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Mercury has been identified as a risk factor for cardiovascular disease among humans. Through diet, mainly fish consumption, humans are exposed to methylmercury, the biomethylated organic form of environmental mercury. As the endothelium is an important player in homeostasis of the cardiovascular system, here, the authors tested their hypothesis that methylmercury activates the lipid signaling enzyme phospholipase A$_2$ (PLA$_2$) in vascular endothelial cells (ECs), causing upstream regulation of cytotoxicity. To test this hypothesis, the authors used bovine pulmonary artery ECs (BPAECs) cultured in monolayers, following labeling of their membrane phospholipids with [^{3}H]arachidonic acid (AA). The cells were exposed to methylmercury chloride (MMC) and then the release of free AA (index of PLA$_2$ activity) and lactate dehydrogenase (LDH; index of cytotoxicity) were determined by liquid scintillation counting and spectrophotometry, respectively. MMC significantly activated PLA$_2$ in a dose-dependent (5 to 15 μM) and time-dependent (0 to 60 min) fashion. Sulfhydryl (thiol-protective) agents, calcium chelators, antioxidants, and PLA$_2$-specific inhibitors attenuated the MMC-induced PLA$_2$ activation, suggesting the role of thiols, reactive oxygen species (ROS), and calcium in the activation of PLA$_2$ in BPAECs. MMC also induced the loss of thiols and increase of lipid peroxidation in BPAECs. MMC induced cytotoxicity in BPAECs as observed by the altered cell morphology and LDH leak, which was significantly attenuated by PLA$_2$ inhibitors. This study established that PLA$_2$ activation through thiols, calcium, and oxidative stress was associated with the cytotoxicity of MMC in BPAECs, drawing attention to the involvement of PLA$_2$ signaling in the methylmercury-induced vascular endothelial dysfunctions.

Keywords Arachidonic Acid, Environmental Cardiovascular Risk, Lipid Signaling, Methylmercury, Phospholipase A$_2$, Vascular Endothelial Cell

For years, mercury (Hg), a heavy metal belonging to the transition element series of the periodic table, has been under intense investigation as a serious environmental pollutant. It is released into the environment from the coal-burning and mining industries, power plants, and other industrial processes (Clarkson, Magos, and Myers 2003; Pleva 1994; Mutter et al. 2004). These anthropogenic activities, along with natural processes such as volcanic action, contribute to the mercury pollution of air, water, and soil (Clarkson, Magos, and Myers 2003; Sarkar et al. 2005; Kuehn 2005). A majority of the mercury to which humans are exposed is methylmercury (MMC), the bioorganic environmental form of mercury (Bellum et al. 2007). Elemental mercury is converted to methylmercury by the microorganisms (bacteria), and methylmercury is bioaccumulated in the aquatic organisms, eventually reaching humans at the top of the food chain (Boening 2000; Dopp et al. 2004).

The severe toxicity of methylmercury and the plethora of adverse biological effects the metal causes have led to its recognition as a ubiquitous environmental toxicant (Maycock and Benford 2007; Monnet-Tschudi et al. 2006). The role of mercury as a possible risk factor in the cardiovascular diseases has been emphasized (Kosta 1991; Virtanen et al. 2007) and elevated body levels of mercury, due to fish consumption by humans, have been attributed as a risk factor in the coronary heart disease (Yoshizawa et al. 2002). An association between the occupational exposure to mercury in mining and refining and risk of cardiovascular diseases has been shown (Boffetta et al. 2001). In humans, exposure to methylmercury due to fish consumption could possibly lead to suppression of the beneficial effects of omega-3 fatty acids on the coronary artery disease (Landmark and Aursnes 2004; Chan and Egeland 2004; Clarkson 2002). Although mercury exposure in humans has been shown to be associated with cardiovascular diseases, detailed studies leading to the understanding of the mechanisms of mercury-induced cardiovascular problems are currently lacking.

The 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. The adverse effects of mercury on the endothelium have been documented. Methylmercury has been shown to cause hypertension in rats (Wakita 1987), and mercury-induced vascular endothelial damage and vasculitis have been documented in humans upon autopsy (Egermayer 2000). Therefore, it is conceivable to hypothesize that methylmercury exerts its toxic effects on the vascular endothelium, which may contribute to the environmental mercury-induced cardiovascular diseases.

In mammalian cells, phospholipases are enzymes that specifically hydrolyze the membrane phospholipids and generate bioactive lipid second messengers, which play an important role in cell signanling (Dennis et al. 1991; Divecha and Irvine 1995). One such enzyme, phospholipase A_2 (PLA_2), is pivotal in regulating signal cascades involving the formation and repair of the phospholipid membrane and formation of inflammatory molecules (Chakraborti 2003). Aside from its job as a housekeeping enzyme (Balsinde, Winstead, and Dennis 2000), PLA_2 catalyzes the hydrolysis of the membrane phospholipid at the *sn*-2 position, generating free unsaturated fatty acid and lysophospholipid (Dennis et al. 1991; Chakraborti 2003). The unsaturated fatty acid released by PLA_2, usually arachidonic acid (AA), is a preferred substrate for the cyclooxygenases (COXs) and lipoxygenases (LOXs), which mediate the formation of bioactive AA metabolites such as the prostaglandins and leukotrienes (Chakraborti 2003). These metabolites of COX and LOX are tightly regulated by PLA_2, and have been identified as crucial players in the inflammatory cascades (Dennis et al. 1991).

PLA_2 has been shown to be activated by several agonists in different systems both in vitro and in vivo (Chakraborti 2003). Roles of PLA_2 and AA metabolites in cardiovascular diseases have been emerging (Lambert, Pedersen, and Poulsen 2006). Unregulated PLA_2 activation mediated by agonists such as environmental toxicants can jeopardize the endothelial function and eventually disrupt the blood vessel homeostasis. As environmental mercury has been implicated in the cardiovascular diseases and earlier, we have shown that mercury (inorganic and organic forms) activates phospholipase D (PLD) and PLA_2 in vascular endothelial cells (ECs) (Hagele et al. 2007; Mazerik et al. 2007). Here, we have hypothesized that methylmercury activates PLA_2 and induces the release of AA, and the activation of PLA_2 contributes to the methylmercury-induced cytotoxicity in the cultured ECs. To test our hypothesis, we investigated whether methylmercury chloride (MMC) could activate PLA_2, induce the release of AA, and regulate the MMC-induced cytotoxicity in our well-established bovine pulmonary artery ECs (BPAECs) in vitro. The current study revealed that MMC induced the release of AA by PLA_2, and PLA_2 played an important role in the regulation of the MMC-induced cytotoxicity 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, minimum essential medium (MEM), and nonessential amino acids were obtained from Gibco Invitrogen (Grand Island, NY). Methylmercury chloride (MMC), *N*-acetyl-l-cysteine (NAC), meso-2,3-dimercaptosuccinic acid (DMSA), ethylene glycol-bis (β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid (EGTA), BAPTA-AM (BAPTA), pyrollidinedithiocarbamate (PDTC), vitamin C, propyl gallate, 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB), and lactate dehydrogenase cytotoxicity assay (LDH) kit were obtained from Sigma Chemical Co. (St. Louis, MO). [^{3}H]arachidonic acid (AA) was obtained from American Radiolabeled Chemicals (St. Louis, MO). Arachidonly trifluoromethyl ketone ($AACOCF_3$) and bromoenol lactone (BEL) were obtained from Cayman Chemical (Ann Arbor, MI). Mn(III)tetrakis(4-benzoic acid)porphyrin chloride (MnTBAP) and quinacrine dihydrochloride were purchased from Calbiochem (San Diego, CA).

Cell Culture

BPAECs were cultured in MEM supplemented with 10% FBS, nonessential amino acids, antibiotics, and growth factor as described previously (Varadharaj et al. 2006; Hagele et al. 2007). Cells in culture were maintained at 37°C in a humidified environment of 5% CO_2–95% air and grown to contact-inhibited monolayers with typical cobblestone morphology. When confluence was reached, cells were trypsinized and subcultured in T 75-cm^2 flasks or 35-mm or 100-mm tissue culture dishes. Confluent cells showed cobblestone morphology under light microscope and stained positive for factor VIII. All experiments were conducted between 5 and 20 passages (75% to 80% confluence).

Assay of Release of Arachidonic Acid and PLA_2 Activation

Release of arachidonic acid (AA) from the cellular membrane phospholipids is widely assayed as an index of PLA_2 activity (Balsinde, Winstead, and Dennis 2000; Mazerik et al. 2007). BPAECs cultured in 35-mm dishes (5×10^5 cells/dish) were labeled with carrier-free [^{3}H]AA (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 MMC at the chosen concentrations (5 to 15 μM) for specified lengths of time (0 to 60 min). When required, cells prelabeled with [^{3}H]AA were pretreated with the selected pharmacological agents/inhibitors for 1 h and then exposed to MMC 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 AA released into the medium, as an index of PLA$_2$ activity, was determined by liquid scintillation counting. The extent of AA released was expressed as DPM of [^{3}H]/dish.

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. (2007). BPAECs were grown to 100% confluence in 100-mm dishes then treated with MEM or MEM containing MMC (1 to 5 μM) for 60 min. When required, cells were pretreated with MEM alone or MEM containing the inhibitor and then treated with MEM alone or MEM containing MMC (10 μM). After incubation, cells were detached by gentle scraping and centrifuged at 5000 $\times$ g for 10 min at 4°C. The cell pellets were then lysed using Triton X-100. Cell lysates were treated with DTNB and the absorbance was determined at 412 nm on a Spectromax plate reader. The levels of total thiols were obtained from a standard curve prepared with GSH and expressed as μg thiols/10^6 cells.

Morphology Assay of Cytotoxicity

Morphological changes in BPAECs grown in 35-mm dishes up to 70% confluence, following their exposure to different concentrations of MMC (0 to 25 μM) in MEM for 1 h at 37°C in a humidified environment of 5% CO$_2$–95% air, were examined as an index of cytotoxicity. Images of cell morphology were digitally captured with the Nikon Eclipse TE2000-S at either 10$\times$ or 100$\times$ magnification.

LDH Assay of Cytotoxicity

BPAECs were grown up to 90% confluence in 35-mm dishes pretreated with MEM alone or MEM containing the chosen PLA$_2$ inhibitor (AACOCF$_3$ 1 μM and BEL 1 and 5 μM) for 1 h, then the cells were exposed to MEM containing MMC (10 μM) for 30 min. At the end of incubation, supernatant was removed, the experiment was terminated with 1 N HCl and LDH release was measured according to the manufacturer's recommendations (Sigma Chemical, St. Louis, MO).

Lipid Peroxidation Determination

Lipid peroxidation in BPAECs, as an index of oxidative stress, following the exposure of cells to different concentrations of MMC, was determined spectrophotometrically as the formation of thiobarbituric acid-reactive substances (TBARS) according to Parinandi, Zwizinski, and Schmid (1991). The extent of MMC-induced formation of TBARS in BPAECs was expressed as absorbance at 532 nm normalized to 1 mg protein of cells at the end of treatment with MEM alone of MEM containing different concentrations of MMC.

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 $p < .05$ indicating significance.

RESULTS

Methylmercury Activates PLA$_2$ in a Dose- and Time-Dependent Fashion

As we have shown earlier that mercury activates PLD in ECs (Hagele et al. 2007) and inorganic mercury activates PLA$_2$ in ECs (Mazerik et al. 2007), here, we investigated whether MMC would induce the activation of PLA$_2$ and release of AA (as an index of PLA$_2$ activation) in BPAECs in a dose-dependent (5 to 15 μM) fashion, following incubation of cells for 30 and 60 min with MMC. MMC significantly caused the activation of PLA$_2$ at 5, 10, and 15 μM concentrations upon treatment of cells for 30 and 60 min respectively, as compared to that in the cells treated with vehicle alone (Figure 1*A*). The significant time-dependant activation of PLA$_2$ in BPAECs upon their treatment with MMC was also clearly evident. At 5 min of treatment, MMC caused a significant activation of PLA$_2$, which further increased at 15, 30, and 60 min of treatment with the compound as compared to the cells treated with the vehicle alone (Figure 1*B*). Overall, these results revealed that organic mercury (methylmercury chloride) was effective in causing a significant dose- and time-dependent activation of PLA$_2$ and AA release in BPAECs.

Thiol-Containing Heavy Metal Chelators Attenuate Methylmercury-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, Morris, and Cronin 2005). Therefore, in order to establish the role of thiols in the MMC-induced PLA$_2$ activation in BPAECs, here, the effects of well-established thiol (sulfhydryl) protective agents (DMSA and PDTC) were investigated. Prior to the exposure of cells to 5 μM MMC, cells were pretreated for 1 h with MEM alone or MEM containing the chosen thiol protective agent(s) (1 mM) and then treated with MMC in the presence of the thiol protective agent(s) for 30 min. DMSA offered effective and significant inhibition of the MMC-induced PLA$_2$ activation and AA release in BPAECs (Figure 2*A*). PDTC also caused effective and significant attenuation of PLA$_2$ activation in ECs induced by methylmercury (Figure 2*B*). Collectively, these results revealed that thiol-protective agents effectively and significantly attenuated the PLA$_2$ activation induced by MMC, suggesting the involvement of cellular thiols.

Antioxidants Attenuate Methylmercury-Induced PLA$_2$ Activation

As we have shown earlier that antioxidants attenuate the methylmercury-induced PLD activation in the vascular ECs (Hagele et al. 2007), here we investigated the effects of the well-established antioxidants on the PLA$_2$ activation in vascular ECs induced by MMC. Prior to exposure of cells to MMC (5 μM),
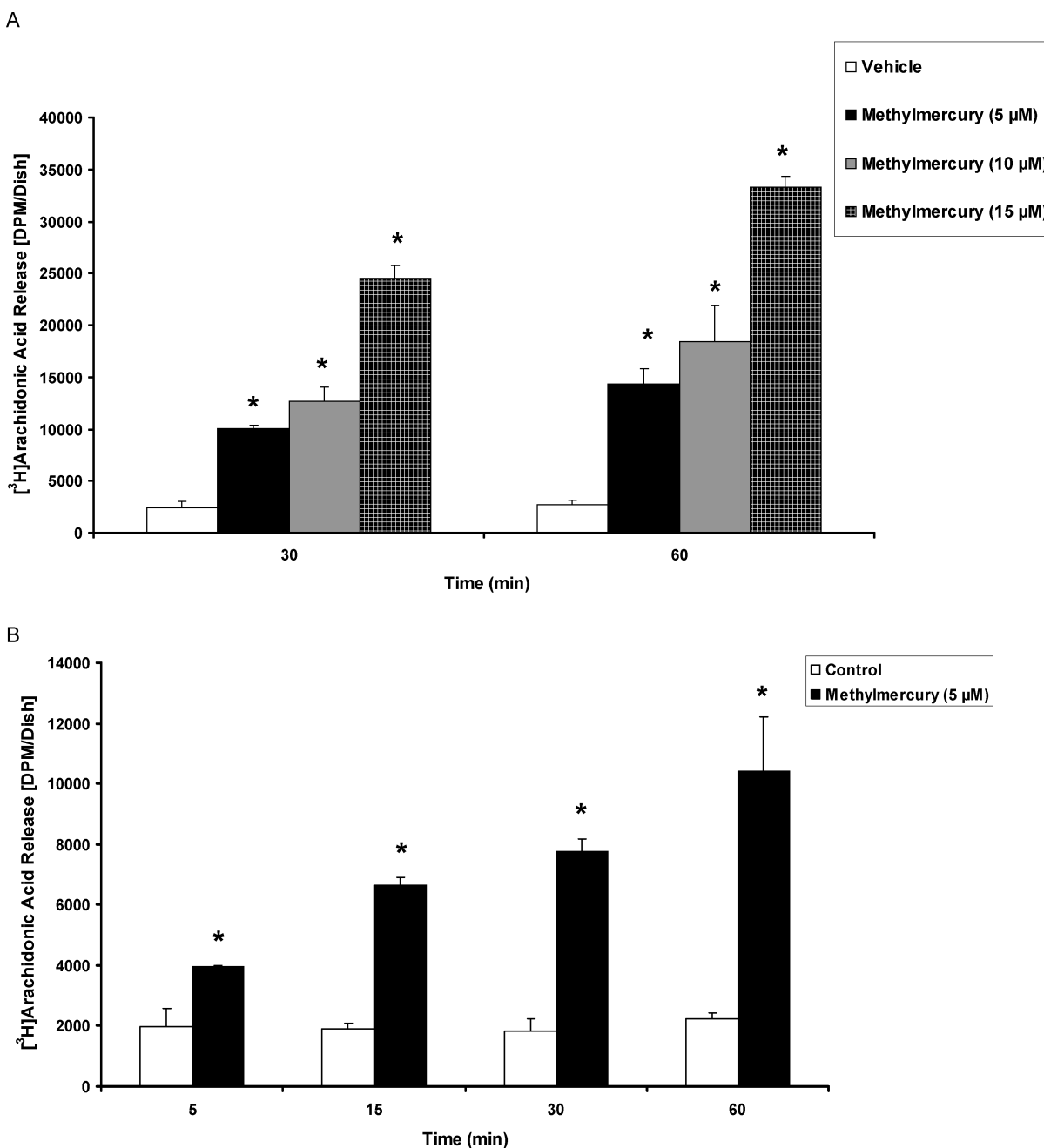
A

B

FIGURE 1

Methylmercury activates PLA_2 in a dose- and time-dependent fashion. BPAECs (5×10^5 cells/35-mm dish) were labeled with [^{3}H]AA (5 μCi) in complete EC medium for 12 to 18 h and then the cells were treated with MEM alone or MEM containing (*A*) MMC (5 to 15 μM) for 30 and 60 min and (*B*) MMC (5 μM) for 5 to 60 min. At the end of incubation, [^{3}H]AA released into the medium was determined as described under Materials and Methods. Data represent mean $\pm$ *SD* of three independent experiments. *Significantly different at $p < .05$ as compared to the vehicle-treated control cells.

cells were pretreated for 1 h with MEM alone or MEM containing the chosen antioxidant (vitamin C 500 μM, NAC 2 mM, propyl gallate 500 μM, and MnTBAP 1 μM) and then treated with MMC in the presence of the antioxidants for 30 min. Vitamin C, a well-established antioxidant, effectively attenuated the MMC-induced PLA_2 activation and release of AA in BPAECs

(Figure 3*A*). NAC, a widely-used thiol antioxidant and protective agent, also caused significant and effective attenuation of the MMC-induced PLA_2 activation in BPAECs (Figure 3*B*). Propyl gallate, another widely used antioxidant, also caused significant and effective attenuation of the MMC-induced PLA_2 activation and release of AA in BPAECs (Figure 3*C*). MnTBAP,
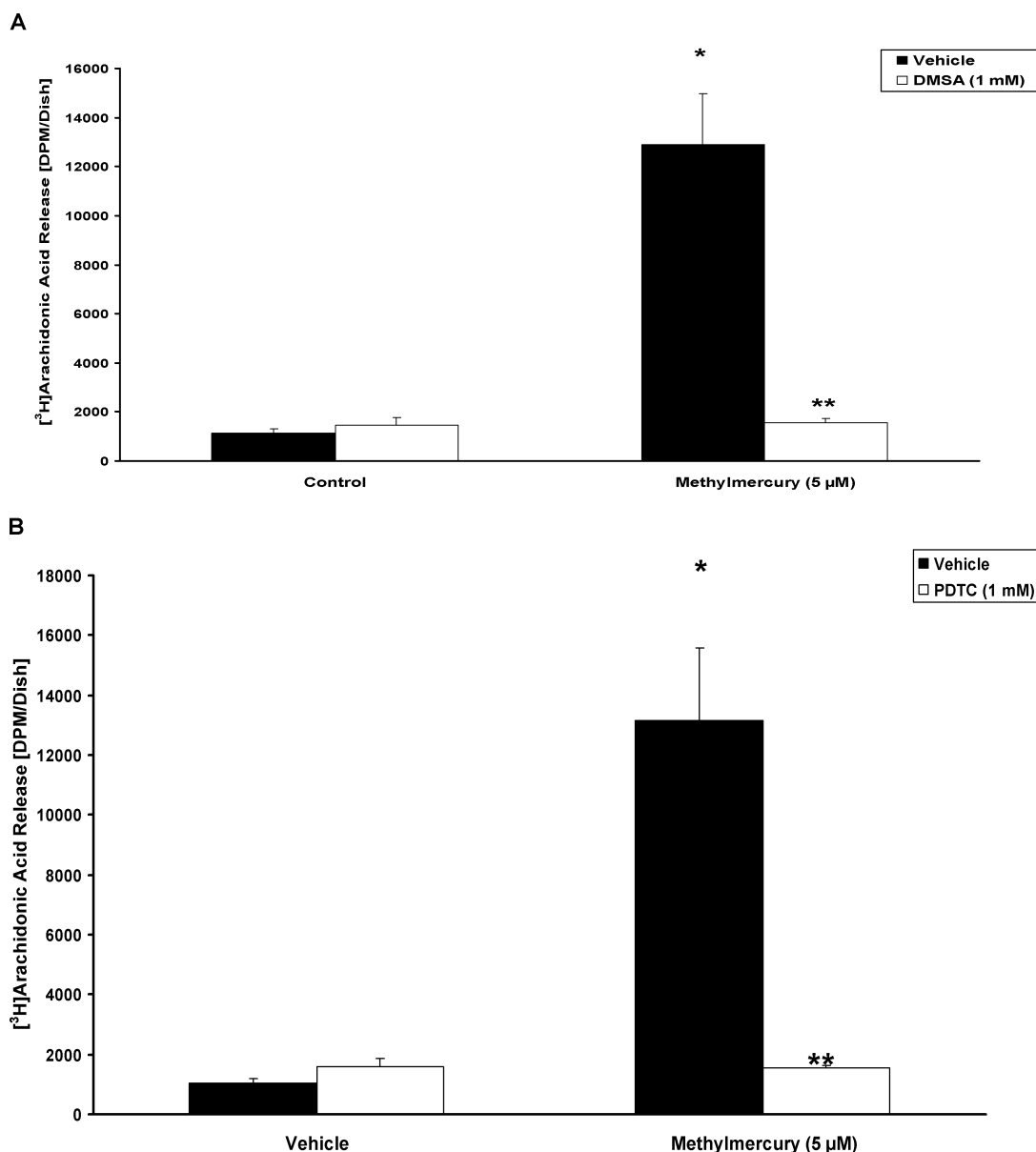
FIGURE 2

Thiol-containing heavy metal chelators attenuate methylmercury-induced PLA$_2$ activation. BPAECs (5×10^5 cells/35-mm dish) were labeled with [^{3}H]AA (5 μCi) in complete EC medium for 12 to 18 h following which the cells were pretreated for 1 h with MEM alone or MEM containing (*A*) DMSA (1 mM) or (*B*) PDTC (1 mM) and then subjected to treatment with MEM alone or MEM containing MMC (5 μM) for 30 min. At the end of incubation, [^{3}H]AA released into the medium was determined as described under Materials and Methods. Data represent mean $\pm$ *SD* of three independent experiments. *Significantly different at $p < .05$ as compared to the cells treated with vehicle alone. **Significantly different at $P < .05$ as compared to the cells treated with MMC alone.

a superoxide dismutase mimetic, effectively attenuated the MMC-induced PLA$_2$ activation in the cells (Figure 3*D*). Overall, the results revealed that the antioxidants effectively attenuated the MMC-induced PLA$_2$ activation in BPAECs, and further suggested the involvement of reactive oxygen species (ROS) in the activation of the enzyme by methylmercury in ECs.

Calcium Chelators Attenuate Methylmercury-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 MMC-induced activation of PLA$_2$ by using the widely known calcium quenchers EGTA (extracellular) and BAPTA (intracellular). Prior to exposure to

MMC (5 μM), cells were pretreated for 1 h with MEM or MEM containing the chosen calcium chelating agent (EGTA 5 mM and BAPTA 500 nM) 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 the MMC-induced PLA$_2$ activation (Figure 4A). The well-known intracellular calcium chelator, BAPTA, also attenuated the MMC-induced PLA$_2$ activation in BPAECs (Figure 4B).
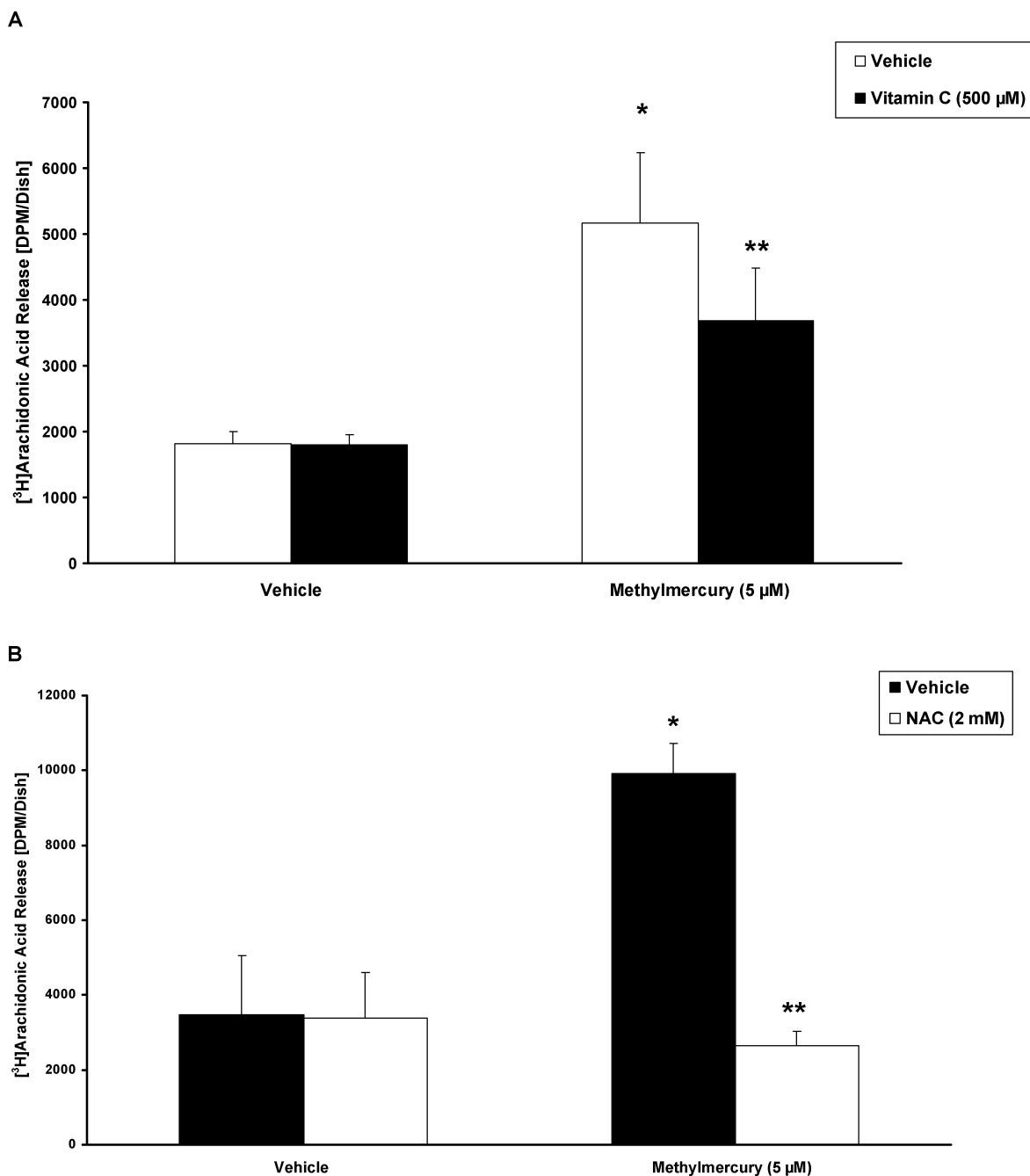
FIGURE 3

Antioxidants attenuate methylmercury-induced PLA$_2$ activation. BPAECs (5×10^5 cells/35-mm dish) were labeled with [^{3}H]AA (5 μCi) in complete EC medium for 12 to 18 h, following which the cells were pretreated for 1 h with MEM alone or MEM containing (*A*) vitamin C (500 μM), (*B*) NAC (2 mM), (*C*) propyl gallate (500 μM), or (*D*) MnTBAP (1 μM), and then subjected to treatment with MEM alone or MEM containing MMC (5 μM) for 30 min. At the end of incubation, [^{3}H]AA released into the medium was determined as described under Materials and Methods. Data represent mean $\pm$ *SD* of three independent experiments. *Significantly different at $p < .05$ as compared the to cells treated with vehicle alone. **Significantly different at $p < .05$ as compared to the cells treated with MMC alone. *(Continued)*
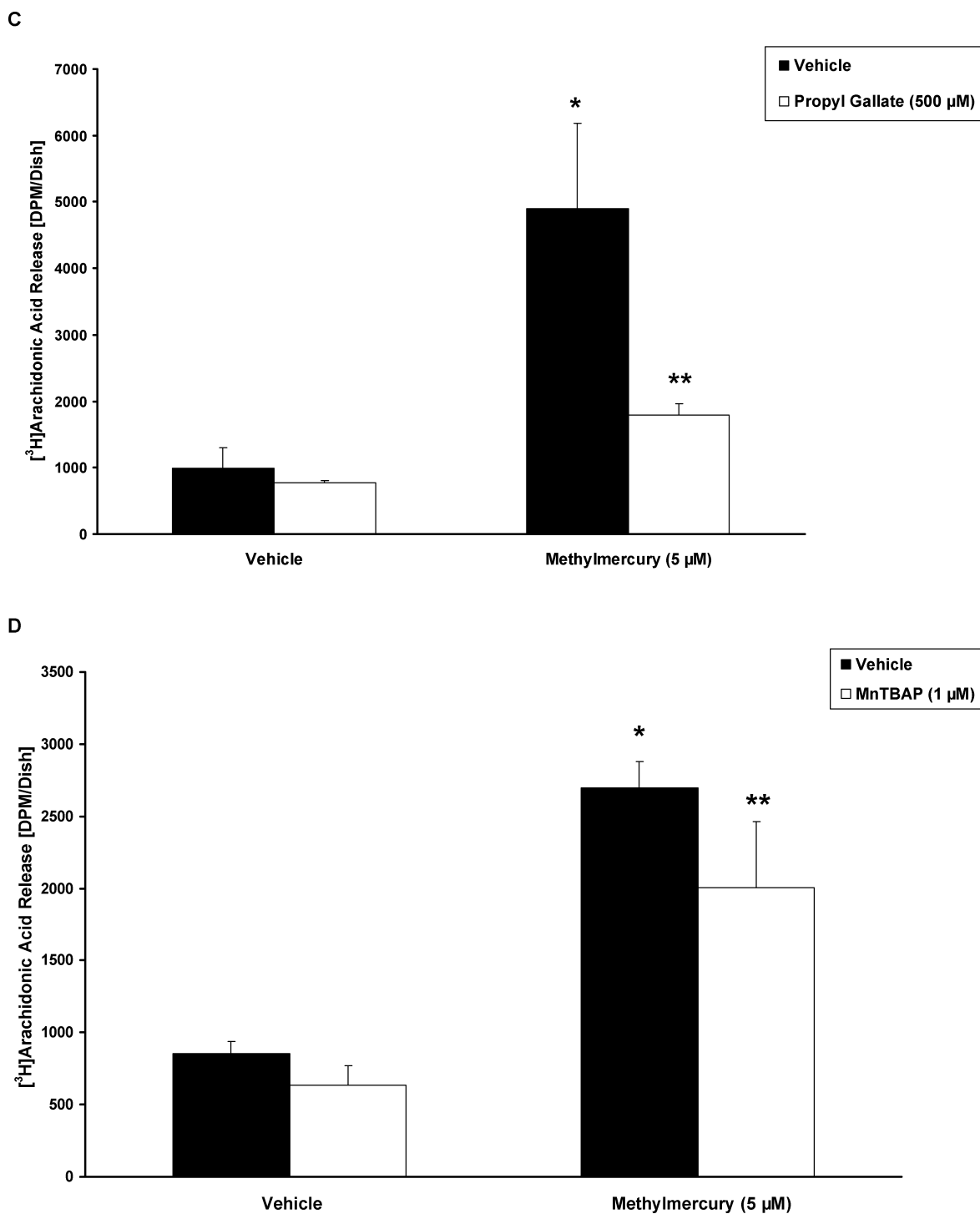
FIGURE 3
(Continued)

These results revealed that calcium chelators effectively and significantly attenuated the MMC-induced PLA$_2$ activation in BPAECs, further suggesting the role of both intracellular and extracellular calcium in the enzyme activation in ECs and also the involvement of cPLA$_2$.

PLA$_2$-Specific Inhibitors Attenuate Methylmercury-Induced PLA$_2$ Activation

As the calcium chelators caused a significant attenuation of the MMC-induced PLA$_2$ activation in the BPAECs, here, we investigated the contribution of cPLA$_2$ in the MMC-induced

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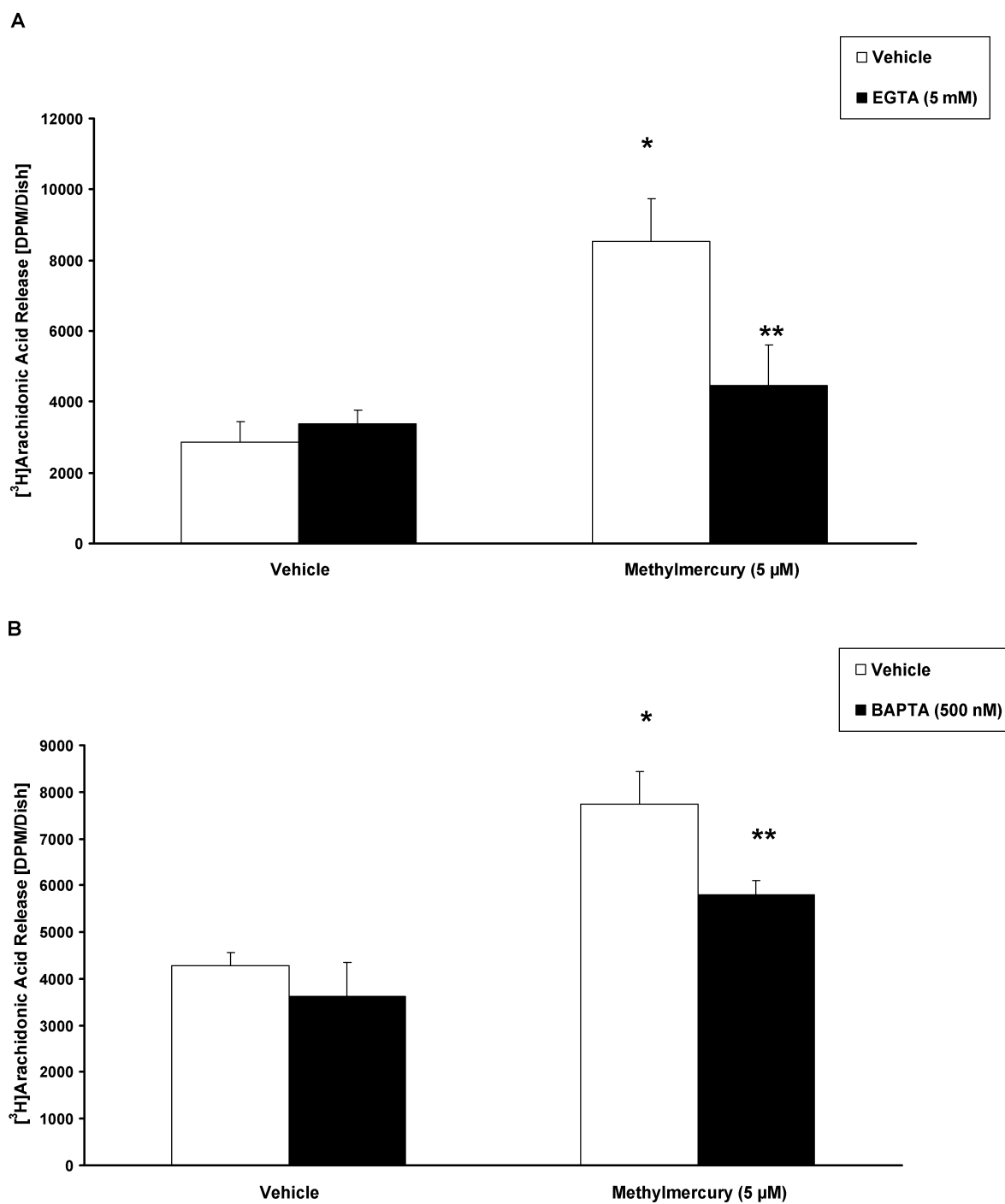
FIGURE 4

Calcium chelators attenuate methylmercury-induced PLA$_2$ activation. BPAECs (5×10^5 cells/35-mm dish) were labeled with [^{3}H]AA (5 μCi) in complete EC medium for 12 to 18 h, following which the cells were (*A*) treated with MEM alone or MEM containing EGTA (5 mM), or MEM containing EGTA (5 mM) and MMC (5 μM) for 30 min. (*B*) BPAECs were pretreated for 1 h with MEM alone or MEM containing BAPTA (500 nM) and then exposed to MEM alone or MEM containing MMC (5 μM) for 30 min. At the end of incubation, [^{3}H]AA released into the medium was determined as described under Materials and Methods. Data represent mean $\pm$ *SD* of three independent experiments. *Significantly different at $p < .05$ as compared to the cells treated with vehicle alone. **Significantly different at $p < .05$ as compared to the cells treated with MMC alone.

release of AA by cells. 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 MMC-induced release of AA by the ECs. Cells were pretreated with MEM alone or MEM containing AACOCF$_3$(5 μM) for 1 h and then treated with MMC (5 μM) in the presence of the inhibitor for 30 min. AACOCF$_3$ slightly but significantly attenuated the MMC-induced AA release by BPAECs (Figure 5A). As the cPLA$_2$-specific inhibitor only slightly inhibited the AA release, the general PLA$_2$ inhibitor, quinacrine, was also tested. Quinacrine also attenuated the MMC-induced PLA$_2$ activation and release of AA in BPAECs (Figure 5B), thus revealing the activation of other isoforms of PLA$_2$ by MMC in ECs. These results confirmed that the MMC activated cPLA$_2$ and thus also contributed to the release of AA by the ECs, but the cPLA$_2$ isoform was not the only form of the enzyme contributing to the AA release in the cells under methylmercury exposure.

N-Acetylcysteine Attenuates Methylmercury-Induced Loss of Cellular Thiols

As it has been established that the cellular thiols are targets for mercury and the current study revealed that thiol protectants attenuated the MMC-induced PLA$_2$ activation in BPAECs, here, we examined the effect of MMC (5 μM) on the levels of total cellular thiols in BPAECs. Cells were treated with MEM alone or MEM containing MMC (1, 3, and 5 μM) for 1 h following which the levels of total cellular thiols were determined. As shown in Figure 6A, MMC caused a significant decrease in the total cellular thiol content as compared to the same in the vehicle-treated control cells. As thiol groups are the targets for attack by mercury and ROS (Hagele et al. 2007; Parinandi et al. 1999), the well-known thiol protectant and antioxidant, NAC (10 mM), was used to investigate the protection against the thiol loss. Cells were pretreated for 1 h with MEM alone or MEM containing NAC, and then exposed to MMC (10 μM) for 1 h. NAC effectively and significantly caused the protection against the MMC-induced loss of the thiol groups in BPAECs. These results further suggested the role of cellular thiols in the MMC-induced PLA$_2$ activation in ECs, and indicated further the involvement of ROS and oxidative stress.

Methylmercury Causes Alterations in Cell Morphology

As it is known that PLA$_2$ is a bioactive lipid signaling enzyme that plays an important role in the EC function (Mazerik et al. 2007), we investigated the effects of MMC on the cell morphology as an index of disruption of cellular structure and cytotoxicity. Cells were treated with MEM alone or MEM containing MMC (2 to 25 μM) and incubated for 30 min. At 30 min, phase-contrast light microscopy examination revealed that MMC caused a marked loss of cell morphology in a dose-dependent manner in BPAECs (Figure 7). These results also confirmed that MMC induced cytotoxicity in BPAECs.

Methylmercury Induces Lipid Peroxidation

Methylmercury has been shown to cause ROS generation and lipid peroxidation in cultured cells and in liver and kidney in vitro (Hagele et al. 2007; Garg and Chang 2006; Shanker and Aschner 2003; Fujimoto et al. 1988; Lin, Huang, and Huang 1996). Also, the results of earlier experiments of the current study revealed that antioxidants significantly attenuated the MMC-induced activation of PLA$_2$ and release of AA in BPAECs, suggesting that MMC induced the formation of ROS in ECs. Therefore, here we investigated the induction of lipid peroxidation in BPAECs upon exposure to MMC. As shown in Figure 8, MMC significantly induced lipid peroxidation in cells even at a concentration of 5 μM (67% increase) at 1 h of treatment as compared to the same in the control untreated cells, which did not appear to change upon increasing the concentration of MMC to 10 μM. Although MMC at a concentration of 25 μM caused a significant increase in lipid peroxidation (40% increase) under identical conditions as compared to the same in the control untreated cells, the extent of lipid peroxidation was lower as observed in the cells exposed to MMC at 5 and 10 μM under identical conditions for 1 h. These results revealed that MMC induced lipid peroxidation in BPAECs, further suggesting the induction of oxidative stress by methylmercury in ECs.

## PLA$_2$ Inhibitors Protect against Methylmercury-Induced Cytotoxicity

As observed in the current study that the cPLA$_2$ inhibitor (AACOCF$_3$) and general PLA$_2$ inhibitor quinacrine significantly inhibited the MMC-induced activation of PLA$_2$ and release of AA in BPAECs, here, we investigated whether PLA$_2$ activation had a role in the MMC-induced cytotoxicity. Because we observed that MMC induced cytotoxicity in BPAECs in a dose-dependent manner as observed from the alterations in cell morphology, here, we used the LDH release assay of cytotoxicity to examine if the inhibitors of PLA$_2$ offered protection against MMC-induced cytotoxicity in BPAECs. Cells were pretreated for 1 h with MEM alone or MEM containing the chosen PLA$_2$ inhibitor (AACOCF$_3$ 1 μM and BEL 1 and 5 μM), then exposed to MMC (10 μM) for 30 min. MMC caused a significant increase in the release of LDH by cells as compared to the cells treated with the vehicle alone. The cPLA$_2$-specific inhibitor AACOCF3 significantly and effectively attenuated the cytotoxicity of MMC (Figure 9A). BEL, an inhibitor selective to calcium-independent PLA$_2$ (iPLA$_2$) also significantly and effectively attenuated the MMC-induced cytotoxicity (Figure 9B). Collectively, these results revealed that MMC induced cytotoxicity in BPAECs, which was attenuated by the PLA$_2$ inhibitors, further suggesting the role of PLA$_2$ in the MMC-induced cytotoxicity in ECs.

DISCUSSION

The results of the present study demonstrated that methylmercury, the environmental organic form of mercury, activated PLA$_2$ and induced the release of AA from the membrane phospholipids of BPAECs in a dose- and time-dependent fashion.

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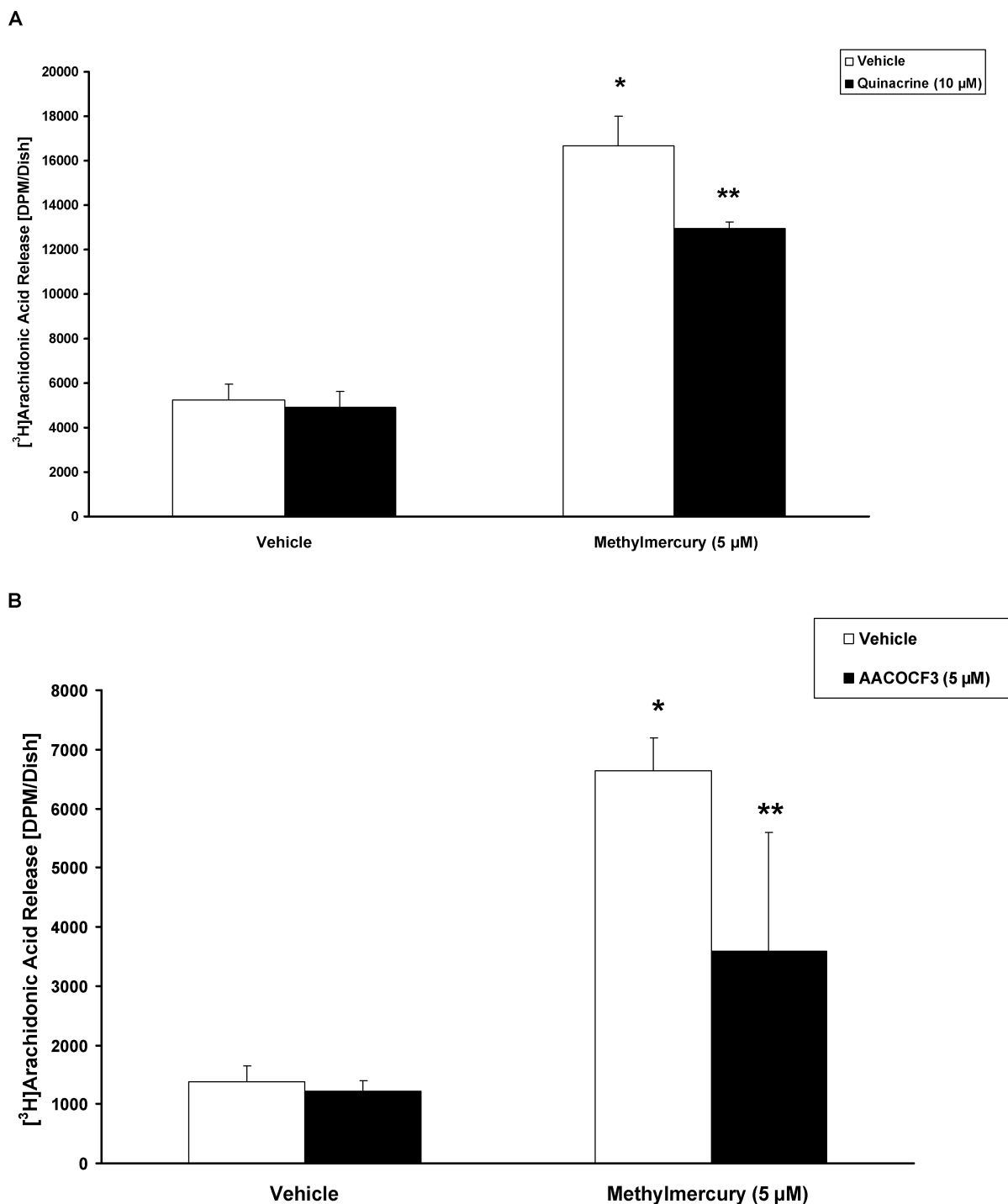
FIGURE 5

PLA_2-specific inhibitors attenuate methylmercury-induced PLA_2 activation. BPAECs (5×10^5 cells/35-mm dish) were labeled with [^{3}H]AA (5 μCi) in complete EC medium for 12 to 18 h, following which the cells were pretreated for 1 h with MEM alone or MEM containing (*A*) the PLA_2-specific inhibitor quinacrine (10 μM) or (*B*) the $cPLA_2$-specific inhibitor, AACOCF$_3$ (5 μM) and then subjected to treatment with MEM alone or MEM containing MMC (5 μM) for 30 min. At the end of incubation, [^{3}H]AA released into the medium was determined as described under Materials and Methods. Data represent mean $\pm$ *SD* of three independent experiments. *Significantly different at $p < .05$ as compared to the cells treated with vehicle alone. **Significantly different at $p < .05$ as compared to the cells treated with MMC alone.
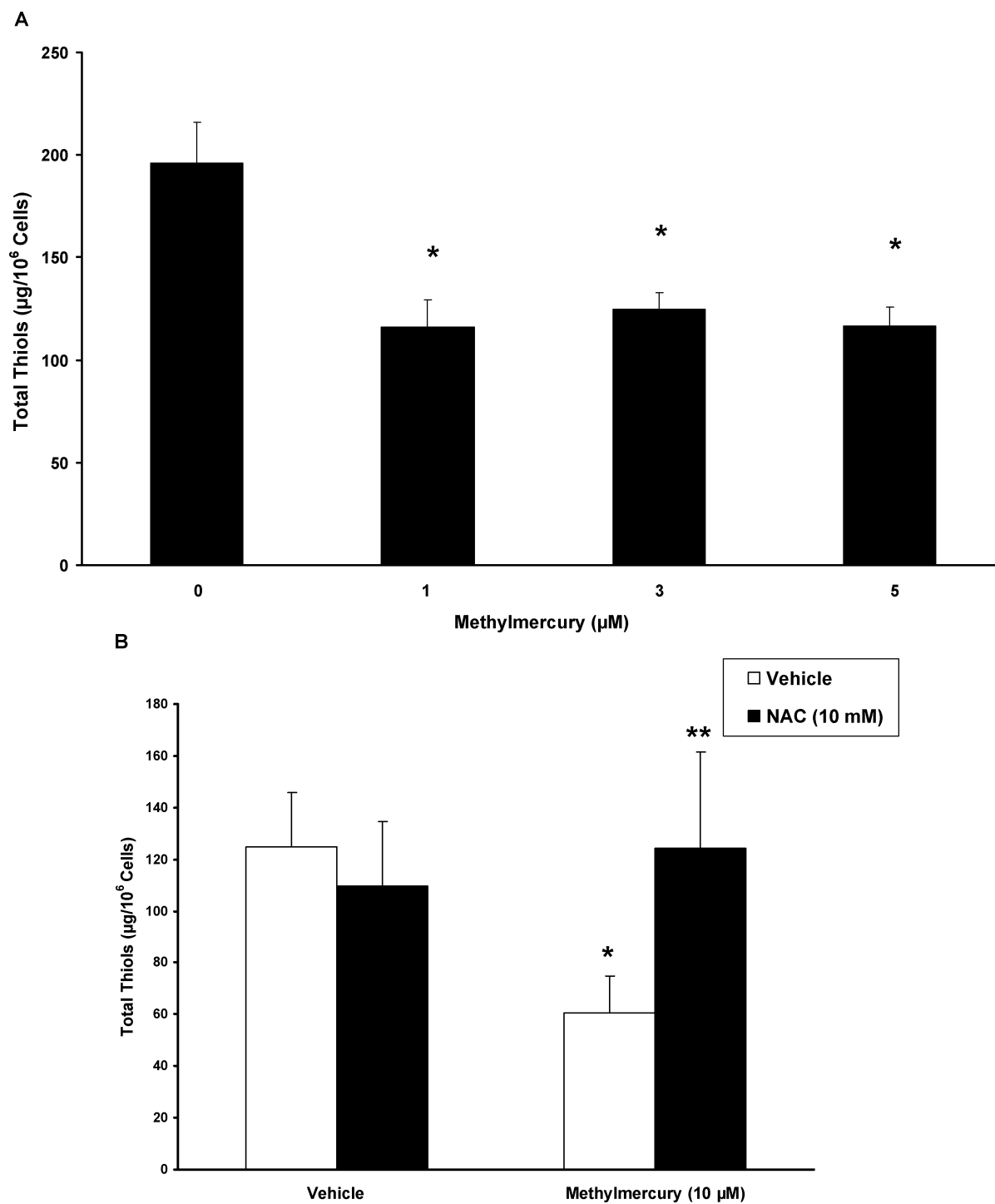
FIGURE 6

N-Acetylcysteine attenuates methylmercury-induced loss of cellular thiols. BPAECs (2×10^6 cells/100-mm dish) were treated with MEM alone or MEM containing MMC (0, 1, 3, and 5 μM) (*A*) for 1 h. BPAECs (2×10^6 cells/100-mm dish) were pretreated with NAC (10 mM) (*B*) for 1 h and then treated with MEM alone or MEM containing MMC (10 μM) for 1 h. After incubation, total cellular thiols were determined spectrophotometrically as described in Materials and Methods. Data represent mean $\pm$ *SD* of three independent experiments. *Significantly different at $p < .05$ as compared to the cells treated with vehicle alone. **Significantly different at $p < .05$ as compared to the cells treated with MMC alone.
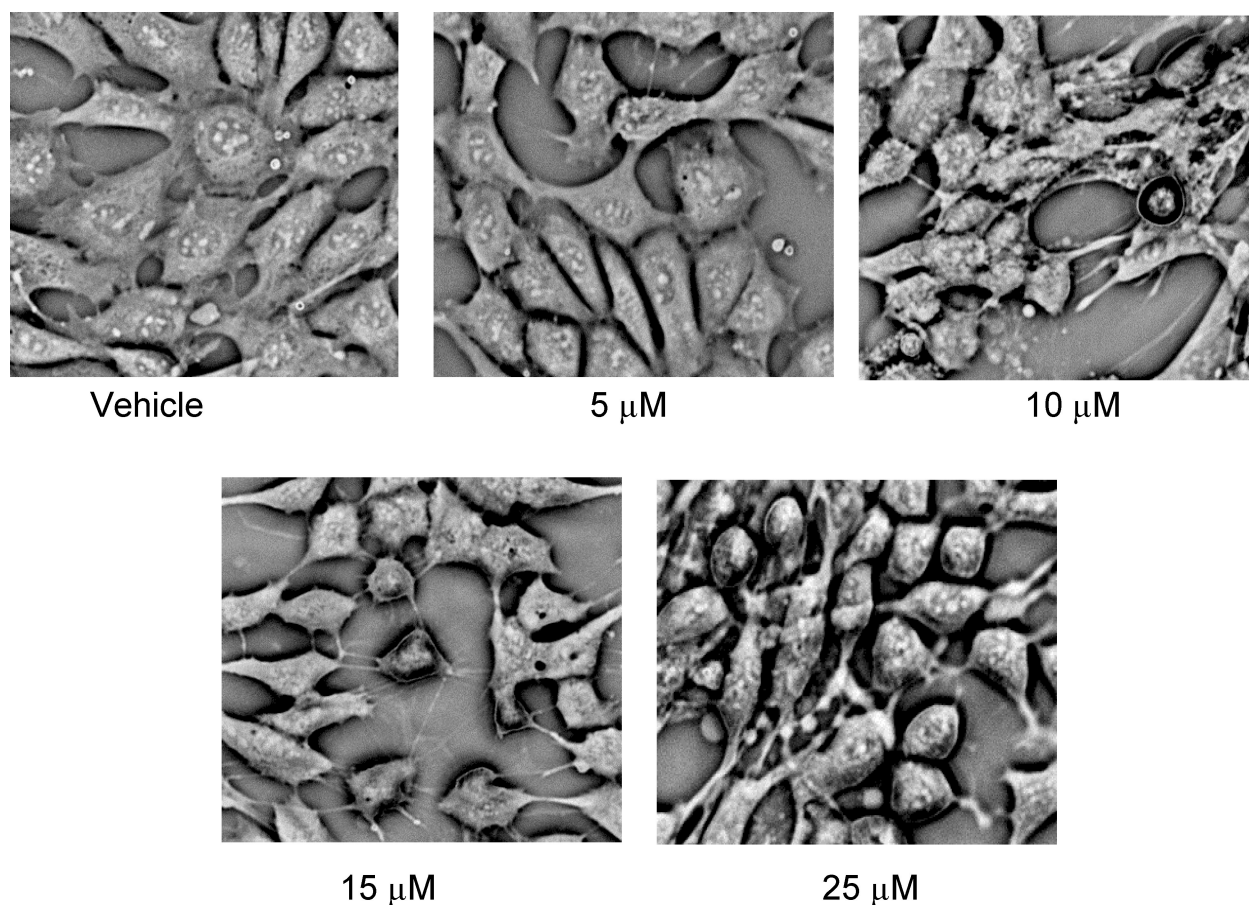
FIGURE 7

Methylmercury causes alterations in cell morphology. BPAECs (5×10^5 cells/35-mm dish) were treated with MEM alone or MEM containing MMC (5 to 25 μM) for 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 $10\times$ as described under Materials and Methods. Each micrograph is a representative picture obtained from three independent experiments conducted under identical conditions.

Furthermore, the study revealed that the methylmercury-induced PLA$_2$ activation in BPAECs was attenuated by the sulfhydryl (thiol) protectants, calcium chelators, and antioxidants indicating the involvement of cellular thiols, calcium, and ROS in the methylmercury-induced activation of PLA$_2$ in ECs. Induction of oxidative stress (lipid peroxidation and loss of cellular thiols) by methylmercury in BPAECs was also evident in the current study. The findings of this study also demonstrated that the cPLA$_2$-specific inhibitor (AACOCF$_3$) (Riendeau et al. 1994) and the iPLA$_2$-specific inhibitor (BEL) not only attenuated the methylmercury-induced activation of PLA$_2$ in BPAECs but also offered protection against the methylmercury-induced cytotoxicity, further establishing the upstream activation of PLA$_2$ and release of AA from the membrane phospholipids, as a causative mechanism of the methylmercury-induced endothelial cytotoxicity.

In the mammalian systems, PLA$_2$s 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) secratory calcium dependent PLA$_2$ (sPLA$_2$) (Lambert, Pedersen, and Poulsen 2006). PLA$_2$ specifically hydrolyzes the *sn*-2 fatty acid ester from the membrane phospholipids, releases the unsaturated fatty acid (typically AA), and forms the lysophospholipid (Balsinde, Winstead, and Dennis 2000). The lipid oxygenases including COXs and LOXs utilize the PLA$_2$-released AA as a preferred substrate and convert it into physiologically active AA metabolites (eicosanoids) such as the prostaglandins and leukotrienes (Balsinde, Winstead, and Dennis 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). The eicosanoids (COX- and LOX-mediated AA metabolites) are potent bioactive lipids and act as key players in inflammation, rendering PLA$_2$ as a crucial lipid signaling enzyme (Dennis et al. 1991; Balsinde, Winstead, and Dennis 2000).

Although the cellular regulation of PLA$_2$ is complex, the regulation of cPLA$_2$ activity has been thoroughly investigated,
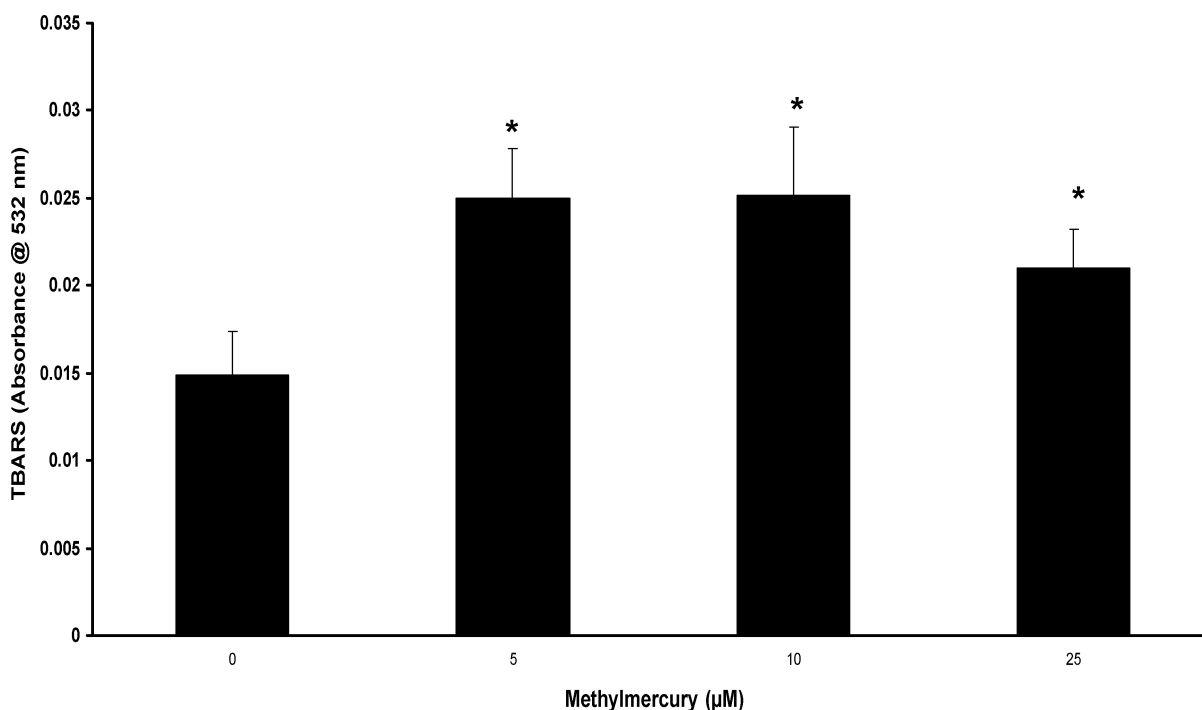
FIGURE 8

Methylmercury induces lipid peroxidation. BPAECs (2×10^6 cells/100-mm dish) were treated with MEM alone or MEM containing MMC (5 to 25 μM) for 1 h and following treatment the extent of lipid peroxidation (formation of TBARS) was determined spectrophotometrically as described in Materials and Methods. The extent of lipid peroxidation was expressed as the absorbance at 532 nm/2×10^6 cells. Data represent mean $\pm$ *SD* of three independent experiments. *Significantly different at $p < .05$ as compared to the cells treated with vehicle alone.

which involves the modulation through the phosphorylation of serine residue of the enzyme catalyzed by different cell signaling kinases, including the mitogen-activated protein kinases, protein kinase A and protein kinase C (Chakraborti 2003). Nonetheless, the regulation of activities of iPLA$_2$ and sPLA$_2$ is yet to be understood in detail. ROS and lipid peroxidation have been identified to activate sPLA$_2$ and iPLA$_2$, leading to the release of AA in the macrophages (Nigam and Schewe 2000; Martinez and Moreno 2001). Oxidants such as hydrogen peroxide have been shown to induce the release of AA from membrane phospholipids of astrocytes through the activation of cPLA$_2$ and iPLA$_2$ (Xu et al. 2003). Overall, these studies have documented that PLA$_2$ activity is regulated by the cellular signaling cascades, ROS, and oxidative stress.

Methylmercury has been extensively studied with respect to its potency to activate PLA$_2$ in neurons and astrocytes (Verity et al. 1994; Aschner 2000; Shanker, Syversen, and Aschner 2003; Shanker et al. 2002). Alterations in calcium regulation and elevation of intracellular calcium levels in cerebellar neurons and NG108-15 cells upon methylmercury treatment have been observed (Limke, Bearss, and Atchison 2004; Sarafian 1993; Marty and Atchison 1998; Hare, McGinnis, and Atchison 1993). The increase in the levels of intracellular calcium and activation of phosphatidylcholine-specific phospholipase C and cPLA$_2$ in MDCK cells, following their exposure to methylmercury, have been documented (Kang et al. 2006). Recently, we have shown that inorganic mercury as mercury chloride induces the release of AA, which is attenuated by calcium chelators in ECs and cPLA$_2$-specific inhibitor, suggesting the inorganic mercury-induced activation of cPLA$_2$ in the ECs (Mazerik et al. 2007). So far, there are no reports made on the methylmercury-induced activation of PLA$_2$ in the vascular ECs. For the first time, the results of the present study established that methylmercury, the predominant toxic form of environmental organic mercury, induced activation of PLA$_2$ and release of AA in vascular ECs, a process that was calcium dependent and ROS and oxidative stress-regulated. Furthermore, the present study also demonstrated that methylmercury induced activation of both the calcium-dependent species of PLA$_2$ (cPLA$_2$) and calcium-independent species of PLA$_2$ (iPLA$_2$) in ECs because the release of AA from the cells was attenuated by the cPLA$_2$-specific inhibitor (AACOCF$_3$) and iPLA$_2$-specific inhibitor (BEL). The mechanism of activation of both cPLA$_2$ and iPLA$_2$ by methylmercury in the vascular ECs, either in an independent manner or in a concerted fashion, needs further investigation.

Methylmercury-induced neurotoxicity in astrocytes and neurons has been shown to operate through the generation of ROS, induction of oxidative stress, and loss of cellular thiols including glutathione (GSH) (Shanker and Aschner 2001; Shanker
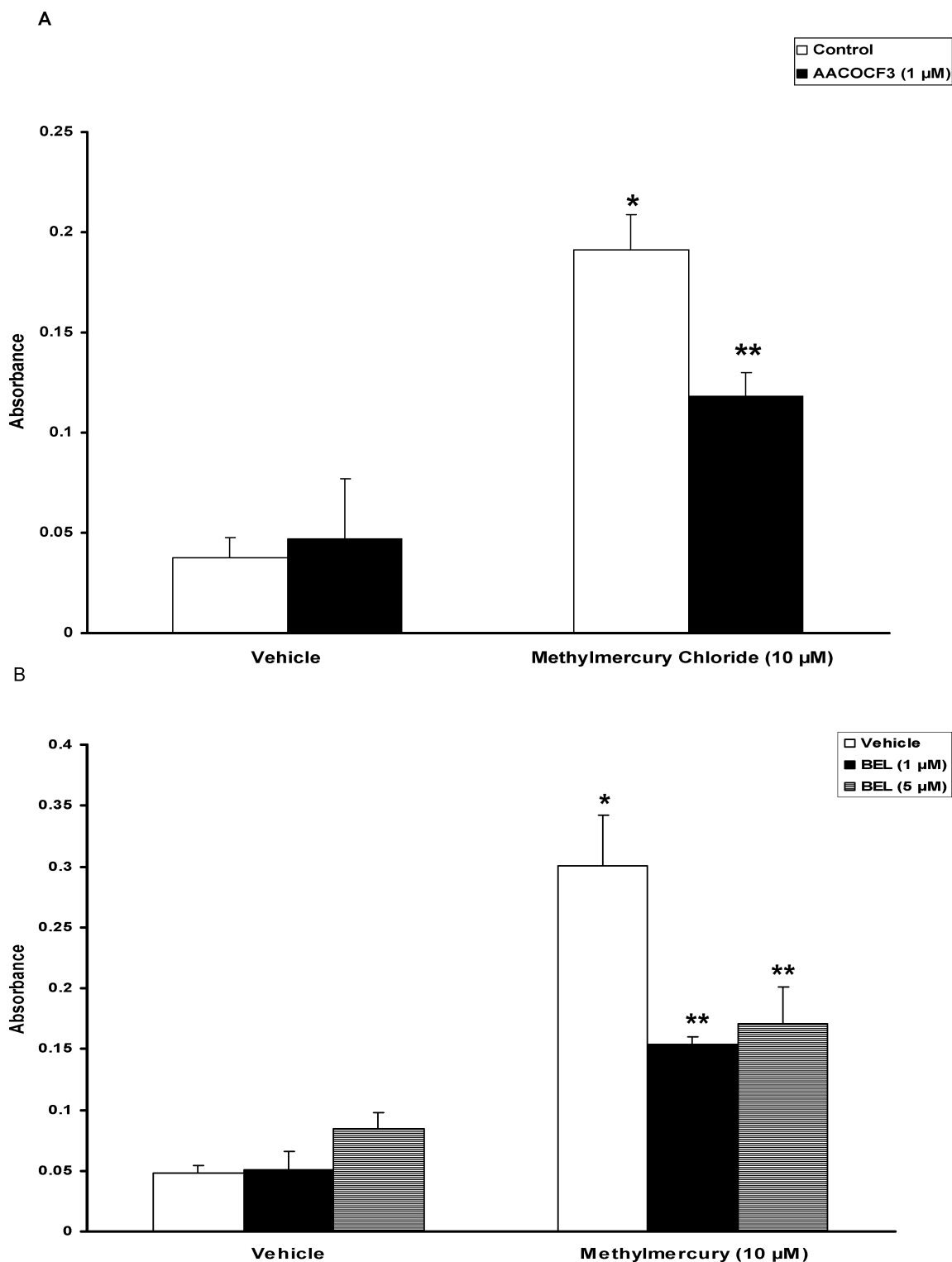
A

B

FIGURE 9

PLA$_2$-specific inhibitors protect against methylmercury-induced cytotoxicity. BPAECs (5×10^5 cells/35-mm dish) were pretreated with MEM alone or MEM containing (*A*) AACOCF$_3$ (1 μM) or (*B*) BEL (1 and 5 μM) for 60 min, following which the cells were treated with MEM alone or MEM containing MMC (10 μM) for 60 min. At the end of incubation, release of LDH into the medium (as an index of cytotoxicity) was determined spectrophotometrically as described under Materials and Methods. The extent of cytotoxicity was expressed as absorbance at 562 nm/35-mm dish. Data represent mean $\pm$ *SD* of three independent experiments. *Significantly different at P < .05 as compared to the cells treated with vehicle alone. **Significantly different at P < .05 as compared to the cells treated with MMC alone.

et al. 2005). Earlier, we have demonstrated that both inorganic (mercury chloride) and organic (methylmercury and thimerosal) forms of mercury cause the activation of another important lipid signaling enzyme, PLD, through the loss of cellular thiols in BPAECs (Hagele et al. 2007). Our recent study has also demonstrated the complete attenuation of the inorganic mercury-induced PLA_2 activation and AA release from BPAECs by the thiol protectants (dithiothreitol [DTT] and NAC) as well as the restoration of cellular thiols (Mazerik et al. 2007), thus suggesting the role of cellular thiols in the inorganic mercury-induced release of AA and activation of PLA_2 in ECs. Along these lines, the results of the present study also revealed that methylmercury, similar to the action of inorganic mercury (mercury chloride) as reported earlier by us, induced the loss of cellular thiols and associated activation of PLA_2 in BPAECs, which were attenuated by the thiol protectant (NAC). Trace heavy metals including mercury have been shown to react with the cellular thiols (Hagele et al. 2007). As observed in the present study, the interaction of methylmercury with cellular thiols and induction of oxidative stress (lipid peroxidation), leading to the activation of PLA_2 and

release of AA, further revealed a possible involvement of the thiol redox-dependant signaling cascade(s) or the direct interaction of organic mercury with the enzyme or both mechanisms.

The role of AA metabolites including the COX-generated prostanoids (prostaglandins, thromboxane, and prostacyclin) has been highlighted in vascular endothelial dysfunction and atherosclerosis (Reiss and Edelman 2006; Bogatcheva et al. 2005). The elevation of intracellular calcium, activation of $cPLA_2$, and release of AA are critical players in the COX-catalyzed formation of AA metabolites in the vascular ECs (Antoniotti et al. 2003). Earlier, we have shown that inorganic mercury (mercury chloride) induces the formation of COX-generated AA metabolites in BPAECs, suggesting the activation of COXs and the formation of the AA-derived inflammatory mediators in ECs upon treatment with inorganic mercury. Moreover, the present study also demonstrated that methylmercury caused lipid peroxidation in BPAECs, which could also lead to the formation of eicosanoid-like inflammatory lipid peroxidation products (TBARS) with potent cell signaling actions in ECs, leading towards activation of PLA_2.

SCHEMA
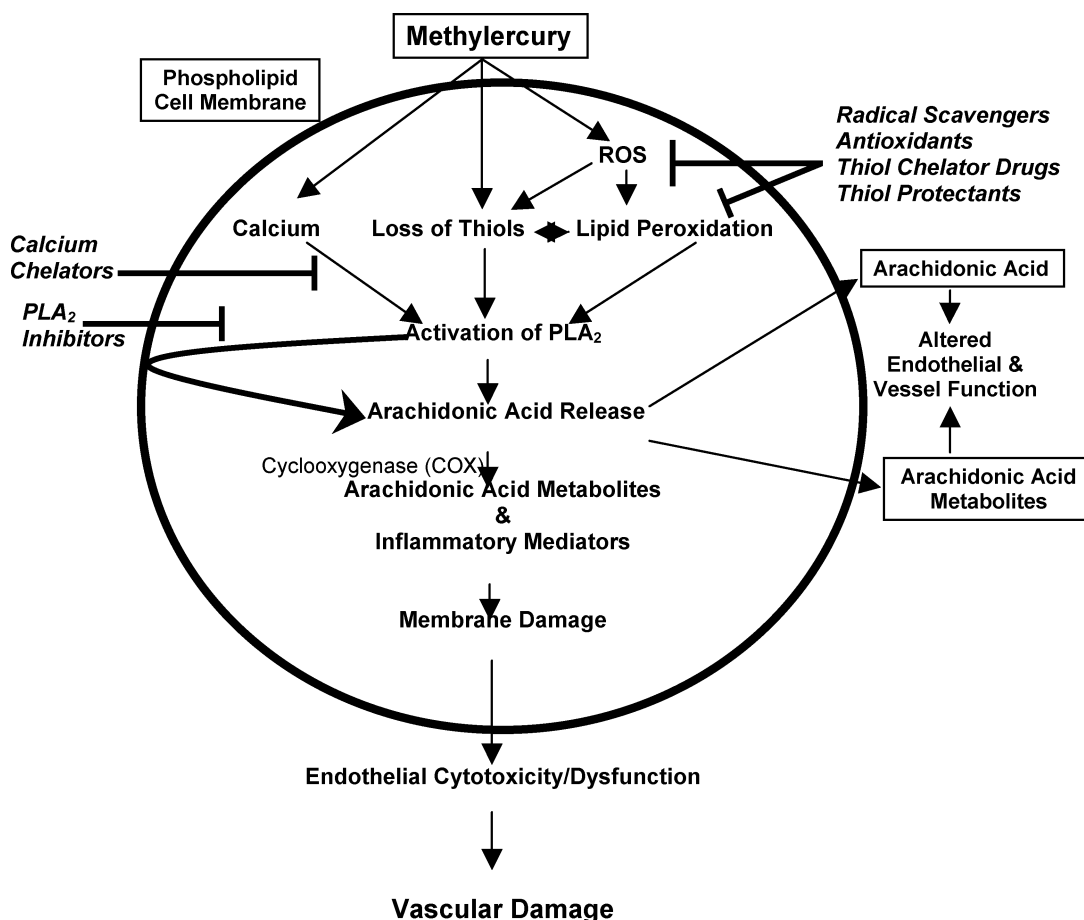
Mechanism of methylmercury-induced activation of PLA_2, release of AA from membrane phospholipids, formation of arachidonic acid metabolites through ROS generation, loss of thiols, and induction of oxidative stress (lipid peroxidation) and their probable roles in endothelial cytotoxicity.

Our earlier study has demonstrated that the inorganic mercury-induced cytotoxicity in BPAECs was protected by the cPLA$_2$-specific inhibitor (AACOCF$_3$), thus identifying a role for cPLA$_2$ in the inorganic mercury-induced EC toxicity (Mazerik et al. 2007). In the present study, we also showed that both the cPLA$_2$-specific inhibitor (AACOCF$_3$) and iPLA$_2$-specific inhibitor (BEL), offered protection against the methylmercury-induced cytotoxicity in BPAECs, indicating the upstream involvement of the activation of PLA$_2$ and release of AA in the methylmercury-induced EC toxicity. Reports have been made on the cytotoxicity of methylmercury in neurons and astrocytes (Verity et al. 1994; Aschner 2000; Shanker et al. 2002, 2003). The widely used PLA$_2$ inhibitor, mepacrine, has been observed to protect against the methylmercury-induced cytotoxicity in the cerebellar granule cells, associating the methylmercury-induced PLA$_2$ activation and cytotoxicity in those neurons (Verity et al. 1994). Furthermore, these documented findings support the results of our current study that the activation of PLA$_2$ (both cPLA$_2$ and iPLA$_2$) played a crucial role in the methylmercury-induced cytotoxicity in BPAECs.

PLA$_2$ and COXs are gaining prominence in vascular diseases and tissue ischemic damage (Hurt-Camejo et al. 2001; Phillis and O'Regan 2003). Moreover, mercury has been implicated as a risk factor in the cardiovascular diseases including myocardial infarction and coronary disease in humans (Yoshizawa et al. 2002; Kim et al. 2005; Nash 2005). Hence, the results of the current study showing that the organic form of environmental mercury (methylmercury) induced the activation of PLA$_2$ and release of AA through calcium signaling, altered thiol-redox status, formation of ROS, and induction of oxidative stress in the vascular ECs accentuate the understanding of mechanisms of the environmental mercury-induced cardiovascular diseases (Schema).

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