Original Research *Highlight article*

Methyl mercury triggers endothelial leukocyte adhesion and increases expression of cell adhesion molecules and chemokines

Joshua Fowler[1], Martin Tsz-Ki Tsui[1,2], Jessica Chavez[1], Safeera Khan[1], Hassan Ahmed[1], Lena Smith[1] and Zhenquan Jia[1]

[1]Department of Biology, University of North Carolina at Greensboro, Greensboro, NC 27410, USA; [2]School of Life Sciences, Chinese University of Hong Kong, Hong Kong SAR 00000, China
Corresponding author: Zhenquan Jia. Email: z_jia@uncg.edu

Impact statement

Environmental exposures to methyl mercury (MeHg) have been epidemiologically related to the development of human cardiovascular disease CVD development. However, its role in development and promotion of atherosclerosis, an initial step in more immediately life-threatening CVDs, remains unclear. Monocyte adhesion to endothelial cells is known a critical first step in the development of atherosclerosis. Our study is the first to report that MeHg plays in the adhesion of monocytes to human microvascular endothelial cells (HMEC-1), and the underlying mechanisms. This study will contribute to our understanding of the detrimental effects involved in MeHg on human health, especially as this exposure exacerbates with the spread of global industrialization.

Abstract

Cardiovascular disease is the leading cause of morbidity, mortality, and health care costs in the USA, and around the world. Among the various risk factors of cardiovascular disease, environmental and dietary exposures to methyl mercury, a highly toxic metal traditionally labeled as a neurotoxin, have been epidemiologically linked to human cardiovascular disease development. However, its role in development and promotion of atherosclerosis, an initial step in more immediately life-threatening cardiovascular diseases, remains unclear. This study was conducted to examine the role that methyl mercury plays in the adhesion of monocytes to human microvascular endothelial cells (HMEC-1), and the underlying mechanisms. Methyl mercury treatment significantly induced the adhesion of monocyte to HMEC-1 endothelial cells, a critical step in atherosclerosis, while also upregulating the expression of proinflammatory cytokines interleukin-6, interleukin-8. Further, methyl mercury treatment also upregulated the chemotactic cytokine monocyte chemoattractant protein-1 and intercellular adhesion molecule-1. These molecules are imperative for the firm adhesion of leukocytes to endothelial cells. Additionally, our results further demonstrated that methyl mercury stimulated a significant increase in NF-κB activation. These findings suggest that NF-κB signaling pathway activation by methyl mercury is an important factor in the binding of monocytes to endothelial cells. Finally, by using flow cytometric analysis, methyl mercury treatment caused a significant increase in necrotic cell death only at higher concentrations without initiating apoptosis. This study provides new insights into the molecular actions of methyl mercury that can lead to endothelial dysfunction, inflammation, and subsequent atherosclerotic development.

Keywords: Methyl mercury, atherosclerosis, endothelial cells, inflammation, adhesion molecules, cytokine monocyte-endothelial cell interactions

Experimental Biology and Medicine **2021; 246: 2522–2532. DOI: 10.1177/15353702211033812**

Introduction

Mercury primarily exists in three forms: inorganic, elemental, and organic. Inorganic mercury is found as salt and poses a risk to human health. Inorganic mercury is primarily found as industrial wastes and, in some cases, in small-scale artisanal gold mining.[1] Elemental mercury is mercury in its purest form, while not safe for humans, it is the most inert form. Organic mercury poses the most direct threat to human health due to its bioavailability. Methylmercury (MeHg) is an extremely potent organic mercury that presents the greatest immediate risk to human health due to its dietary and environmental prevalence and bioavailability. While mercury runoff from industry is primarily airborne and inorganic, it can fall to the earth through precipitation and collect in rivers, streams, and ponds. Bacteria

are then able to ingest the mercury and through biomethylation, transform the toxin into methyl mercury.[2] Human are then able to follow the food chain upwards as bacteria are ingested by plankton, which is ingested by fish, which are ingested by larger fish which biomagnifies mercury concentration in the tissues of these animals. Indeed, as the mercury bioaccumulates in these organisms, they become a greater risk to human consumption. Tuna, Snapper, and other large, carnivorous fish are of particular risk as regular in the human diet, as they have bioaccumulated large concentrations of mercury.[3]

Typically, MeHg exposure has been linked to neurotoxicity and neurodegenerative disorders. Acute MeHg toxicity and its links to neurodegeneration have been seen epidemiologically for many years. In the 1950s, a great deal of industrial runoff entered the coastal waters of Japan and found its way into the food chain. Those people who's diet incorporated the infected fish and other wildlife of the surrounding waters began to exhibit ataxia, sensory disturbances, dysarthria, auditory disturbances, and involuntary tremors. This led to the discovery of MeHg as a neurotoxic agent.[4] While a great deal of information exists on the neurotoxic impacts of MeHg, a literature gap exists that fails to explain the toxic effects of MeHg in other organ systems.

The prevalence and impact of MeHg toxicity on the cardiovascular system are a new area of research. Epidemiological evidence from sites such as the Amazon rainforest, Finland, and the Faroe Islands have shown evidence that links diets high in MeHg to increased incidence or risk of cardiovascular disease.[5–7] In Finland, a study was done to link the dietary intake of high-MeHg fish to cardiovascular disease (CVD) in 1833 men between age 42 and 60 who had no previous incidence of CVD. Within the time frame of the study, 73 of the men experienced an acute myocardial infarction (AMI) within two to seven years. Seventy-eight of the men died, of which 18 died of CHD and 24 from CVD. The study used hair mercury concentration and urinary mercury excretion, which correlated with fish intake. Final results indicated a 2.1-fold increase in the risk of AMI after attenuating the effect of other factors like family history and smoking habits.[8]

In the Amazon rainforest, gold mining and hydroelectric damming of rivers release Hg into the environment, where it methylates and begins biomagnifying in fish. Nearby Amazonian tribes rely heavily on fish as a dietary source of protein and thus are subjected to increased exposure to MeHg.[9] Studies by Grotto *et al.* included blood, hair, and plasma sampling from 108 individuals living along a major Amazonian tributary susceptible to environmental MeHg contamination. Analysis of catalase, glutathione (GSH), glutathione peroxidase (GPx), and ALA-D biomarkers indicated a negative correlation between blood, hair and plasma MeHg concentrations and levels of oxidative stress biomarkers. This indicates a positive correlation between MeHg exposure and oxidative stress which can lead to CVD.

Studies in the Faroe Islands conducted by Sorensen *et al.* evaluated the effects of prenatal exposure to MeHg on blood pressure in childhood. The rationale was that early development of hypertension was a risk factor for the development of CVD. A sample size of 1022 single births was examined and cross-referenced with maternal hair mercury levels and umbilical cord blood mercury concentration. Children were reevaluated at age 7 for changes in blood pressure. Statistical analysis showed an average increase of 13.9 mmHg to the diastolic blood pressure and an average increase of 14.6 mmHg to systolic blood pressure.

As described above, MeHg is typically considered a neurotoxin. Recently it has been implicated as a potential promoter of atherosclerosis in humans. However, its underlying mechanisms have not been discovered. As toxins can be transported through the body in blood, the effect of MeHg on endothelial cells is an important target to research. Recent studies have shown that endothelial dysfunction induced by inflammation promotes monocyte adhesion and migration to the subendothelial space, contributing to atherosclerosis.[10–12] Atherosclerosis develops due to an inflammatory pathway, which then promotes the expression of adhesion molecules, like intercellular adhesion molecule (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1) resulting in in the capture and migration of circulating monocytes to endothelial cells.[13] These adhesion molecules are further stimulated by other cytokines like IL-1β and TNF-α, a powerful inducer of the NF-kB pathway.[14] Monocytes naturally express complementary cell-surface β_2-integrins, like MAC-1 and LFA-1, and VLA-4 which stabilize this adhesion process by binding to ICAM-1 and VCAM-1, respectively.[13,15] This cell-surface binding and adhesion allows for the migration of monocytes into the intima of a blood vessel.

While traditionally labeled a neurotoxin, MeHg has been epidemiologically linked to CVD pathologies; however, its role in development and promotion of atherosclerosis, an initial step in more immediately life-threatening CVDs, remains unclear. We hypothesized that MeHg stimulates the adhesion of monocytes due to chemokines and adhesion molecules expressed. Human microendothelial cells (HMEC-1) are a well-characterized cell line that retains many important properties of endothelial cells. For example, they retain many of the chemotactic, adhesion molecules, and inflammatory biomarkers found in other endothelial cell lines. This study took place to investigate the role of MeHg-induced endothelial dysfunction and damage.

Materials and methods

Cell culture

EA.hy926 (ATCC® CRL-2922™) cells were cultured in 8 mL of Gibco glucose-rich Dulbecco's Modified Eagle Media (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. HMEC1 (ATCC ® CRL-3243™) cells were grown in GenDepot® MCDB 131 media, supplemented with 10 mM L-glutamine, 10 ng/mL endothelial growth factor (EGF), 1 μg/mL hydrocortisone, and 10% FBS. THP-1 cells were grown in RPMI media supplemented with 10% FBS and 1% penicillin/streptomycin.

eLUCIdate™ RAW Nf-kB reporter cells were cultured in DMEM supplemented with 5% glutamate and 1% penicillin/streptomycin. Cell lines were grown in Cellstar® Filtered Cap 75 cm^2 cell-culture treated, screw cap flasks in incubators set to 37°C, and 5% CO_2. Media was renewed every two to three days and cells were split into a new passage at 85–95% confluence.

Cell treatment

Cells were treated using a mixture of Sigma-Aldrich/Millipore Sigma® Hanks Balanced Salt Solution (HBSS) containing calcium, magnesium, glucose, and methyl mercury in dose-dependent concentrations of 0.1 μM, 1.0 μM, and 2.0 μM for varying time intervals. Cells were split into 100 mm × 10 mm Corning® Cell culture plates and allowed to grow to 85% confluence. Plates were then decanted of their media and rinsed two times with 1× PBS; 6 mL of treatment was pipetted onto the adherent cells and plates were returned to the incubator at 37°C and 5% CO^2.

Antioxidant enzyme lysate

Cells were treated with MeHg using the methods described above. After 24 h of incubation, cells were rinsed with PBS two times to remove any remaining media; 3 mL of trypsin was then added per 75 cm^2 of growth surface area. Cells were then incubated for 4 min, and trypsin was neutralized with the addition of 6 mL of FBS-containing growth media. This cell suspension was centrifuged for 10 min at 1000 r/min to create a cell pellet. The cell pellet was then resuspended in 1 mL of PBS, transferred to an Eppendorf tube, and centrifuged again at 5000 r/min for 5 min to rinse any remaining media. The resulting pellet was resuspended in 300 μL of Tissue Buffer containing KH_2PO_4/K_2HPO_4 with 2 mM EDTA. The mixture was sonicated four times at 15-s intervals. The resulting solution was centrifuged at 13,000 r/min for 5 min and the supernatant collected and tested for antioxidant enzymes.

Monocyte adhesion assay

The critical first step in the development of atherosclerosis is inflammation or dysfunction-induced monocyte adhesion to endothelial cells.[10–12] We measured the degree of this adhesion caused by MeHg exposure by performing a monocyte adhesion assay. HMEC-1 cells were treated with 0.1, 1.0, and 2.0 μM MeHg in HBSS then subsequently treated with calcein-labeled THP-1 monocytes. Cells were cultured to 85% confluence in their respective media, described above, and treated with varying concentrations of 0.1–2.0 μM methyl mercury for 4 h. About 1.5 h prior to the end of treatment, THP-1 monocytes were counted using a hemocytometer. Approximately 2.5 million cells per plate were then collected and centrifuged at 1000 r/min for 7 min. The resulting cell pellet was resuspended in 10 mL of RPMI media containing 0.5% FBS and 10% Pen/Strep. Next, 7.5 μL of calcein was added to the THP-1 monocytes for labeling before allowing them 1 h to incubate. Following incubation of treatment cells, the treatment media was decanted, and the cells washed with 1× phosphate-buffered saline, twice. Labeled THP-1 monocytes were centrifuged at 1000 r/min for 7 min and then suspended in 6 mL/plate of RPMI media as mentioned above. Then, cells were then incubated for 1 h with calcein-labeled THP-monocytes. The cells were washed with cold 1× PBS, removing any unbound monocytes, then remaining cells were removed using a cell scraper and 1× PBS. The resulting mixture was transferred to a Costar® transparent 96-well plate and read at 496 nm/520 nm.

Protein concentration assay

Protein was measured using 10 μL of lysate diluted in 790 μL of DI H20; 200 μL of Bio-Rad® Protein Assay Dye Reagent was then be added. The mixture will be vortexed and transferred to Cuvettes. Absorbance will be measured using the Beckman-Coulter® DU800 spectrophotometer at 595 nm with 1.48 mg/dL Bovine Serum Albumin as a standard.

Glutathione assay

EAhy926 and HMEC1 cells were grown to 90% confluence in treated cell culture plates and exposed to MeHg and HgCl concentrations mentioned above at varying time intervals. An antioxidant enzyme lysate was produced, and 10 μL of the lysate was then mixed with GSH buffer and HPO_3. This solution was centrifuged at 13,000 r/min for 5 min. The solution was then combined with GSH buffer in a separate Eppendorf tube and incubated with 100 μL of O-phthalaldehyde (OPT) for 15 min in a dark room; 250 μL of the resulting solution was pipetted to a black Costar® 96-well plate, and the absorbance was measured by excitation at 250 nm and emission at 420 nm. A blank of GSH buffer and a positive sample containing 100 μL of GSH standard was also measured in the plate. The GSH content of samples was quantified using a standard curve previously created by our lab.

NAD(P)H quinone dehydrogenase 1 assay

EAhy926/HMEC1 cells were grown to confluence, treated, and an antioxidant enzyme lysate was created using methods, described previously. A reaction master mixture was created using 15 mL of Tris-HCl/NQO1 buffer, 45 μL of 50 mM nicotinamide adenine dinucleotide phosphate (NADPH), and 60 μL of dichlorophenolindopehnol (DCPIP); 4 μL of the collected antioxidant lysate was then combined with 696 μL of the created master mix in a cuvette, and absorbance measured using the Beckman-Coulter ® DU800 spectrophotometer at 600 nm. The rate kinetics of DCPIP reduction elucidated NQO1 activity.

RNA extraction

HMEC1 cells were cultured in their appropriate media in Corning® cell culture treated plates until 90% confluent. The media was then decanted, and the cells were treated with varying concentrations of MeHg and HgCl as described above for varying time frames. After completion of the treatment, cell plates were rinsed twice with 1× PBS to ensure any remaining treatment media does not remain.

Cells were treated with 1 mL of TRIzol® and pipetted into 1 mL Eppendorf tubes; 200 μL of chloroform was added, the solution was agitated and then transferred to a centrifuge at 12,000 RCF for 15 min. The top aqueous phase was combined with 500 μL of isopropanol and mixed well before being centrifuged again at 12,000 RCF for 10 min. The resulting pellet was washed and centrifuged at 7400 RCF for 5 min twice before resuspending the pellet in 10–15 μL of DEPC.

cDNA synthesis

Cells were cultured, treated, and RNA was extracted using the methods stated above. The resulting RNA was quantified using a Thermo Scientific™ Nanodrop 2000 and diluted to a 500 ng/μL concentration; 2 μL of dilute RNA was then combined with 5 μL of 5× Buffer, 1.25 μL of ddNTP, 1.25 μL of Random Primer, 0.625 μL of MMLV-reverse transcriptase, and 14.875 μL of DEPC water. The 25 μL solution was converted to cDNA using the Applied Biosystems™ Veriti™ 96-Well Thermal Cycler.

Quantitative real-time polymerase chain reaction

Adhesion of monocytes to ECs is regulated by several critical chemotactic and inflammatory cytokines, as well as the presence of vascular adhesion molecules on the cell surface. Using qRT-PCR, we probed HMEC-1 cells treated with different concentrations of MeHg for changes in gene expression of these molecules. Levels of IL-8, IL-6, ICAM, and CCL2 in HMEC-1 cells treated for 4 h with 0–2.0 μM MeHg were quantified using qRT-PCR. Cells were cultured and treated, and RNA was extracted and converted to cDNA using the methods stated above. The resulting cDNA was probed for IL-8, IL1β, IL-6, and MCP-1 using GAPDH as a housekeeping gene. This was done by combining 1 μL of cDNA with 10 μL of Power SYBR ® Green PCR Master Mix, 2 μL of 1:10 diluted cDNA, 2 μL of 5 μM Forward Primer, 2 μL of 5 μM Reverse Primer, and 5 μL of DEPC. The Applied Biosystems™ QuantStudio3™ Real-Time PCR system was run for 40 cycles. Each cycle encompassed a 95°C phase for 15 s, a 58°C phase for 1 min, and a 60°C phase for 15 s. In order to quantify gene expression, comparative threshold values were evaluated.

Human primer sequences

The primer sequences are listed as follows: GAPDH (Forward: 5′-AGAACGGGAAGCTTGTCATC-3′, Reverse: 5′-GGAGGCATTGCTGATGATCT-3′). IL-8 (Forward: 5′-CTCTGTGTGAAGGTGCAGTT-3′, Reverse: 5′-AAACTTCTCCACAACCCTCTG-3′). CCL2 (Forward: 5′-GCTCAGCCAGATGCAATCAA-3′, Reverse: 5′-GGTTGTGGAGTGAGTGGTCAAG-3′). IL-6 (Forward: 5′-TACCCCCAGGAGAAGATTCC-3′, Reverse: 5′-GCCATCTTTGGAAGGTTCAG-3′). ICAM (Forward: 5′-GGCTGGAGCTGTTTGAGAAC-3′, Reverse: 5′-ACTGTGGGGTTCAACCTCTG- 3′). GCLC (Forward 5′-ACCATCATCAATGGGAAGGA-3′, Reverse: 5′-GCGATAAACTCCCTCATCCA-3′). NQO1 (Forward: 5′-TTACTATGGGATGGGGTCCA-3′, Reverse: 5′-TCTCCCATTTTTCAGGCAAC-3′).

NF-kB luciferase assay

Studies show that NF-κB-mediated (Nuclear factor kappa B) transcription of the previously described cytokines and adhesion molecules plays an important role in atherosclerotic pathology.[16–18] Activation of this signaling pathway results in a greater magnitude of leukocyte adhesion to endothelial cells. We chose to examine whether MeHg exposure is implicated in the activation of this pathway by using the eLUCidate NF-κB reporter cell line. This cell line has been well characterized and has been widely used to detect changes in the NF-κB transduction pathway. These cells contain a Renilla luciferase reporter gene under direct transcriptional control of the upstream NF-κB response element in DNA. By introducing coelenterazine as a substrate, we were able to induce luminescence in living cells. The intensity of this luminescence directly correlates with NF-κB pathway activation. eLUCIdate™ RAW 264 NF-kB report cell line cells were grown using previously mentioned media in 6-well plates. Cells were treated with various concentrations of MeHg for 4 h. Following treatment, cells were washed twice with 1 mL of cold PBS to remove any excess media. Cells were then lysed with lysis buffer on a shaker table for ∼20 min. Finally, 2 μL of coelenterazine was added to catalyze the luminescent reaction. Luminescence was ready using the Synergy™ 2 Multi-Mode Microplate reader.

7-Aad/annexin V-PI flow cytometry. Cells were cultured and treated as described previously. Confluent cells were then gently washed two times with cold PBS to remove any remaining media. A cell scraper was used to remove any remaining cells, and the subsequent cell suspension was transferred to a microcentrifuge tube and centrifuged at 7000 r/min for 5 min. The supernatant was discarded, and cells were resuspended in 1× annexin-binding buffer to a concentration of 2.0×10^5 cells/100 μL. Following resuspension, 2.5 μL of FITC-AnnexinV and 1 μL of 7-AAD were added per 100 μL of suspension. The solution was incubated for 15 min at room temperature before addition of 400 μL of binding buffer. The analysis was done using the Guava EasyCyte Flow Cytometry system.

Statistical analysis. All data were subjected to analysis of variance (ANOVA) using GraphPad Prism® software and are expressed as mean ± SEM. Significant treatment differences were subjected to the Student *t* tests and $P < 0.05$ was considered different.

Results

Monocyte binding in HMEC-1 endothelial cells increases in a dose-dependent manner in response to treatment with methyl mercury. As shown in Figure 1, at 2.0 μM and 1.0 μM, HMEC-1 cells displayed a significant ($P < 0.05$) ∼3.4-fold and∼1.7-fold increase in monocyte adhesion, respectively, when compared with control cells cultured in HBSS media alone; 0.1 μM treatments showed no significant change in monocyte binding compared to

control. TNF-α, a known promotor of monocyte binding, was used a positive control and displayed a significant ($P < 0.05$) ~3.4-fold increase in monocyte adhesion (Figure 1).

Levels of inflammatory cytokine gene expression in HMEC-1 cells following methyl mercury exposure. As shown in Figure 2(a), IL-8 levels of gene expression increased significantly ($P < 0.05$) by 18-fold in 1.0 μM treatment group and appear near significantly ($P = 0.06$) upregulated by 163-fold in 2.0 μM treatment group. Expression of IL-6 significantly ($P < 0.05$) increased by 4-fold in 1.0 μM treatments and by 17.7 in 2.0 μM treatments (Figure 2(b)). CCL2 levels significantly ($P < 0.05$) increased in 2.0 μM treatments by 63.7-fold (Figure 2(c)). Finally, ICAM expression also significantly increased ($P < 0.05$) by 14-fold in 2.0 μM treatments and near significant increase ($P = 0.052$) of ~2-fold at 1.0 μM treatment (Figure 2(d).

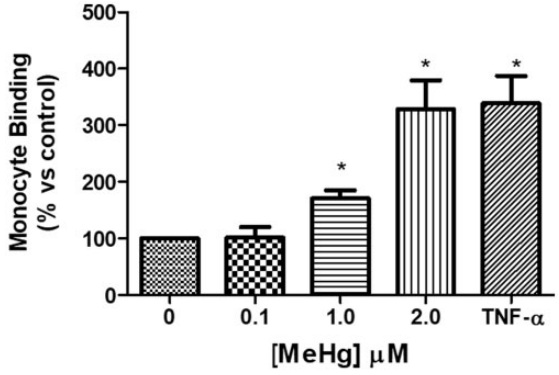

Figure 1. HMEC-1 cells were treated with 0.1, 1.0, and 2.0 μM MeHg for 4 h in Hanks' balanced salt solution (HBSS). Cells were then co-treated with calcein-labeled THP-1 monocytes and absorbance was read at 570 nm via Biotech Synergy 2 plate reader. All data represent mean ± SEM ($n = 4$, *$P < 0.05$ vs. control.).

Examination of activation of the NF-kB pathway due to methyl mercury exposure. As shown in Figure 3, cells treated with 2.0 μM MeHg for 4 h showed a significant ($P < 0.05$) increase by 4.5-fold in luminescence. This finding indicates that MeHg promotes the binding of NF-κB transcription factors to response elements on DNA, inducing the transcription of the luciferase reporter gene, as well as a potential transcription of any downstream cytokines. Lipopolysaccharide, a known activator of the NF-kB pathway, was used as a positive control and induced a significant ($P < 0.05$) 8.6-fold increase in luminescence (Figure 3).

Phase II antioxidant levels show no significant changes in response to methyl mercury treatments. Studies have implicated phase II cellular antioxidant enzymes, such as glutathione (GSH) and NAD(P) H dehydrogenase (quinone)-1 (NQO-1) as important drivers of ROS-mediated adhesion molecule expression in endothelial cells.[19] Both GSH and NQO1 are important cellular antioxidants in the cells that protect them against oxidative damage from reactive oxygen species (ROS). qRT-PCR

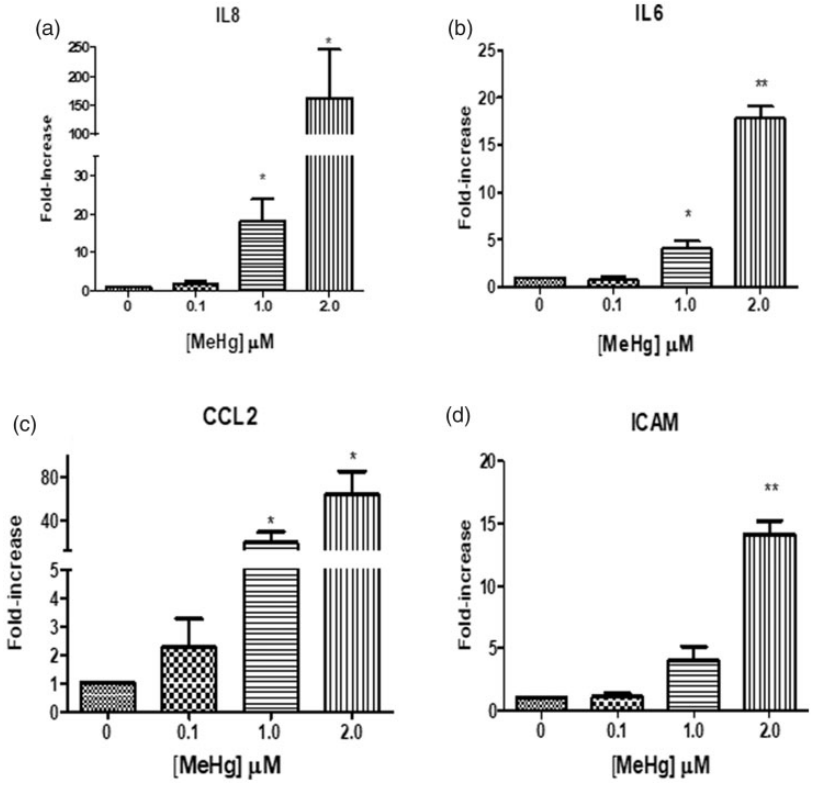

Figure 2. HMEC-1 cells were treated with 0.1, 1.0, and 2.0 μM MeHg for 4 h in HBSS. RNA was isolated, converted to cDNA, and probed for IL-8 (a), IL-6 (b), CCL2 (c), and ICAM (d) using SYBRgreen qRT-PCR reagents via QuantStudio 3. GAPDH was used as a housekeeping gene. All data represent mean ± SEM ($n = 3$, *$P < 0.05$ vs. control. **$P < .01$ vs. control).

was performed to measure the relative gene expression of gamma-glutamyl cysteine ligase (GCL) and NQO1 in cells treated for 4 h with 0–2.0 μM. GCLc is an important precursor molecule involves in the synthesis of GSH. Treatment showed no significant ($P < 0.05$) changes in the gene expression of GCLc (Figure 4(a)), or NQO1 (Figure 4(b)) in HMEC-1 cells. To further validate these findings, we employed EAhy926 cells, an additional human endothelial cell line. EAhy926 cells cultured for 24 h with 0.1, 1.0, and 2.0 μM MeHg in HBSS were measured for total protein levels as a standard before quantifying changes in antioxidant enzymes. As shown in Figure 5, the levels of GSH and NQO1 (nmol/mg protein) showed no significant changes (P <0.05) in response to any methyl mercury treatment concentrations.

Methyl mercury effect on cell viability in HMEC-1 cells. Cell viability of HMEC-1 cells was determined using flow cytometric analysis. Cells were fluorescently co-stained with FITC-labeled annexin V and propidium iodide (PI) as markers of apoptosis and necrosis, respectively. This procedure is provided in the "Materials and Methods" chapter. HMEC-1 cells treated for 4 h with 0–2.0 μM MeHg were dyed with 7-AAD and Annexin V/PI and examined via Guava EasyCyte. No significant cytotoxicity was observed at 1 μM MeHg (Figure 6(d)). However, MeHg at 2.0 μM showed a significant ($P < 0.05$) decrease in cell viability when compared to control (Figure 6(a)). As shown in Figure 6(b) and (c), MeHg treatments at both 1.0 and 2.0 μM concentrations showed no significant changes in early or late apoptosis. However, rates of cell necrosis significantly increased in the 2.0 μM treatment group (Figures 6(d) and 7).

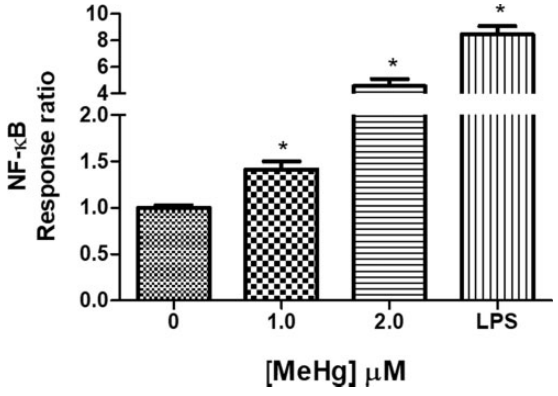

Figure 3. NF-kB RAW cells were treated with 0.1, 1.0, and 2.0 μM of MeHg in HBSS for 4 h. Coelenterazine was then added as a substrate at a concentration of 1 μM, and luminescence was read using the Biotech Synergy 2 plate reader. LPS at 10 μg/mL was used as a positive control. All data represent mean ± SEM ($n = 3$. *$P < 0.05$).

Discussion

Mercury consumption has long been implicated in a range of human health problems. More recently, greater research has been conducted into the roles of mercury on human cardiovascular health including increased risk of hypertension, myocardial infarction, cardiac arrhythmia and carotid artery obstruction, among others.[20] However, its role in development and promotion of atherosclerosis, an initial step in more immediately life-threatening CVDs, remains unclear. In the present study, we demonstrated for the first time that exposure to MeHg *in vitro* caused a significant increase in monocyte binding in HMEC-1 cells, which would contribute to atherosclerotic development and subsequent CVD. Further, we identified increasing trends in gene expression of several inflammatory cytokines and adhesion molecules in cells treated with organic mercury as well as changes in NF-kB signaling pathway activation in macrophage-derived reporter cell lines.

Currently, the literature lacks the support that directly links MeHg exposure to monocyte adhesion and subsequent atherosclerotic development. The adhesion of circulating monocytes to vascular endothelial cells is the initiating step in the development of atherosclerosis.[13–15] Binding of these cells to an endothelial lining promotes migration into the subendothelial space followed by differentiation into macrophages, a process facilitated by contact with monocyte chemoattractant protein 1 (MCP-1/CCL2).[13–15] These macrophages scavenge surrounding molecules before eventually becoming foam cells, which play an important role and in the early stage development of atherosclerotic lesion formation.[21,22] Thus, determining the extent of monocyte adhesion to endothelial cells

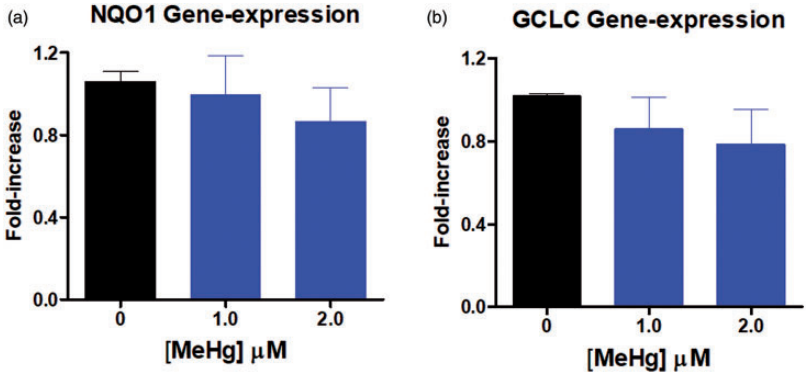

Figure 4. HMEC-1 cells treated for 4 h with 0.1, 1.0, and 2.0 μM MeHg in HBSS. RNA was isolated, converted to cDNA, and probed for GCLC (a) and NQO1 (b) using SYBRgreen qRT-PCR reagents via Applied Biosystems QuantStudio 3. Data were calculated against GAPDH as a housekeeping gene. All data represent mean ± SEM ($n = 5$). (A color version of this figure is available in the online journal.)

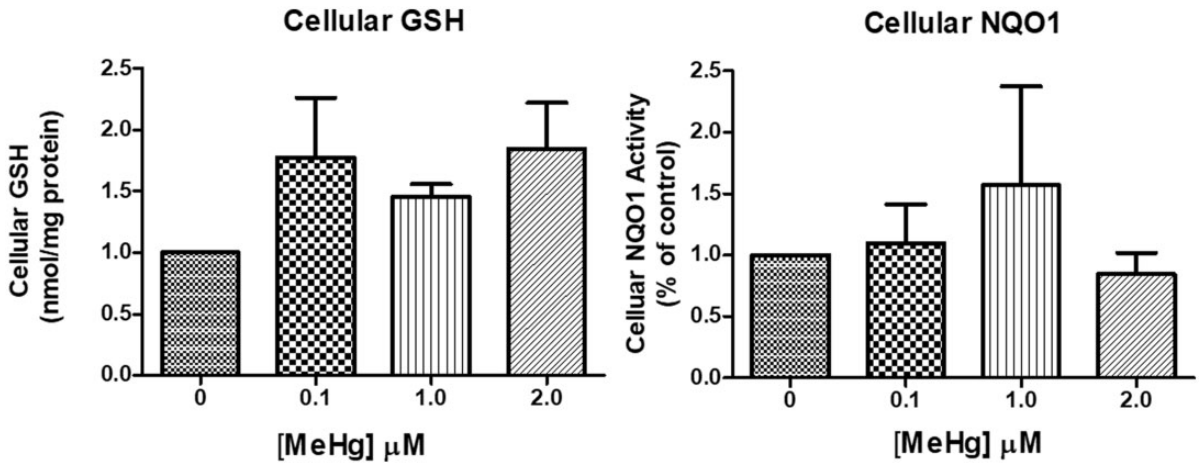

Figure 5. Left panel: EAhy926 cells treated for 24 h with 0.1, 1.0, and 2.0 μM MeHg in HBSS for 24 h. GSH was calculated as a ratio of GSH to total protein per sample. Right panel: EAhy926 cells treated for 24 h with 0.1, 1.0, and 2.0 μM MeHg for 24 h. NQO1 was calculated using the rate of consumption of NADPH. Samples were examined using DU-800 spectrophotometer. All data represent mean ± SEM.

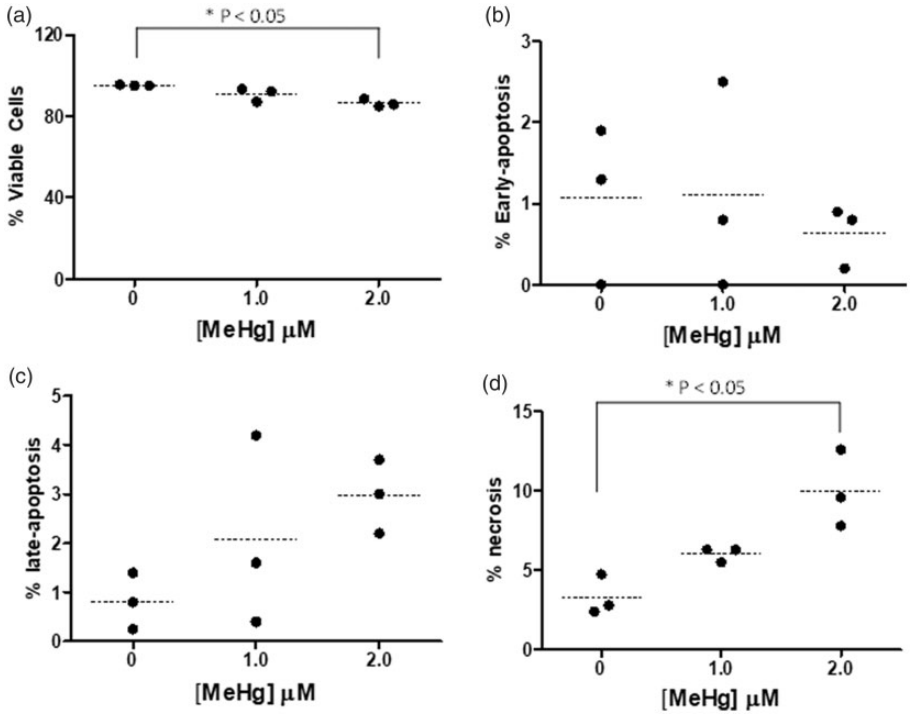

Figure 6. HMEC-1 cells were treated for 4 h with 0.1, 1.0, and 2.0 μM MeHg in HBSS for 4 h. 7-AAD and Annexin/PI were used as dyes to determine the difference between viable cells (a), early-apoptotic cells (b), late-apoptotic cells (c) and necrotic cells (d). Cell viability was determined using flow cytometric analysis via Guava EasyCyte ($n = 3$; $^*P < 0.05$ vs. control).

promoted by MeHg is critical to understanding the link between organic mercury exposure and CVD. We report, for the first time, observance of an increase in monocyte adhesion following exposure to organic mercury. In our study, treatment of both 1.0 μM and 2.0 μM concentrations of MeHg yielded a significant increase in monocyte adhesion (Figure 1). This finding lends support to the hypothesis that organic mercury exposure can promote atherosclerotic development by recruitment of monocytes. Our finding is consistent with a previous study by Furieri *et al.*,[23] showing that MeHg may directly target vascular endothelial cells causing dysfunction. Furieri *et al.*, who conducted research on coronary arteries in rats exposed to MeHg, have shown that even low levels of exposure induced vasoconstriction and decreased nitric oxide bioavailability. This suggests that organic mercury exposure could disrupt contraction and relaxation in heart tissue, resulting in hypertension and promoting CVD.[23] This aligns with evidence gathered linking fish consumption to elevated blood mercury levels and increased incidence of hypertension, angina, and myocardial infarction.[22]

The pro-inflammatory cytokines such as IL-8 and IL-6, adhesion molecules presented on endothelial cells (ICAM-1), as well as cellular contact with MCP-1/CCL2

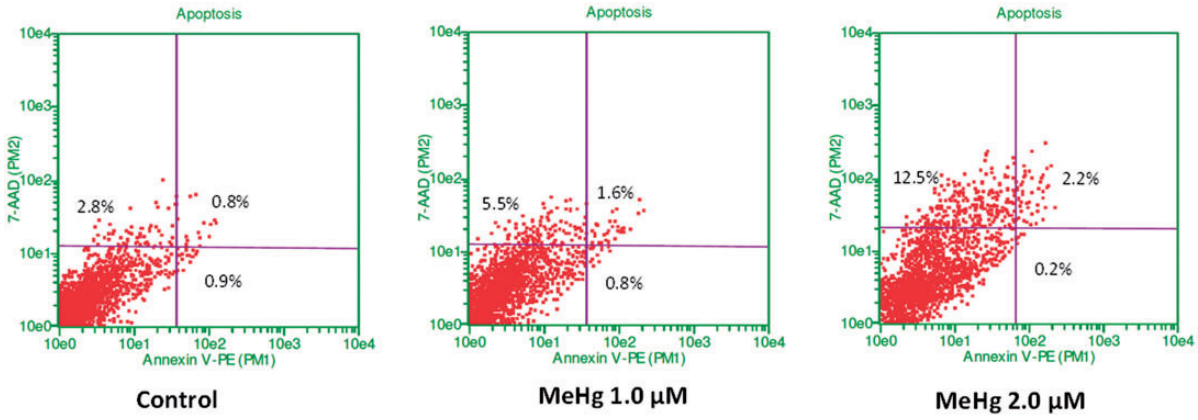

Figure 7. Representative flow cytometric analysis of control HMEC-1 cells and cells exposed to MeHg at 1.0, and 2.0 µM. HMEC-1 cells were treated for 4 h with 0.1, 1.0, and 2.0 µM MeHg in HBSS for 4 h. Bottom left represents healthy cells, top left represents necrosis, top right represents late-apoptosis, and bottom right represents early apoptosis. (A color version of this figure is available in the online journal.)

have been suggested to play a crucial role in regulating recruitment of monocytes to the endothelial cells.[21,24] Levels of ICAM-1 in plasma have been linked with a pro-inflammatory response while also mediating immune cell adhesion to endothelial cells.[25] MCP-1/CCL2 is known to facilitate monocyte binding and differentiation into macrophages. This chemotactic protein binds to CCR2 receptors on migrating monocytes and induces differentiation. Studies conducted on human occlusive aortas have demonstrated that the presence of IL-8 and MCP-1 promotes immune cell recruitment and perpetuates inflammatory reactions that can lead to vessel wall destruction.[26] Another member in the interleukin chemokine family, IL-6, acts as a signaling cytokine and has been implicated in inflammation-mediated human pathologies. Studies conducted in mice not only suggest IL-6 as a marker for atherosclerosis but indicate it may contribute to atherosclerotic lesion development.[27] Further, increases in IL-6 and ICAM-1 concentration in apparently healthy men have been linked with an increased incidence of repeat myocardial infarctions.[28,29] Finally, studies conducted on human arterial atherosclerotic walls have found a marked increase in concentrations of both IL-6 and IL-8.[30]

The importance of the above pro-inflammatory cytokines and adhesion molecules in atherosclerotic development makes them an ideal target for evaluating the links between MeHg exposure and CVD. Our results showed a significant upregulation of ICAM-1, IL-6, and MCP-1/CCL2 in the 1.0 µM and 2.0 µM MeHg treatment groups (Figure 2). These results suggest that MeHg-induced monocytes binding to the endothelial cells may be partially mediated by expressions of chemokines and adhesion molecules, which further strengthen the hypothesis that MeHg exposure contributes to atherosclerosis through monocyte adhesion, migration, and differentiation in the subendothelial space. Consistent with our results in vascular endothelial cells, previous literature in other organ systems lends credence to the idea that mercury exposure can also increase these chemotactic cytokines in the different types of cells. For example, experiments conducted on human glial cells found an increase in IL-6 production after 8 and 16-h MeHg treatments with concentrations as low as 1.25 µM.[31] Further studies performed on macrophages demonstrated increases in both IL-6 and IL-8 following treatment with varying doses of MeHg at times between 3 and 10 h in human U937 macrophages.[32] These results in other organ systems echo our findings in endothelial cells that MeHg can contribute to the inflammatory response by upregulations of pro-inflammatory cytokines.

Previous studies have demonstrated that activation of nuclear factor-kappaB (NF-kB) is essential for the expression of adhesion molecules and chemokines that are critically involved in leukocyte adhesion to endothelium.[33,34] Molecular targets of identifying NF-κB activation include proinflammatory cytokines (e.g., IL-8, IL-6, IL-2, etc.), adhesion molecules (e.g., VCAM-1 and ICAM-1), some chemokines and also cell cycle regulators.[35] Changes in the activation of this pathway can strengthen the link between MeHg exposure, endothelial dysfunction, and atherosclerosis. Identification of shifts in this pathway can grant insight into MeHg pathology. To identify the potential activation of the NF-kB pathway, we utilized the eLUCIdate™ NF-kB reporter macrophages that are designed to luminance with the addition of coelenterazine as a substrate in the event of NF-kB activation. Our results demonstrated a significant increase in the 2.0 µM MeHg treatment group (Figure 3), suggesting that NF-κB signaling is involved in the MeHg-induced monocyte binding to endothelial cells.

Reactive oxygen species (ROS) have been identified as another contributor to monocyte adhesion in endothelium and atherosclerotic development. While ROS in normal physiological concentrations serves as signaling molecules, an overabundance of these compounds can trigger endothelial damage, promote the production of adhesion molecules and inflammatory cytokines, and induce cell death in oversaturated foam-cell macrophages.[36] This is done by oxidation of circulating low-density lipoproteins which themselves can stimulate expression of ICAM-1, initiating the previously mentioned cascade of monocyte binding, differentiation, and terminal foam-cell apoptosis.[37] Normal regulation of these intracellular ROS is handled

by phase II antioxidant enzymes such as glutathione (GSH). Analysis of the ratio between oxidized glutathione (GSSG) to its reduced form of GSH is indicative of oxidative damage, and decreases in intracellular GSH levels can suggest increased ROS generation.[19] Additionally, the enzyme NADP(H) dehydrogenase [quinone]-1 (NQO1) is a player in intracellular ROS regulation. Studies conducted propose that upregulation of NQO1 production serves as an adaptive response to intracellular oxidative stress by performing the two-electron reduction of quinones and scavenging superoxide radicals.[38] Research has provided support to the idea that MeHg exposure alters levels of these enzymes are contributing to ROS-mediated cellular dysfunction. Evaluation of Wistar rats dosed for 100 days with low levels of MeHg found a significant decrease in GSH antioxidants in blood plasma.[39] Further studies have corroborated this evidence in the nervous system as well as provided evidence of MeHg-induced ROS generation in the cardiovascular system.[40,41] Our results conducted on EAhy 926 cardiac endothelial cells and HMEC-1 endothelial cells showed no significant changes in GSH or NQO1 in the expression of these gene markers by MeHg exposure (Figures 4 and 5). Longer treatment time would be needed to clarify this effect.

A reasonable explanation for no activation patterns in antioxidant responses to MeHg can be found in literature and pertains to the possible confounding signals between the NF-kB activation by MeHg and Nrf2 signaling pathways.[39–44] Nrf2 can induce various antioxidant genes like GSH and NQO1, which are typically present following MeHg toxicity.[45,46] Although activation of this pathway by MeHg is not fully understood, it has been demonstrated that Nrf2 binds to KEAP-1 molecules, which contain several cysteine residues. MeHg is able to bind to these residues covalently, which may promote the signaling pathway. Experimental results have shown that neuroblastoma cells with elevated levels of Nrf2 expression have an increased resistance to MeHg toxicity when compared to those with standard levels of expression.[46] Interestingly, literature also exists that suggests the p65 transcription factor of the NF-kB pathway could negatively impact Nrf2 activation by competing for the transcriptional co-activator (CREB-binding protein)-p300 complex (CBP).[42,43] Conversely, studies conducted on different cell lines have yielded evidence that NF-kB and Nrf2 serve to promote each other. Experiments conducted in human acute myeloid leukemia cells displayed that NF-kB transcription factors could potentially induce transcription of Nrf2.[44] In this context, the result of our studies showing no significant changes in mediating GSH and NQO1 antioxidants and gene expression of these molecules in endothelial cells could be a result of the aforementioned confounding signals between the NF-kB activation by MeHg and the possible Nrf2 signaling pathways. Therefore, future work to dissect this relationship could utilize siRNA to blow Nrf2 expression, or *in vivo* studies could employ the use of Nrf2-knockout animals. As the crosstalk between these two pathways is complex and cell-specific, it is possible that NF-kB activation by MeHg in endothelial cells could confound downstream expression mediated by the Nrf2/Keap1 pathway of antioxidant regulation.

Previous studies have lent credence to the fact that necrosis versus apoptotic cell death can contribute to chemotactic and inflammatory cytokine release into the intracellular matrix, thus perpetuating atherosclerotic development.[47] Apoptotic death is an important process by which unwanted cells activate self-detrimental pathways to induce death to maintain constant size and regulate cell production.[48–50] Necrosis, on the other hand, appears to be invoked by extrinsic sources causing acute cellular dysfunction. Recent studies using TEM analysis of human atherosclerotic lesions have shown that a great deal of cell death in these tissues is due to necrosis.[50–52] Thus, it would be beneficial to determine the type of cell death occurring as a result of MeHg exposure, as necrotic cell death could further bolster the induced inflammatory response. Our studies using flow cytometric analysis of HMEC-1 cells with 7-AAD and Annexin V/PI showed a significant decrease in cell viability at 2.0 μM concentrations, specifically, a significant increase in necrotic cell populations without invoking apoptosis (Figure 6). This indicates that high concentrations of MeHg can cause severe damage to cellular membranes, increasing the presence of the aforementioned inflammatory cytokines and chemotactic molecules, contributing to atherosclerosis. However, 1.0 μM concentrations of MeHg showed no significant changes in cell death type despite upregulating expression of the same chemotactic and inflammatory markers. Future experiments must be performed to explore whether MeHg induced necrosis was significantly contributing to atherosclerotic development both *in vitro* and *in vivo*.

Epidemiological studies show that environmental Hg exposure has been closely related to the occurrence and development of human cardiovascular disease CVD, especially atherosclerosis. The adhesion of monocytes to endothelial cells is the key first step in the development of atherosclerosis. Our study reported for the first time that methylmercury significantly induces monocyte-endothelial cell adhesion at low concentrations. Although the findings within vitro exposure may not be directly extended to the whole animal or human cardiovascular system *in vivo*, this research will help us understand the clinically adverse effects of methylmercury on human health, especially as this exposure increases with the spread of global industrialization.

It would be pertinent to note that blood concentrations of Hg (chiefly MeHg) have been reported up to ~1 μM (~200 ng Hg/mL) in human subjects following accidental exposure to MeHg, and only decreased to ~0.13 μM after three months of clearance.[53] A study conducted identifying the concentrations of mercury found in fish samples destined for human consumption by X-ray absorption found levels as low as 0.4 μM and as high as 6.0 μM.[54] However, this mercury was found in various forms including conformations already bound to thiol groups. Further consideration must be taken to factor in the bioaccumulation of mercury in physiological systems. With a half-life of ~70 days, it is possible for mercury levels to increase

steadily with repeated consumption of mercury-contaminated foods.[55] Additional factors involved in our decision to use these concentrations include other studies conducted *in vitro* spanning concentrations of 0.1 μM to 20 μM.[56] With this knowledge, MeHg concentrations from 0.1 μM to ~1.0 μM were used in this study, which we considered to be acutely non-lethal, physiologically relevant, and certainly achievable *in vivo*.[53]

To summarize this study, we demonstrated for the first time that *in vitro* MeHg exposure to HMEC-1 cells causes a significant increase in monocyte binding, a quality that would contribute to atherosclerotic development and subsequent CVD. A significant increase in the expression of inflammatory and chemotactic cytokines including ICAM-1, IL-6, IL-8, and MCP-1/CCL2 was also observed, which would contribute to monocyte adhesion and binding. Additionally, observation of the activation of NF-kB suggests that MeHg-induced adhesion of monocytes to endothelial cells is associated with this pathway, which can further facilitate the upregulation of cytokines and adhesion molecules. This study provides new insight into the molecular actions of MeHg that can lead to endothelial injury, inflammation, and subsequent atherosclerosis. These data will contribute to our understanding of the detrimental effects involved in MeHg on human health, especially as this exposure exacerbates with the spread of global industrialization.

AUTHORS' CONTRIBUTIONS

ZJ designed the experiments. JF, JC, SF, HA, and LS completed the experiments and processed the experimental data. JF, MTK, and ZJ drafted the manuscript. JF, MTK, and ZJ contributed to analysis and interpretation of results.

DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

FUNDING

The study was financially supported by a Giant Steps Research Development Grant from University of North Carolina at Greensboro.

ORCID iD

Zhenquan Jia https://orcid.org/0000-0002-5187-1549

REFERENCES

1. Pastinszky I. [Pigmentation disorders of the face in internal diseases. *Orv Hetil* 1973;**114**:150–2]
2. Rice KM, Walker EM, Jr., Wu M, Gillette C, Blough ER. Environmental mercury and its toxic effects. *J Prev Med Public Health* 2014;**47**:74–83
3. Ikem A, Egiebor NO. Assessment of trace elements in canned fishes (mackerel, tuna, salmon, sardines and herrings) marketed in Georgia and Alabama (United States of America). *J Food Compos Anal* 2005;**18**:771–87
4. Harada M. Minamata disease: methylmercury poisoning in Japan caused by environmental pollution. *Crit Rev Toxicol* 1995;**25**:1–24
5. Grotto D, Valentini J, Fillion M, Passos CJS, Garcia SC, Mergler D, Barbosa Jr F. Mercury exposure and oxidative stress in communities of the Brazilian amazon. *Sci Total Environ* 2010;**408**:806–11
6. Larsen TJ, Jørgensen ME, Larsen CVL, Dahl-Petersen IK, Rønn PF, Bjerregaard P, Byberg S. Whole blood mercury and the risk of cardiovascular disease among the greenlandic population. *Environ Res* 2018;**164**:310–5
7. Zhang Y, Xu C, Fu Z, Shu Y, Zhang J, Lu C, Mo X. Associations between total mercury and methyl mercury exposure and cardiovascular risk factors in us adolescents. *Environ Sci Pollut Res Int* 2018;**25**:6265–72
8. Salonen JT, SeppäNen K, NyyssöNen K, Korpela H, Kauhanen J, Kantola M, Tuomilehto J, Esterbauer H, Tatzber F, Salonen R, Intake of mercury from fish, lipid peroxidation, and the risk of myocardial infarction and coronary, cardiovascular, and any death in Eastern Finnish men. *Circulation* 1995;**91**:645–55
9. Marques RC, Dórea JG, Bastos WR, de Freitas Rebelo M, de Freitas Fonseca M, Malm O. Maternal mercury exposure and neuro-motor development in breastfed infants from Porto Velho (amazon), *Brazil. Int J Hygiene Environ Health* 2007;**210**:51–60
10. Mayer K, Merfels M, Muhly-Reinholz M, Gokorsch S, Rosseau S, Lohmeyer J, Schwarzer N, Krull M, Suttorp N, Grimminger F, Seeger W. Omega-3 fatty acids suppress monocyte adhesion to human endothelial cells: role of endothelial PAF generation. *Am J Physiol Heart Circ Physiol* 2002;**283**:H811–8
11. Chen JW, Chen YH, Lin FY, Chen YL, Lin SJ. Ginkgo biloba extract inhibits tumor necrosis factor-alpha-induced reactive oxygen species generation, transcription factor activation, and cell adhesion molecule expression in human aortic endothelial cells. *Arterioscler Thromb Vasc Biol* 2003;**23**:1559–66
12. Carluccio MA, Siculella L, Ancora MA, Massaro M, Scoditti E, Storelli C, Visioli F, Distante A, De Caterina R. Olive oil and red wine antioxidant polyphenols inhibit endothelial activation: antiatherogenic properties of Mediterranean diet phytochemicals. *Arterioscler Thromb Vasc Biol* 2003;**23**:622–9
13. Mestas J, Ley K. Monocyte-endothelial cell interactions in the development of atherosclerosis. *Trends Cardiovasc Med* 2008;**18**:228–32
14. Marui N, Offermann MK, Swerlick R, Kunsch C, Rosen CA, Ahmad M, Alexander RW, Medford RM. Vascular cell adhesion molecule-1 (VCAM-1) gene transcription and expression are regulated through an antioxidant-sensitive mechanism in human vascular endothelial cells. *J Clin Invest* 1993;**92**:1866–74
15. Solovjov DA, Pluskota E, Plow EF. Distinct roles for the alpha and beta subunits in the functions of integrin alphaMbeta2. *J Biol Chem* 2005;**280**:1336–45
16. Read MA, Whitley MZ, Williams AJ, Collins T. NF-kappa B and I kappa B alpha: an inducible regulatory system in endothelial activation. *J Exp Med* 1994;**179**:503–12
17. Brown K, Gerstberger S, Carlson L, Franzoso G, Siebenlist U. Control of I kappa B-alpha proteolysis by site-specific, signal-induced phosphorylation. *Science* 1995;**267**:1485–8
18. Ouchi N, Kihara S, Arita Y, Okamoto Y, Maeda K, Kuriyama H, Hotta K, Nishida M, Takahashi M, Muraguchi M, Ohmoto Y, Nakamura T, Yamashita S, Funahashi T, Matsuzawa Y. Adiponectin, an adipocyte-derived plasma protein, inhibits endothelial NF-kappaB signaling through a cAMP-dependent pathway. *Circulation* 2000;**102**:1296–301
19. Tavakoli S, Asmis R. Reactive oxygen species and thiol redox signaling in the macrophage biology of atherosclerosis. *Antioxid Redox Signal* 2012;**17**:1785–95
20. Houston M. The role of mercury in cardiovascular disease. *J Cardiovasc Dis Diagnos* 2014;**2**:18
21. Libby P. Inflammation in atherosclerosis. *Nature* 2002;**420**:868–74
22. Kim YN, Kim YA, Yang AR, Lee BH. Relationship between blood mercury level and risk of cardiovascular diseases: results from the fourth Korea national health and nutrition examination survey (KNHANES IV) 2008-2009. *Prev Nutr Food Sci* 2014;**19**:333–42
23. Furieri LB, Galán M, Avendaño MS, García-Redondo AB, Aguado A, Martínez S, Cachofeiro V, Bartolomé MV, Alonso MJ, Vassallo DV. Endothelial dysfunction of rat coronary arteries after exposure to low

concentrations of mercury is dependent on reactive oxygen species. *Br J Pharmacol* 2011;**162**:1819–31
24. Boisvert WA, Santiago R, Curtiss LK, Terkeltaub RA. A leukocyte homologue of the IL-8 receptor CXCR-2 mediates the accumulation of macrophages in atherosclerotic lesions of LDL receptor-deficient mice. *J Clin Invest* 1998;**101**:353–63
25. Lawson C, Wolf S. ICAM-1 signaling in endothelial cells. *Pharmacol Rep* 2009;**61**:22–32
26. Koch AE, Kunkel SL, Pearce WH, Shah MR, Parikh D, Evanoff HL, Haines GK, Burdick MD, Strieter RM. Enhanced production of the chemotactic cytokines interleukin-8 and monocyte chemoattractant protein-1 in human abdominal aortic aneurysms. *Am J Pathol* 1993;**142**:1423–31
27. Huber SA, Sakkinen P, Conze D, Hardin N, Tracy R. Interleukin-6 exacerbates early atherosclerosis in mice. *Arterioscler Thromb Vasc Biol* 1999;**19**:2364–7
28. Ridker PM, Rifai N, Stampfer MJ, Hennekens CH. Plasma concentration of interleukin-6 and the risk of future myocardial infarction among apparently healthy men. *Circulation* 2000;**101**:1767–72
29. Ridker PM, Hennekens CH, Roitman-Johnson B, Stampfer MJ, Allen J. Plasma concentration of soluble intercellular adhesion molecule 1 and risks of future myocardial infarction in apparently healthy men. *Lancet* 1998;**351**:88–92
30. Rus HG, Vlaicu R, Niculescu F. Interleukin-6 and interleukin-8 protein and gene expression in human arterial atherosclerotic wall. *Atherosclerosis* 1996;**127**:263–71
31. Chang JY. Methylmercury causes glial IL-6 release. *Neurosci Lett* 2007;**416**:217–20
32. Yamamoto M, Khan N, Muniroh M, Motomura E, Yanagisawa R, Matsuyama T, Vogel CF. Activation of interleukin-6 and -8 expressions by methylmercury in human U937 macrophages involves RelA and p50. *J Appl Toxicol* 2017;**37**:611–20
33. Boyle EM Jr., Kovacich JC, Canty TG Jr., Morgan EN, CE, Verrier ED, Pohlman TH. Inhibition of nuclear factor-kappa B nuclear localization reduces human E-selectin expression and the systemic inflammatory response. *Circulation* 1998;**98**:II282–8
34. Nallasamy P, Si H, Babu PV, Pan D, Fu Y, Brooke EA, Shah H, Zhen W, Zhu H, Liu D, Li Y, Jia Z. Sulforaphane reduces vascular inflammation in mice and prevents TNF-alpha-induced monocyte adhesion to primary endothelial cells through interfering with the NF-kappaB pathway. *J Nutr Biochem* 2014;**25**:824–33
35. Liu T, Zhang L, Joo D, Sun S-C. NF-κB signaling in inflammation. *Sig Transduct Target Ther* 2017;**2**:1–5
36. Cai H, Harrison DG. Endothelial dysfunction in cardiovascular diseases: the role of oxidant stress. *Circ Res* 2000;**87**:840–4
37. Kita T, Kume N, Minami M, Hayashida K, Murayama T, Sano H, Moriwaki H, Kataoka H, Nishi E, Horiuchi H, Arai H, Yokode M. Role of oxidized LDL in atherosclerosis. *Ann N Y Acad Sci* 2001;**947**:199–205; discussion 05–6
38. Li L, Dong H, Song E, Xu X, Liu L, Song Y. Nrf2/ARE pathway activation, HO-1 and NQO1 induction by polychlorinated biphenyl quinone is associated with reactive oxygen species and PI3K/AKT signaling. *Chem Biol Interact* 2014;**209**:56–67
39. Grotto D, de Castro MM, Barcelos GR, Garcia SC, Barbosa F, Jr. Low level and Sub-chronic exposure to methylmercury induces hypertension in rats: nitric oxide depletion and oxidative damage as possible mechanisms. *Arch Toxicol* 2009;**83**:653–62
40. Kaur P, Aschner M, Syversen T. Glutathione modulation influences methyl mercury induced neurotoxicity in primary cell cultures of neurons and astrocytes. *Neurotoxicology* 2006;**27**:492–500
41. Lemos NB, Angeli JK, Faria Tde O, Ribeiro Junior RF, Vassallo DV, Padilha AS, Stefanon I. Low mercury concentration produces vasoconstriction, decreases nitric oxide bioavailability and increases oxidative stress in rat conductance artery. *PLoS One* 2012;**7**:e49005
42. Yu M, Li H, Liu Q, Liu F, Tang L, Li C, Yuan Y, Zhan Y, Xu W, Li W, Chen H, Ge C, Wang J, Yang X. Nuclear factor p65 interacts with Keap1 to repress the Nrf2-ARE pathway. *Cell Signal* 2011;**23**:883–92
43. Wardyn JD, Ponsford AH, Sanderson CM. Dissecting molecular cross-talk between Nrf2 and NF-kappaB response pathways. *Biochem Soc Trans* 2015;**43**:621–6
44. Rushworth SA, Zaitseva L, Murray MY, Shah NM, Bowles KM, MacEwan DJ. The high Nrf2 expression in human acute myeloid leukemia is driven by NF-kappaB and underlies its chemo-resistance. *Blood* 2012;**120**:5188–98
45. Kensler TW, Wakabayashi N, Biswal S. Cell survival responses to environmental stresses via the Keap1-Nrf2-ARE pathway. *Annu Rev Pharmacol Toxicol* 2007;**47**:89–116
46. Hwang GW. Role of intracellular defense factors against methylmercury toxicity. *Biol Pharm Bull* 2012;**35**:1881–4
47. Stoneman VE, Bennett MR. Role of apoptosis in atherosclerosis and its therapeutic implications. *Clin Sci* 2004;**107**:343–54
48. Nicholson DW, Thornberry NA. Apoptosis. Life and death decisions. *Science* 2003;**299**:214–5
49. Thompson CB. Apoptosis in the pathogenesis and treatment of disease. *Science* 1995;**267**:1456–62
50. Jia Z, Misra HP. Exposure to mixtures of endosulfan and zineb induces apoptotic and necrotic cell death in SH-SY5Y neuroblastoma cells, in vitro. *J Appl Toxicol* 2007;**27**:434–46
51. Martinet W, Schrijvers DM, De Meyer GR. Necrotic cell death in atherosclerosis. *Basic Res Cardiol* 2011;**106**:749–60
52. Gu Z, Kaul M, Yan B, Kridel SJ, Cui J, Strongin A, Smith JW, Liddington RC, Lipton SA. S-nitrosylation of matrix metalloproteinases: signaling pathway to neuronal cell death. *Science* 2002;**297**:1186–90
53. Gupta M, Bansal JK, Khanna CM. Blood mercury in workers exposed to the preparation of mercury cadmium telluride layers on cadmium telluride base. *Ind Health* 1996;**34**:421–5
54. Harris HH, Pickering IJ, George GN. The chemical form of mercury in fish. *Science* 2003;**301**:1203
55. Clarkson TW, Magos L, Myers GJ. The toxicology of mercury–current exposures and clinical manifestations. *N Engl J Med* 2003;**349**:1731–7
56. Antunes Dos Santos A, Appel Hort M, Culbreth M, Lopez-Granero C, Farina M, Rocha JB, Aschner M. Methylmercury and brain development: a review of recent literature. *J Trace Elem Med Biol* 2016;**38**:99–107

(Received May 11, 2021, Accepted July 2, 2021)