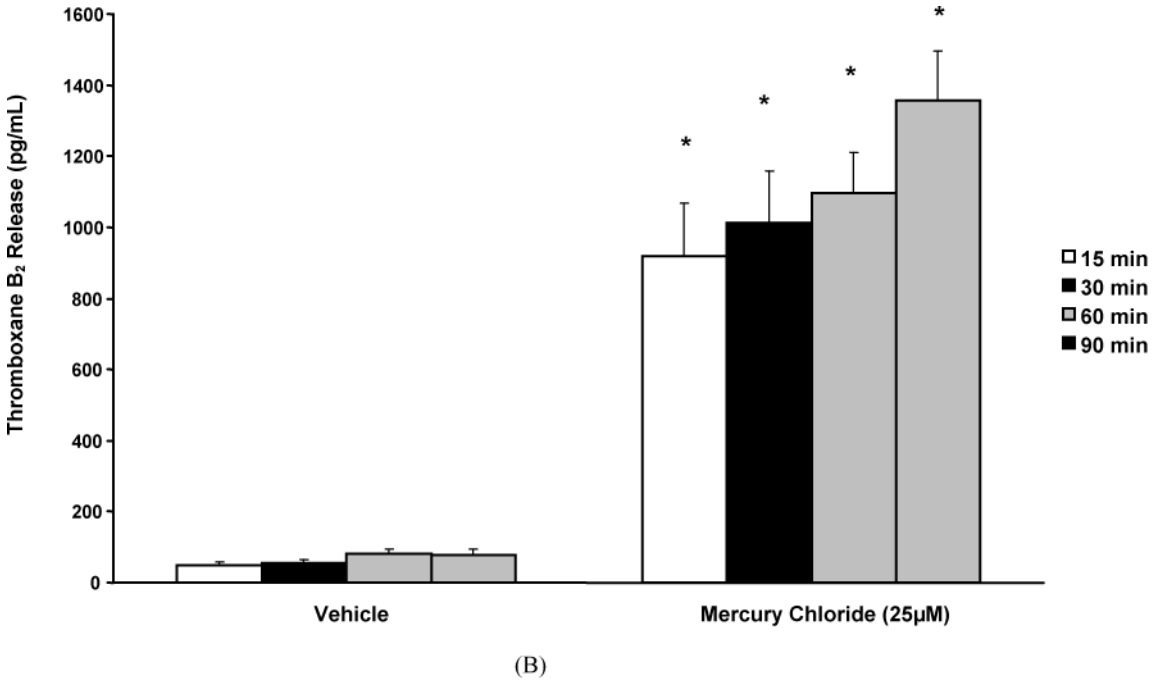
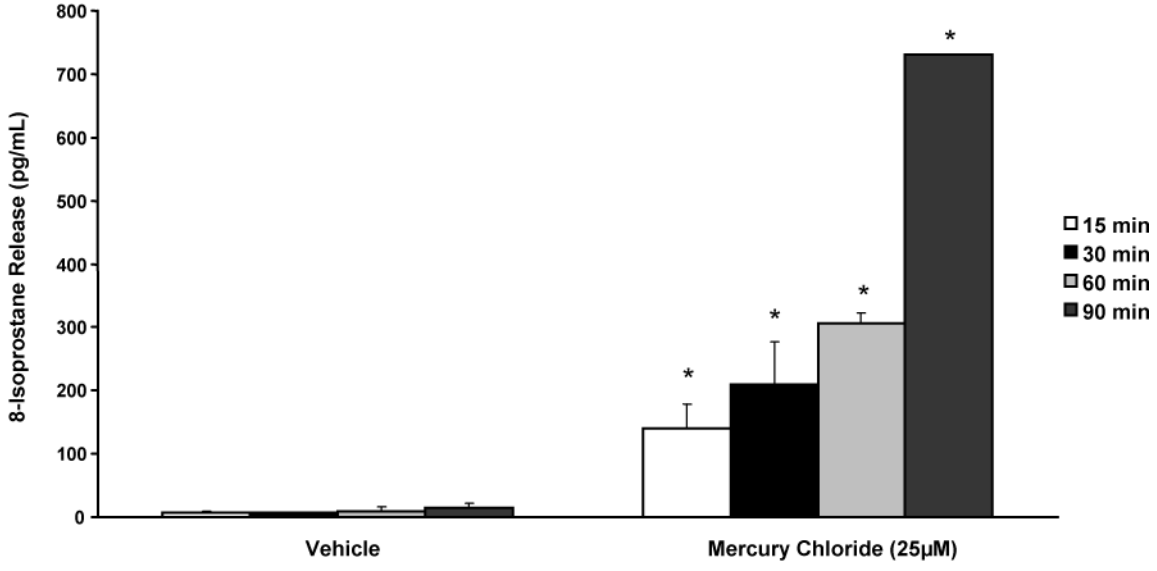


FIGURE 6 **Mercury induces release of COX-mediated formation of arachidonic acid metabolites. BPAECs (5 × 10^5 cells/35-mm dish) were treated with MEM alone or MEM containing mercury chloride (25 μM) for different time periods (15 to 90 min). At the end of the incubation period, the release of total prostaglandins (A), thromboxane-B2 (B), and 8-isoprostane (C) 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. *(Continued)***



(B)



(C)

FIGURE 6 **(Continued)**

BPAECs in a dose- and time- dependent fashion. The study also showed that the mercury-induced PLA_2 activation in BPAECs was attenuated by metal chelating agents, thiol protectants, and calcium chelators, indicating the complexing of mercury with the cellular targets, involvement of cellular thiols, and the role of calcium in the mercury-induced release of arachidonic acid from the cells. This study also demonstrated that the $cPLA_2$-specific inhibitor ($AACOCF_3$) (Riendeau et al. 1994) attenuated the mercury-induced release of arachidonic acid by BPAECs, further suggesting the activation of $cPLA_2$ by inorganic mercury (mercury chloride). Furthermore, it was also evident from the current results that mercury chloride induced the formation of COX-generated arachidonic acid metabolites in a time-dependent manner. Overall, this study demonstrated that inorganic mercury induced the activation of PLA_2 resulting in the release of arachidonic acid and formation of its metabolites in the vascular ECs.

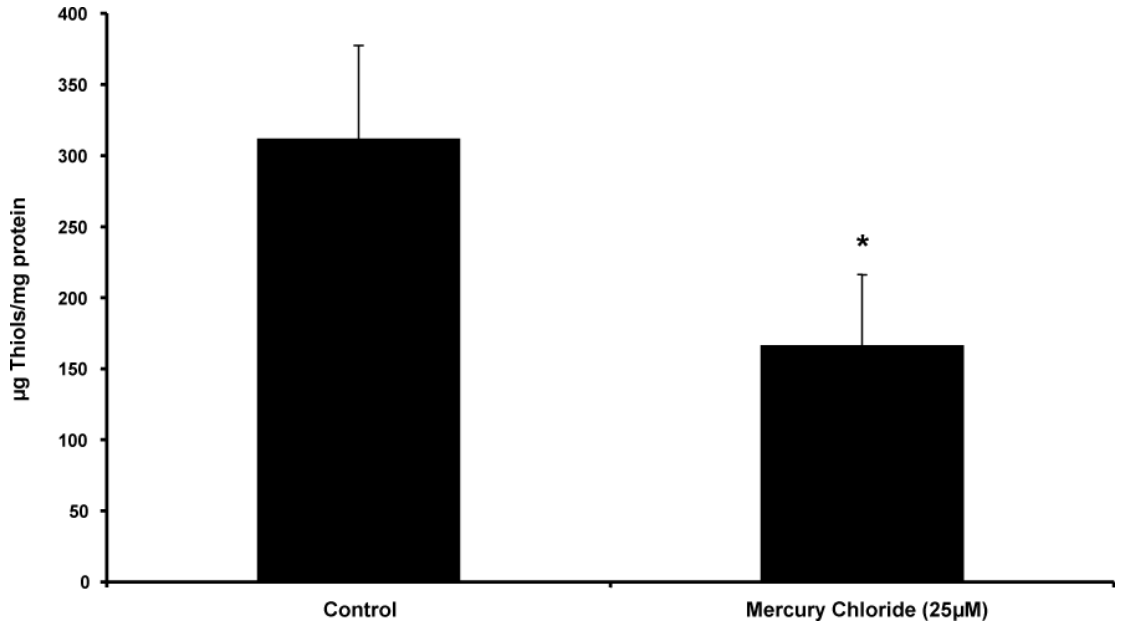


FIGURE 7 Mercury causes the decrease of cellular thiols. BPAECs (2×10^6 cells/100-mm dish) were treated with MEM alone or MEM containing mercury chloride (25 μM) for 60 min. After incubation, total thiols were determined spectrophotometrically as described in Materials and Methods. Data represent means ± S.D. of three independent experiments. *Significantly different at $P < 0.05$ as compared to cells treated with vehicle alone.

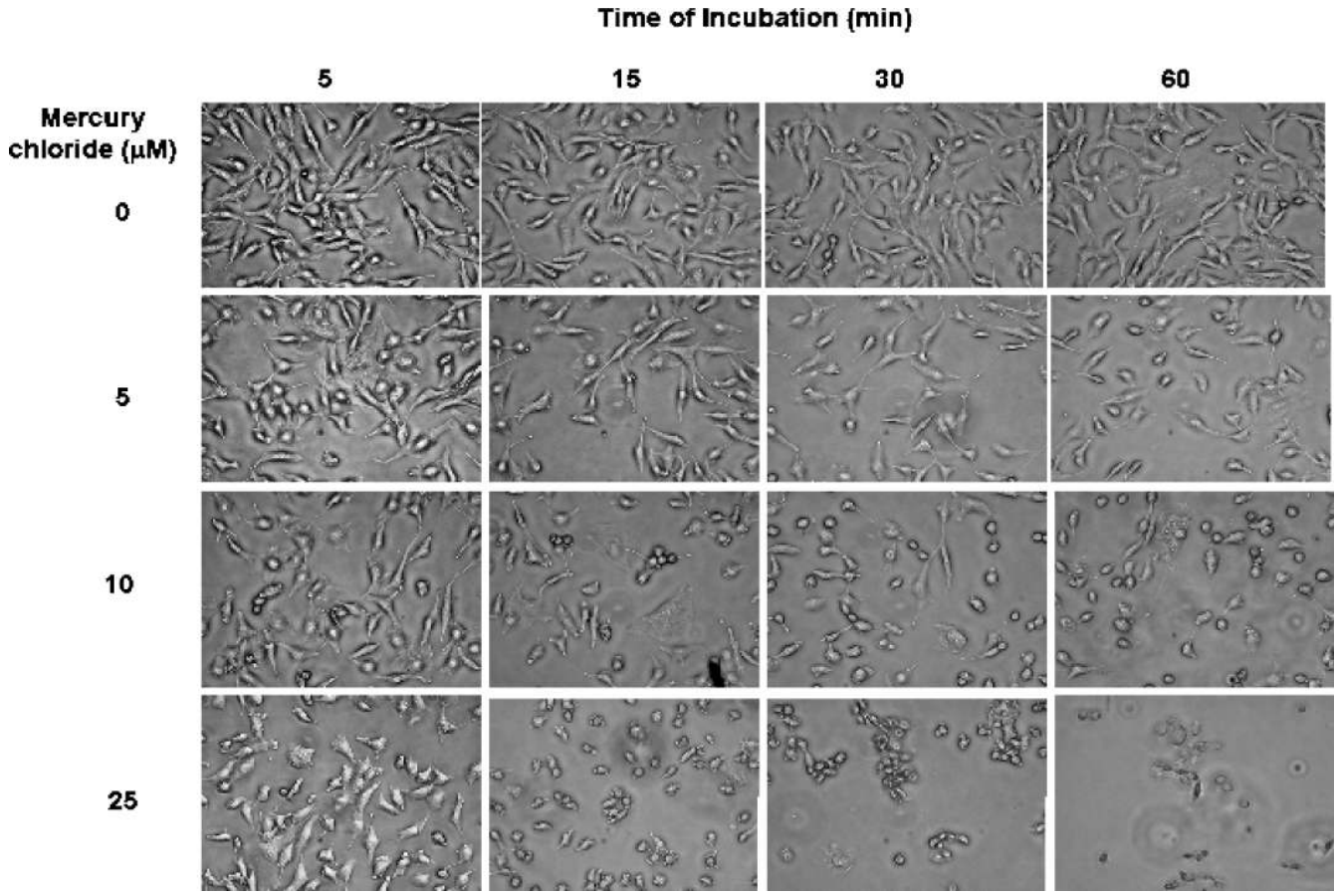


FIGURE 8 Mercury induces alterations in cell morphology. BPAECs (5×10^5 cells/35-mm dish) were treated with MEM alone or MEM containing mercury chloride (5, 10, and 25 μM) for different time periods (5 to 60 min). At the end of the incubation period, the medium was replaced with PBS containing 0.5% glucose and the cells were examined under light microscope at a magnification of 10× as described under Materials and Methods. Each micrograph is a representative picture of three independent experiments conducted under identical conditions.

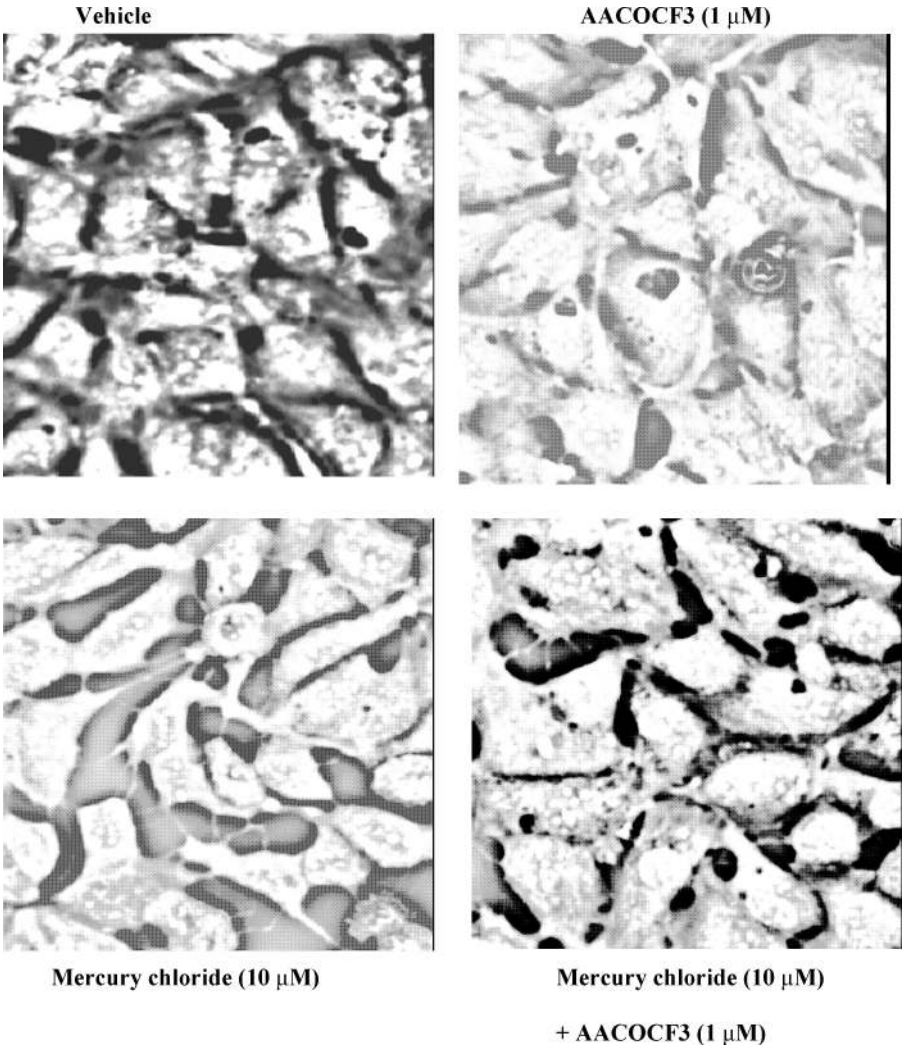


FIGURE 9 **cPLA2 inhibitor protects against mercury-induced alterations in cell morphology. BPAECs (5×10^5 cells/35-mm dish) were pretreated with MEM alone or MEM containing $AACOCF_3$ (1 μM) for 60 min, following which the cells were treated with MEM alone or MEM containing mercury chloride (10 μM) for 60 min. At the end of incubation, the medium was replaced with PBS containing 0.5% glucose and the cells were examined under light microscope at a magnification of 100× as described under Materials and Methods. Each micrograph is a representative picture of three independent experiments conducted under identical conditions.**

PLA_2s in mammalian systems are broadly divided into three major classes: (1) cytosolic calcium-dependent PLA_2 ($cPLA_2$), (2) intracellular calcium-independent PLA_2 ($iPLA_2$), and (3) secretory calcium-dependent PLA_2 ($sPLA_2$) (Lambert et al. 2006). PLA_2 acts on the *sn*-2 fatty acid esterified in the membrane phospholipids to release the unsaturated fatty acid and to generate the lysophospholipid (Balsinde et al. 2000). The free arachidonic acid thus generated acts as a substrate for COXs and LOXs and gets converted into physiologically active eicosanoids including the prostaglandins and leukotrienes (Balsinde et al. 2000). The lysophospholipid with the alkyl group at the *sn*-1 position, which is also generated from the membrane phospholipid upon the action of PLA_2, is converted into the platelet-activating factor (PAF). As both the eicosanoids (COX- and LOX-mediated arachidonic acid metabolites) are potent bioactive lipids and key players in inflammation, PLA_2 is regarded as an important lipid signaling enzyme (Dennis et al. 1991; Balsinde et al. 2000).

Regulation of PLA_2 appears to be complex. The activity of $cPLA_2$ has been extremely studied and shown to be regulated through phosphorylation of serine, which is mediated by nitrogen-activated protein kinases, protein kinase A, and protein kinase C (Chakraborti 2003). However, the regulation of activities of $iPLA_2$ and $sPLA_2$ is not thoroughly understood. Lipid peroxidation has been shown to simulate the activity of $sPLA_2$ (Nigam and Schewe

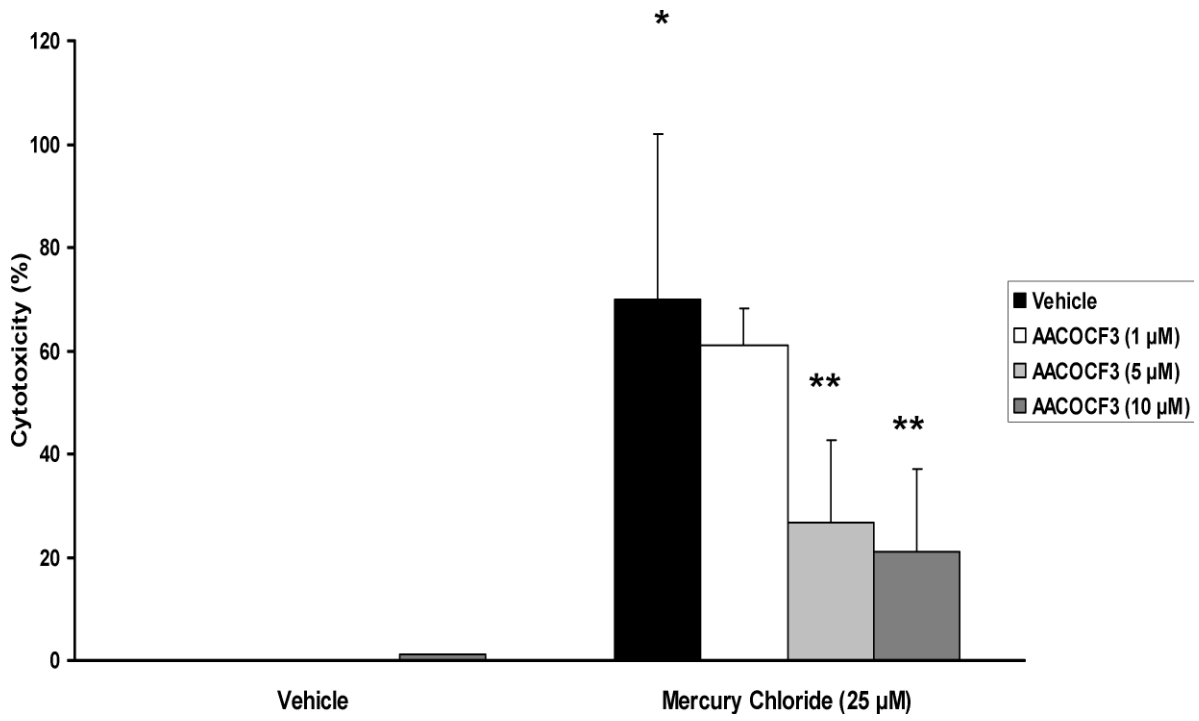


FIGURE 10 **cPLA2 inhibitor protects against mercury-induced cytotoxicity. BPAECs (5×10^5 cells/35-mm dish) were pretreated with MEM alone or MEM containing $AACOCF_3$ (1, 5, and 10 μM) for 60 min, following which the cells were treated with MEM alone or MEM containing mercury chloride (10 μM) for 60 min. At the end of incubation, cytotoxicity was assayed as described under Materials and Methods. Cytotoxicity was calculated as the% cells that had taken up trypan blue. 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 mercury chloride alone.**

2000). Reactive oxygen species have been shown to activate $iPLA_2$ and cause release of arachidonic acid in macrophages (Martinez and Moreno 2001). Oxidant (hydrogen peroxide)-mediated release of arachidonic acid by astrocytes has been demonstrated due to activation of $cPLA_2$ and $iPLA_2$ (Xu et al. 2003). Taken together, these studies have revealed that PLA_2 activity is regulated by cellular signaling cascades, ROS, and oxidative stress.

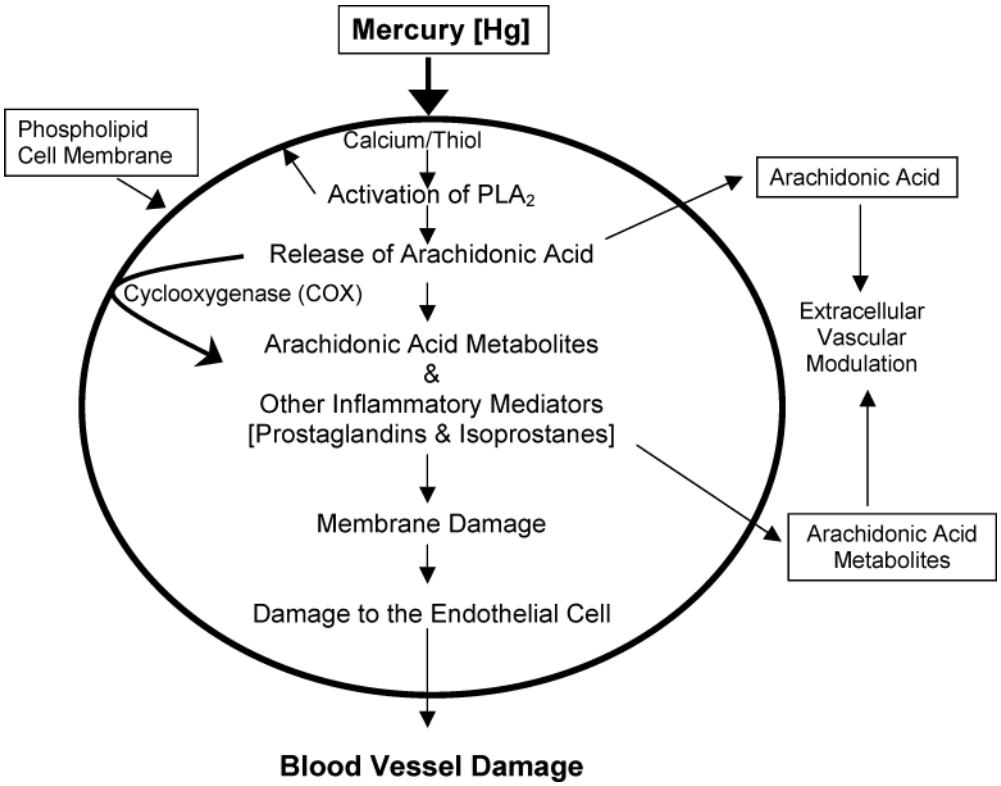
Among the toxic mercury compounds, the ability of only methylmercury in activating PLA_2 in neurons and astrocytes has been reported (Verity et al. 1994; Aschner 2000; Shanker et al. 2002, 2003). Dysregulation of calcium and increases in intracellular calcium in cerebellar granule cells (neurons) induced by methylmercury have been reported (Limke et al. 2004; Sarafian 1993; Marty and Atchison 1998). Methylmercury has been shown to increase intracellular concentrations of calcium in NG108–15 cells (Hare et al. 1999). Both the elevation of intracellular calcium and activation of phosphatidylcholine-specific phospholipase C and $cPLA_2$ by methylmercury in MDCK cells have been demonstrated (Kang et al. 2006). However no reports have been made so far on the mercury-induced activation of PLA_2 in the vascular ECs. The results of the present study had shown that mercury chloride induced the release of arachidonic acid, which was attenuated by calcium chelators in BPAECs and $cPLA_2$-specific inhibitors, suggesting the inorganic mercury-induced activation of $cPLA_2$ in the ECs. In addition, the partial inhibition of the inorganic mercury-induced arachidonic acid release from BPAECs as observed in the present study also suggested the activation of calcium-independent intracellular PLA_2 ($iPLA_2$) in ECs.

Methylmercury has been shown to cause neurotoxicity in astrocytes and neurons through the generation of ROS, induction of oxidative stress, and loss of cellular thiols including GSH (Shanker and Aschner 2001; Shanker et al. 2005). Recently, we have shown that both inorganic (mercury chloride) and organic (methylmercury and thimerosal) forms of mercury cause the activation of phospholipase D through the loss of cellular thiols in BPAECs (Hagele et al. 2006). Also, the results of the present study demonstrated the complete attenuation of the mercury chloride-induced arachidonic acid release from BPAECs by the thiol

protectants (DTT and NAC) and loss of cellular thiols induced by mercury chloride. This observation strongly suggested the role of cellular thiols in mercury chloride-induced release of arachidonic acid and activation of PLA_2 in ECs. Heavy metals including mercury have been shown to react with the cellular thiols (Hagele et al. 2006). The interaction of inorganic mercury with cellular thiols causing the release of arachidonic acid, as noticed in the present study, might have involved the thiol-dependant signaling events or the direct interaction of inorganic mercury with the enzyme or both.

Arachidonic acid metabolites including the COX-generated prostanoids (prostaglandins, thromboxane, and prostacyclin) have been identified as important inflammatory mediators in vascular endothelial dysfunction and atherosclerosis (Reiss and Edelman 2006; Bogatcheva et al. 2005). Influx of calcium, activation of $cPLA_2$, and release of arachidonic acid have been shown to play a crucial role in COX-mediated generation of arachidonic acid metabolites in the vascular ECs (Antoniotti et al. 2003). The results of the current study clearly revealed the mercury chloride-induced formation of COX-generated arachidonic acid metabolites in BPAECs and further suggested the activation of COXs and formation of arachidonic acid-derived inflammatory mediators in ECs under inorganic mercury exposure. Nevertheless, the present study also suggested the activation of COXs in addition to the activation of PLA_2 in ECs by inorganic mercury.

Our current study clearly revealed that mercury chloride-induced cytotoxicity in BPAECs (alterations in cell morphology and membrane damage) was protected by the $cPLA_2$ inhibitor ($AACOCF_3$), thus establishing a role of $cPLA_2$ in the inorganic mercury-induced cytotoxicity in ECs. Cytotoxicity of methylmercury has been reported in neurons and astrocytes (Verity et al. 1994; Aschner 2000; Shanker et al. 2002, 2003). Mepacrine, a well-established PLA_2 inhibitor, has been shown to protect against methylmercury-induced cytotoxicity in the cerebellar granule cells as observed from the trypan blue uptake study, suggesting an association between the methylmercury-induced PLA_2 activation and cytotoxicity in those cells (Verity et al. 1994). These reports further support our current findings that $cPLA_2$ activation also regulated the induction of cytotoxicity in ECs under mercury exposure.



SCHEME 1 **Mechanism of mercury-induced activation of PLA_2, release of arachidonic acid from membrane phospholipids, and formation of arachidonic acid metabolites and their probable role in endothelial and vasculotoxicity.**

The role of phospholipases including PLA_2 and COXs in vascular diseases and ischemic tissue injury is becoming increasingly evident (Hurt-Camejo et al. 2001; Phillis and O'Regan 2003). Furthermore, mercury has been implicated as a risk factor in myocardial infarction, coronary disease, and cardiovascular disease among humans (Yoshizawa et al. 2002; Kim et al. 2005; Nash 2005). Therefore, the results of the current study that inorganic mercury (mercury chloride) induced the release of arachidonic acid through activation of PLA_2 and mediated the formation of COX-mediated inflammatory arachidonic acid metabolites in ECs have profound implications in the understanding of mechanisms of mercury-induced cardiovascular diseases (Scheme 1).

REFERENCES

Antoniotti, S., Fiorio, P. A., Pregnolato, S., Mottola, A., Lovisolo, D., and Munaron, L. 2003. Control of endothelial cell proliferation by calcium influx and arachidonic acid metabolism: a pharmacological approach. *J. Cell Physiol.* 197(3):370–378.

Aschner, M. 2000. Astrocyte swelling, phospholipase A2, glutathione and glutamate: interactions in methylmercury-induced neurotoxicity. *Cell Mol Biol. (Noisy-le-grande)* 46(4):843–854.

Balsinde, J., Winstead, M. V., and Dennis, E. A. 2000. Phospholipase A2 regulation of arachidonic acid mobilization. *FEBS Lett* 531:2–6.

Blanusa, M., Varnai, V. M., Piasek, M., and Kostial, K. 2005. Chelators as antidotes of metal toxicity: therapeutic and experimental aspects. *Curr. Med. Chem.* 12(23):2771–2794.

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

Boffetta, P., Sallsten, G., Garcia-Gomez, M., Pompe-Kirn, V., Zaridze, D., Bulbulyan, M., Caballero, J. D., Ceccarelli, F., Kobal, A. B., and Merler, E. 2001. Mortality from cardiovascular disease and exposure to inorganic mercury. *Occup. Environ. Med.* 58:461–466.

Bogatcheva, N. V., Sergeeva, M. G., Dudek, S. M., and Verin, A. D. 2005. Arachidonic acid cascade in endothelial pathobiology. *Microvasc. Res.* 69:107–127.

Chakraborti, S. 2003. Phospholipase A2 isoforms: a perspective. *Cell Signal* 15:637–665.

Chan, H. M., and Egeland, G. M. 2004. Fish consumption, mercury exposure, and heart disease. *Nutr. Rev.* 62:68–72.

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

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

Dennis, E. A., Rhee, S. G., Billah, M. M., and Hannun, Y. A. 1991. Role of phospholipases in generating lipid second messengers in signal transduction. *FASEB J* 5:2068–2077.

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

Dopp, E., Hartman, L. M., Florea, A. M., Rettenmeier, A. W., and Hirner, A. V. 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.

Hagele, T. J., Mazerik, J. N., Gregory, A., Kaufman, B., Magalang, U., Kuppusamy, M., Marsh, C. B., Kuppusamy, P., and Parinandi, N. L. 2006. Mercury activates vascular endothelial cell phospholipase D through thiols and oxidative stress. *Int. J. Toxicol.* 26(1): 57–69.

Hare, M. F., McGinnis, K. M., and Atchison, W. D. 1993. Methylmercury increases intracellular concentrations of Ca^{++} and heavy metals in NG108–15 cells. *J. Pharmacol. Exp. Ther.* 266(3):1626–1635.

Hurt-Camejo, E., Camejo, G., Peilot, H., Oorni, K., and Kovanen, P. 2001. Phospholipase A2 in vascular disease. *Circ. Res.* 89:298–304.

Kang, M. S., Jeong, J. Y., Seo, J. H., Jeon, H. J., Jung, K. M., Chin, M. R., Moon, C. K., Bonventre, J. V., Jung, S. Y., and Kim, D. K. 2006. Methylmercury-induced toxicity is mediated by enhanced intracellular calcium through activation of phosphatidylcholine-specific phospholipase C. *Toxicol. Appl. Pharmacol.* 216(2):206–215.

Kim, D. S., Lee, E. H., Yu, S. D., Cha, J. H,., and Ahn, S. C. 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.

Kuehn, B. 2005. Medical Groups Sue EPA Over Mercury Rule. *JAMA.* 294:415–416.

Lambert, I. H., Pedersen, S. F., and Poulsen, K. A. 2006. Activation of PLA2 isoforms by cell swelling and ischemia/hypoxia. *Acta Physiol.* 187:75–85.

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

Limke, T. L., Bearss, J. J., and Atchison, W. D. 2004. Acute exposure to methylmercury causes Ca2 +dysregulation and neuronal death in rat cerebellar granule cells through an M3 muscarinic receptor-linked pathway. *Toxicol. Sci.* 80:60–68.

Martinez, J., and Moreno, J. 2001. Role of Ca2+-independent phospholipase A_2 on arachidonic acid release induced by reactive oxygen species. *Arch. Biochem. Biophys.* 392(2):257–262.

Marty, M. S., and Atchison, W. D. 1998. Elevations of intracellular Ca2+ as a probable contributor to decreased viability in cerebellar granule cells following acute exposure to methylmercury. *Toxicol. Appl. Pharmacol.* 150(1):98–105.

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

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

Nigam, S., and Schewe, T. 2000. Phospholipase A2s and lipid peroxidation. *Biochim. Biophys. Acta* 1488:167–181.

Parinandi, N. L., Scribner, W. M., Vepa, S., Shi, S., and Natarajan, V. 1999. Phospholipase D activation in endothelial cells is redox sensitive. *Antioxid. Redox. Signal* 1(2):193–210.

Phillis, J. W., and O'Regan, M. H. 2003. The role of phospholipases, cyclooxygenases, and lipoxygenases in cerebral ischemic/traumatic injuries. *Crit. Rev. Neurobiol.* 15:61–90.

Pleva, J. 1994. Dental mercury—a public health hazard. *Rev. Environ. Health* 10:1–27.

Reiss, A. B., and Edelman, S. D. 2006. Recent insights into the role of prostanoids in atherosclerotic vascular disease. *Curr. Vasc. Pharmacol.* 4:395–408.

Riendeau, D., Guay, J., Weech, P. K., Laliberte, F., Yergey, J., Li, C., Desmarais, S., Perrier, H., Liu, S., Nicoll-Griffith, D., and Street, I. P. 1994. Arachidonyl trifluoromethyl ketone, a potent inhibitor of 85-kDa Phospholipase A_2, blocks production of arachidonate and 12-hydroxyeicosatetraenoic acid by calcium ionophore-challenged platelets. *J. Biol. Chem.* 269(22):15619–15624.

Sarafian, T. A. 1993. Methylmercury increases intracellular Ca2+ and inositol phosphate levels in cultured cerebellar granule neurons. *J. Neurochem.* 61(2):648–657.

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

Shanker, G., and Aschner, M. 2001. Identification and characterization of uptake systems for cystine and cysteine ion cultured astrocytes and neurons: evidence for methylmercury-targeted disruption of astrocyte transport. *J. Neurosci. Res.* 66:998–1002.

Shanker, G., Mutkus, L. A., Walker, S. J., and Aschner, M. 2002. Methylmercury enhances arachidonic acid and cytosolic phospholipase A2 expression in primary cultures of neonatal astrocytes. *Mol. Brain Res.* 106:1–11.

Shanker, G., Syversen, T., and Aschner, M. 2003. Astrocyte-mediated methylmercury Neurotoxicity. *Biol. Trace Elem. Res.* 95(1):1–10.

Shanker, G., Syversen, T., Aschner, J. L., and Aschner, M. 2005. Modulatory effect of glutathione status and antioxidants on methylmercury-induced free radical formation in primary cultures of cerebral astrocytes. *Mol. Brain Res.* 137:11–22.

Valko, M., Morris, H., and Cronin, M. T. 2005. Metals, toxicity and oxidative stress. *Curr. Med. Chem.* 12(10):1161–1208.

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

Verity, M. A., Sarafian, T., Pacifici, E. H. K., and Sevanian, A. 1994. Phospholipase A_2 stimulation by methylmercury in neuron culture. *J. Neurochem.* 62:705–714.

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

Xu, J., Yu, S., Sun, A. Y., and Sun, G. Y. 2003. Oxidant-mediated AA release from astrocytes involves $cPLA_2$ and $iPLA_2$. *Free Radic. Biol. Med.* 34(12):1531–1543.

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