ROLE OF REACTIVE OXYGEN SPECIES, MITOGEN-ACTIVATED PROTEIN KINASES AND SIGNALING CASCADE IN MERCURY IMMUNOTOXICITY

by

SANG HYUN KIM

(Under the Direction of Raghubir P. Sharma)

ABSTRACT

Mercury is a widespread metal in the environment and consequently there are large populations that are currently exposed to low levels of mercury as a result of ubiquitous environmental factors. Mercury is highly toxic and moderate levels of exposure to mercury can cause immune system alterations and decrease host resistance to viral infections in mice. Although mercury is broadly sulfhydryl reactive, surprisingly little is understood as to the specific cellular targets and biochemical pathways targeted by this metal. The objectives of the present studies were to define the role of immune effector functions in mercury-induced immunotoxicity and related signaling cascade.

Mercury interferes with lipopolysaccharide (LPS)-mediated immune response. We show here that mercury inhibited nitric oxide (NO) production and altered inflammatory cytokine expressions. This study indicates that mercury suppresses NO synthesis by inhibition of the nuclear factor κB pathway and modulates cytokine expression by p38 mitogen-activated protein kinase (MAPK) activation in J774A.1 macrophages.

Mercury generates reactive oxygen species (ROS) and lipid peroxidation, suggesting that the generated ROS is involved in mercury-induced cytotoxicity. Mercury decreased mitochondrial transmembrane potential and increased ROS generation, and consequent depletion of GSH and lipid peroxidation, which is the major cause of mercury-induced cytotoxicity. In addition, alterations of calcium homeostasis are also involved in mercury-induced cytotoxicity.

Alteration of ROS regulates MAPK, one of the most important members in control cell signaling. The results of these studies revealed that mercury-induced ROS regulates MAPKs and these MAPKs are important mediator of mercury immunotoxicity. Mercury altered T lymphocyte population in spleen and thymus. Mercury altered the expression of inflammatory cytokines, c-myc, and major histocompatibility complex II in liver, kidney, spleen, and thymus. Results indicated that decreases in T lymphocyte populations in immune organs and altered cytokine gene expression contribute to the immunosuppressive effects of mercury. Additionally, mercury augment subtoxic dose of LPS-mediated proinflammatory cytokine expression by altering GSH regulated p38 MAPK in liver.

Taken together, the results of these studies further strengthen the role of oxidative stress in mercury immunotoxicity. We have also identified a new mechanism by which mercury-induced ROS regulate MAPK-mediated cytokine expression.

INDEX WORDS: Mercury; Immnotoxicity; Reactive oxygen species; Mitogen-activated protein kinase, Tumor necrosis factor α

ROLE OF REACTIVE OXYGEN SPECIES, MITOGEN-ACTIVATED PROTEIN KINASES AND SIGNALING CASCADE IN MERCURY IMMUNOTOXICITY

by

SANG HYUN KIM

B.S., The Woosuk University, South Korea, 1995

M.S., The Woosuk University, South Korea, 1997

A Dissertation Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

DOCTOR OF PHILOSOPHY

ATHENS, GEORGIA

2003

ROLE OF REACTIVE OXYGEN SPECIES, MITOGEN-ACTIVATED PROTEIN KINASES AND SIGNALING CASCADE IN MERCURY IMMUNOTOXICITY

by

SANG HYUN KIM

Approved:

Major Professor: Raghubir P. Sharma

Committee: Corrie C. Brown
Julie A. Coffield
Anant V. Jain
Oliver (Wan-I) Li

Electronic Version Approved:

Maureen Grasso
Dean of the Graduate School
The University of Georgia
May 2003

DEDICATION

I dedicate this dissertation to my family, my wife Suck-Hee and my son Brian (Young-Whan). Their unconditional love, support and understanding were integral to the completion of my studies. I also dedicate this dissertation to my father, who showed the true love and taught the way of living as good person. Dad, your soul still lives in my heart.

ACKNOWLEDGEMENTS

I would like to extend my sincere gratitude to my major professor, Dr. Raghubir P. Sharma. He has been an inspiration for me and his excellent guidance is reflected in the accomplishments that I have made at the University of Georgia. His commitment to success has kept me focused and will continue to inspire me in my future endeavors. I am truly honored to have worked under his guidance.

I also extend my thanks to my committee members Dr. Corrie C. Brown, Dr. Julie A. Coffield, Dr. Anant V. Jain and Dr. Oliver (Wan-I) Li. I am extremely grateful for their invaluable advice and confidence.

The cooperative efforts of the faculty and staff of the Department of Physiology and Pharmacology, and Program of Toxicology were invaluable in the completion of this dissertation. Heartfelt appreciation goes to all my friends and my colleagues, Victor Johnson, Quanren He, Jiyoung Kim, Neera Gopee, Marcin Osuchowski and Neelesh Sharma. To Lynn McCoy and Linda Black, their assistance and kind consideration will be never forgotten.

A special heart-felt thank goes out to Dr. Tae-Yong Shin of the Woosuk University and Dr. Hyung-Min Kim of Wonkwang University. Dr. Shin is like a father to me. He and his family helped me enormously at critical stage in my developing career and for this I am eternally grateful. Dr. Kim has been an inspiration for me. From my first step as a graduate student, he encouraged me and showed the way of scientist and researcher.

My loving mother Kab-Soon, brother Jin-Woo and sisters Hyang-Kyu, Jin-Kyoung and Sun-Hee, though across the miles, were never far from my heart and mind. They

instilled in me a passion for knowledge and assured me that anything was possible. Without their love and support, I would not have the strength to continue.

Last but certainly not least, I would like to thank to my family, my wife Suck-Hee and my son Brian (Young-Whan) for the never-ending love and supports. I needed this from you and could not have achieved this prestigious goal otherwise. She always has a confidence for me and cheered me up when I was frustrated. She sacrificed herself to assist me and raised our son as a sweet child. I love you.

TABLE OF CONTNETS

LIST OF TABLES

Page

LIST OF FIGURES

Page

CHAPTER 1

INTRODUCTION AND SIGNIFICANCE

Mercury is widespread in the environment and chronic exposure to low levels of mercury is common due to contamination of food and drinking water supplies. Mercury is highly toxic and moderate levels of exposure to mercury can cause neurotoxic manifestations, immune system alterations, and decrease host resistance to viral infections in mice (Christensen et al., 1996;Gerstner and Huff, 1977;Wild et al., 1997). Prolonged exposure to subclinical concentrations of mercury also increases susceptibility to a host of viruses (Koller, 1975). Mercury exposure has also been shown to increase serum IgG, and IgE levels in mice after subcutaneous injection (Pietsch et al., 1989;Prouvost-Danon et al., 1981) and to induce auto-antibody formation in the form of anti-glomerular basement membrane in rats (Sapin et al., 1977).

Recently, there has been growing concern regarding the human health effects of low level of environmental mercury contamination. Although mercury is broadly sulfhydryl reactive, surprisingly little is understood as to the specific cellular targets and/or biochemical pathways targeted by the metal. Clinical studies on industrial workers exposed to mercury levels within World Health Organization guidelines have also shown immune system abnormalities in the absence of gross neurological dysfunction (Perlingeiro and Queiroz, 1994). Thus, the immune system may be an important target for mercury intoxication as well as a particularly revealing sentinel organ system in which to investigate the biochemistry of low level mercury exposure.

Oxidative stress evokes many intracellular events including regulation of cytokines and apoptosis (Ueda et al., 2002). Reactive oxygen species is a known potent activator of nuclear factor κB, a transcription factor controlling the expression of many immune related genes. Mitogen-activated protein kinases are involved in all aspects of immune responses,

from the initiation phase of innate immunity, to activation of adaptive immunity, and to cell death when immune function is complete (Dong et al., 2002). The possibility for interaction between mercury-induced changes in oxidative stress and the production of cytokines in leukocytes requires investigation.

Studies have demonstrated the impact of mercury on both oxidative stress and cell death but information is lacking on the intermediate signaling cascade. Given the continued use of mercury in medical and industrial processes, a better understanding of the mechanisms of mercury immunotoxicity will dramatically improve human health risk assessment.

The objective of the research comprising this dissertation was to test the hypothesis **that the immune toxicity of low level of mercury is defined by interplay between oxidative stress induced by mercury and the activation of mitogen-activated protein kinases and inflammatory cytokines in leukocytes.** To fulfill this objective the following specific aims were pursued.

1) determine the role of mercury on nitric oxide production, proinflammatory cytokines, and transcriptional signaling in murine macrophages.

2) investigate the signaling cascade of calcium, oxidative stress, mitogen-activated protein kinases, and caspase on mercury induced cytotoxicity in murine macrophages.

3) investigate the impact of mercury-induced cell death signaling in T and B lymphocytes

4) examine the role of mercury on lymphocyte phenotype population and apoptosis signaling in vivo.

5) determine the impact of mercury on endotoxin-induced inflammatory response and cell signaling pathways in mice.

Reference

Christensen MM, Ellermann-Eriksen S, Rungby J, and Mogensen SC (1996) Influence of mercuric chloride on resistance to generalized infection with herpes simplex virus type 2 in mice. *Toxicology* **114**:57-66.

Dong C, Davis RJ, and Flavell RA (2002) MAP kinases in the immune response. *Annu.Rev.Immunol.* **20**:55-72.

Gerstner HB and Huff JE (1977) Clinical toxicology of mercury. *J.Toxicol. Environ.Health* **2**:491-526.

Koller LD (1975) Methylmercury: effect on oncogenic and nononcogenic viruses in mice. *Am.J.Vet.Res.* **36**:1501-1504.

Perlingeiro RC and Queiroz ML (1994) Polymorphonuclear phagocytosis and killing in workers exposed to inorganic mercury. *Int.J.Immunopharmacol.* **16**:1011-1017.

Pietsch P, Vohr HW, Degitz K, and Gleichmann E (1989) Immunological alterations inducible by mercury compounds. II. HgCl2 and gold sodium thiomalate enhance serum IgE and IgG concentrations in susceptible mouse strains. *Int.Arch.Allergy Appl.Immunol.* **90**:47-53.

Prouvost-Danon A, Abadie A, Sapin C, Bazin H, and Druet P (1981) Induction of IgE synthesis and potentiation of anti-ovalbumin IgE antibody response by HgCl2 in the rat. *J.Immunol.* **126**:699-792.

Sapin C, Druet E, and Druet P (1977) Induction of anti-glomerular basement membrane antibodies in the Brown-Norway rat by mercuric chloride. *Clin.Exp.Immunol.* **28**:173-179.

Ueda S, Masutani H, Nakamura H, Tanaka T, Ueno M, and Yodoi J (2002) Redox control of cell death. *Antioxid.Redox.Signal.* **4**:405-414.

Wild LG, Ortega HG, Lopez M, and Salvaggio JE (1997) Immune system alteration in the rat after indirect exposure to methyl mercury chloride or methyl mercury sulfide. *Environ.Res.* **74**:34-42.

CHAPTER 2

LITERATURE REVIEW

Mercury, the metal

Mercury (Hg) is widespread in the environment and consequently there are large populations that are currently exposed to low levels of mercury as a result of ubiquitous environmental factors. The major source of mercury is the natural degassing of the earth's crust, including rivers and the ocean, and this source is estimated to produce the order of 2700 to 6000 tons per year. Among metals, mercury is unique in that it is found in the environment in several physical and chemical forms and is one of the best-illustrated metal that its diversity of effects caused by different chemical species (WHO, 1990;WHO, 1991). On the basis of chemical forms, there are three types of mercury: elemental, inorganic, and organic compounds, each having characteristic toxicokinetics and health effects. At room temperature, elemental mercury exists as a liquid. As a result of its high vapor pressure, this form of mercury is released into the environment as mercury vapor. Mercury also exists as a cation with an oxidation state of 1+ (mercurous) or 2+ (mercuric). In occupational and environmental settings, the most common cationic form of mercury encountered is the mercuric form, which may have a valence of 1+ or 2+, depending on whether the mercuric ion is covalently bonded to a carbon atom of an organic side group, such as an alkyl group. With respect to organic forms of mercury, methylmercury is the most frequently encountered organic mercuric compound in the environment. It forms mainly as the result of methylation of inorganic (mercuric) forms of mercury by microorganisms in soil and water. Due to industrialization and changes in the environment during the twentieth century, humans and animals are exposed to numerous chemical forms of mercury, including elemental mercury vapor (Hg^0), inorganic mercurous (Hg^+) and mercuric (Hg^{2+}) compounds, and organic mercuric ($R\text{-}Hg^+$ or R-Hg-R; where R represents any organic ligand) compounds (Fitzgerald

and Clarkson, 1991). Inasmuch as mercury is ubiquitous in the environment, it is nearly impossible for most humans to avoid exposure to some form or forms of mercury on a regular basis. All forms of mercury cause toxic effects in a number of tissues and organs, depending on the chemical form of mercury, the level of exposure, the duration of exposure, and the route of exposure.

Use and exposure

Human activities significantly redistribute mercury and release it into the environment. They allow mercury that was formerly unavailable to the biosphere to be mobilized and carried to new areas via air and water (Klaassen CD, 1996). In spite of the known toxicity of mercury, this metal is still used in many places. Mercury is used in vaccine as thimerosal, dental amalgam, thermometer, cleaning solutions, batteries, pesticide, insecticide, and chemical industries (EPA, 1999).

The mercury is used in medicine as thimerosal, phenylmercuric acetate, phenylmercuric nitrate, mercuric acetate, mercuric nitrate, merbromin, and mercuric oxide yellow form. Thimerosal is the mercury-based vaccine preservative (Clements et al., 2001). In the 1990's the number of common childhood vaccines using thimerosal greatly increased. At the same time the use of thimerosal increased, there has been an increase in the number of reported cases of autism. Autism is a neurological disorder that usually manifests itself in the first two years of life (Bernard et al., 2001). It is characterized by sensory, neurological and behavioral problems. Thimerosal is no longer being used in new vaccines but critics fear old stores of vaccines may still harbor the preservative. Thimerosal has toxic effects on vital mammalian enzymes and enzyme systems. It inhibits sodium channels in sensory neurons

(Song et al., 2000), inhibits calcium uptake in skeletal muscle sarcoplasmic reticulum and rat cerebellar microsomes by inhibiting the Ca^{2+}-ATPase (Sayers et al., 1993), and interacts with the Ca^{2+} release channel ryanodine receptor from skeletal muscle sarcoplasmic reticulum (Abramson et al., 1995). Thimerosal induces chromosomal aberrations in human lymphocytes (Lynch and Parry, 1993;Marrazzini et al., 1994;Sbrana et al., 1993). Thimerosal disrupts calcium homeostasis by histamine-induced Ca^{2+} release in intact HeLa cells (Montero et al., 2001), by sensitizing inositol 1,4,5-trisphosphate receptors in cerebellar microsomes (Mezna and Michelangeli, 1997).

Humans can be exposed to environmental mercury via all three routes of exposure: inhalation, ingestion, and dermal. The most likely routes of exposure are inhalation of inorganic mercury vapor after a spill or during a manufacturing process, or ingestion of methylmercury from contaminated fish. The fetus of a mother who eats contaminated fish can be exposed to methylmercury via the mother's blood, and an infant can be exposed by ingestion of breast milk. The organic forms of mercury are primarily neurotoxins. Therefore exposure can damage the brain and nervous system. The developing brain of a fetus or child is especially vulnerable to organic mercury exposure. Inorganic forms of mercury primarily affect the kidney, but are also neurotoxins. Other organs and systems of the body can be harmed by exposure to mercury.

One of the major exposure routes of mercury is dental amalgam. As early as the 7th century, the Chinese used a "silver paste" containing mercury to fill decayed teeth. By the early 1800's, the use of a mercury/silver paste as a tooth filling material was being popularized in England and France. Some dentists expressed concerns that the Hg/silver mixture (amalgam) expanded after setting, frequently fracturing the tooth or protruding

above the cavity preparation, and thereby prevented proper jaw closure. Other dentists were concerned about mercurial poisoning; because mercury exposure resulted in many overt side effects, including dementia and loss of motor coordination. However, for economic reasons dental amalgam is still used and estimates of annual mercury usage by US dentists range from approximately 100,000 kg in the 1970's to 70,000 kg today. Mercury fillings continue to remain the material preferred by 92% of US dentists for restoring posterior teeth. More than 100 million mercury fillings are placed each year in the US (Lorscheider et al., 1995a;Lorscheider et al., 1995b).

Another major exposure routes of mercury are fish. Mercury falls from the air and can get into surface water, accumulating in streams and oceans. Bacteria in the water cause chemical changes that transform mercury into methylmercury that can be toxic. Fish absorb methylmercury from water as they feed on aquatic organisms. Nearly all fish contain trace amounts of methylmercury, which are not harmful to humans. However, long-lived, larger fish such as Shark, Swordfish, King mackerel, Tilefish that feed on other fish accumulate the highest levels of methylmercury and pose the greatest risk to people who eat them regularly (FDA, 2001). Mercury caused one of the most tragedies by industrial water pollution in Japan. Methylmercury poisoning caused Minamata disease, which was caused by eating contaminated fish, which has killed more than 100 people and paralyzed several thousand people around Minamata Bay, Japan in 1956 (Eto, 1997;Jouany, 1998). The cause of Minamata disease was confirmed that sediments (containing more than 600 ppm of Hg) or fish (at least 20 ppm) at the bay. The Minamata disease has provided many lessons, which have shaped the scientific field in environmental research.

Mercury toxicokinetics

The toxicity of various forms of mercury is related to cationic mercury per se whereas solubility, biotransformation, and tissue distribution are influenced by the valence state and an anionic component (Berlin M, 1986). Metallic or elemental mercury evaporates to mercury vapor at ambient air temperatures, and causes most human exposure by inhalation. Mercury vapor readily diffuses across the alveolar membrane and being lipid soluble it has affinity for the red blood cells and the central nervous system. Gastrointestinal absorption of inorganic mercury from food is less than 15 percent in mice and about 7 percent in human, whereas absorption of methyl mercury is of the order of 90 to 95 percent (Berlin M, 1986). Injection of mercury chloride produces necrosis in kidney (Gritzka and Trump, 1968). Cellular changes include fragmentation and disruption of the plasma membrane, vesiculation and disruption of the endoplasmic reticulum and other cytoplasmic membranes, dissociation of polysomes and loss of ribosomes, mitochondrial swelling with appearance of amorphous intramatrical deposits, and condensation of nuclear chromatin. Although exposure to a high dose of mercuric chloride is directly toxic, chronic low dose exposure to mercury induce an immunological disease. This form of chronic mercury injury to human is clinically the most common form of mercury-induced toxicity.

Human toxicity of inorganic mercury

Acute systemic poisoning appears seldom to have caused disturbances of the eye, but in 3 young people bilateral mydriasis, hyperemia of optic nerveheads and retinal venous distension with rapid decrease in visual activity have been reported (Grant W.M., 1986). Acute systemic mercurialism may be fatal within a few minutes, but death by uremic

poisoning is usually delayed 5-12 days. The symptoms after ingestion of inorganic mercury are: 1) Burning pain, sense of constriction, and ashen discoloration of the mucous membrane in mouth and pharynx, occurring immediately after the ingestion of corrosive mercury. 2) Within a few minutes intense epigastric pain, followed by diffused abdominal pain and associated with almost continuous vomiting of mucoid material, which frequently contains blood and shreds of mucous membrane. 3) Severe purging, with liquid, bloody feces and considerable tenesmus. 4) Metallic taste, excessive salivation and thirst. 5) A rapid, weak pulse, shallow breathing, pallor, prostration, collapse, and death (Gosselin, 1984).

Inorganic, ionic mercury can produce severe types of acute toxicity. Precipitation of mucous membrane proteins by mercuric salts results in an ashen-gray appearance of the mucosa of the mouth, pharynx and intestine and also causes intense pain, which may be accompanied by vomiting. Systemic toxicity may begin within a few hours after exposure to mercury and last for days. A strong metallic taste is followed by stomatitis with gingival irritation, foul breath and loosening of the teeth. The most serious and, unfortunately, the most frequently encountered systemic effect of inorganic mercury is renal toxicity. Renal tubular necrosis occurs after acute exposure, leading to oliguria or anuria (Gilman, 1985).

Mercuric chloride directly affects the human placental syncytiotrophoblast microvillous membrane. Mercuric chloride alters the facilitated diffusion of alpha-aminoisobutyric acid into vesicles of this membrane in μM concentration. Mercuric chloride abolishes temporal kinetics of alpha-aminoisobutyric acid transport, inducing an initial increase in alpha-aminoisobutyric acid transport (Goodman et al., 1983). Segregational errors of chromosomes were studied in human lymphocytes fibroblasts exposed to mercuric chloride. The cells were exposed to mercuric chloride only during a limited period of the

pre-DNA synthetic stage of the cell cycle or from that stage up to mitosis. In the lymphocytes a clear increase of C-mitotic figures for mercury chloride was observed. Segregational errors were, however, much more important after the shorter exposure period (Verschaeve et al., 1984).

Mercury, host defense and NO

Some bacteria have developed a high level of resistance to antibiotics, and bacterial resistance to environmental toxins suggests there is a correlation between mercury pollution and the organisms' increasing resistance to antibiotics (Roberts, 1998a;Roberts, 1998b). A common mercury resistance gene that produces a mercury detoxifying enzyme in many of the bacteria, which suggest that the organisms' resistance to antibiotics is most likely attributable to an environmental influence, possibly the presence of mercury contamination in the soil and the intermittent presence of mercury. Bacteria naturally develop resistance to toxins in their environment. They adapt, and survive, by acquiring genes that allow them to resist toxins. The mercury-resistant genes and antibiotic-resistant genes are found together on mobile genetic elements. The reservoir of resistant bacteria may contribute another threat to the future efficacy of antibiotics needed to treat infections and illnesses in humans (Greated et al., 2002;Sabry et al., 1997).

LPS is a toxic component of bacteria and leads to the secretion of variety of immunomodulatory molecules such as NO and pro-inflammatory cytokines in macrophages and other immune cells. NO is readily produced by LPS, and manifests its biological actions via a wide range of chemical reactions. NO is a small, relatively stable, free radical gas that swiftly diffuses into the cells and cell membranes where it reacts with molecular targets. The

precise reactions, which are realized in any biological setting, depend on the concentration of NO achieved and often on subtle variations in the composition of the intra and extracellular milieu. NO is produced by NO synthase that catalyses L-arginine to NO. Three distinct forms of NOS are identified: a neuronal form (nNOS) in the brain, an endothelial form (eNOS) in vascular endothelium and an inducible form (iNOS) in macrophages (Bredt et al., 1991;Xie et al., 1992). Both nNOS and eNOS are constitutively expressed in cells; however, iNOS is regulated by transcriptional activation of the gene.

Mercury decreases host resistance to several viral infections, and affects one of the important immunologic parameter, NO, in mice (Christensen, Ellermann-Eriksen, Rungby, and Mogensen, 1996;Gregory and Wing, 2002;Silbergeld et al., 2000). Low and non-cytotoxic levels of mercury decrease NO in murine splenic macrophages (Tian and Lawrence, 1996), and inhibit NO and iNOS expression in pancreatic islet β cells (Eckhardt et al., 1999).

Mercury and ROS

Reactive oxygen species (ROS), such as H_2O_2, superoxide (O_2^-) and the hydroxyl radical (OH·), are generated in cells by several pathways. Cellular energy metabolism is based on the production of ATP through the electron-transport reaction in which O_2 accepts electrons and H^+ and then is eventually reduced to water. In addition to this standard reaction, leakage of a single electron being transferred to O_2 is possible, thus resulting in the production of O_2^-. Electron transport through the mitochondrial respiratory chain is extraordinarily efficient, and normally the vast majority of O_2 is consumed. However, 1-2% of electrons are leaked to generate O_2^- in reactions mediated by coenzyme Q and ubiquinone

and its complexes. Thus, mitochondria are believed to be a major site of ROS production (Boveris and Chance, 1973). Cells have antioxidant systems to protect themselves against dangerous ROS. Although ROS have been considered only to damage cells, accumulating evidence shows that oxidative stress evokes many intraculluare events, such as proliferation, gene expression, cell-cycle arrest, and apoptosis (Nakamura et al., 1997).

The toxicity of mercury and its ability to react with and deplete free sulfhydryl groups are well known. The decrease in free sulfhydryl groups lead to the formation of an oxidative stress, resulting in tissue-damaging effects. The administration of mercury to rats results in nephrotoxic acute renal failure (Gstraunthaler et al., 1983). Mercury causes a depletion of glutathione in renal tubules, and also a reduction in the activities of SOD, catalase, and glutathione peroxidase, enzymes responsible for the protection of cells against the peroxidative action of superoxide anion and hydroperoxides. Thus, nephrotoxicity is mediated by mercury-induced alterations in membrane integrity via the formation of ROS and the perturbation of antioxidant defense mechanisms.

ROS is known to mediate mercury-induced cell death in many cell types. Mercury inhibits human monocyte function by formation of ROS (InSug et al., 1997). Mercury potentiates ROS generation and decreases glutathione accompanied by decreases of mitochondrial transmembrane potential. These mercury-induced low thiol reserve and ROS formation activate death-signaling pathways.

Administration of mercury to rats results in increased hydrogen peroxide formation, glutathione depletion, and lipid peroxidation in kidney mitochondria (Lund et al., 1993). Moreover mercury induces alterations in mitochondrial calcium homeostasis. The mercury-

induced hydrogen peroxide formation occurs principally at the ubiquinone-cytochrome b region of the mitochondrial respiratory chain.

Another important role of mercury-induced ROS formation for cell death is the activation of caspase cascade by ROS. Mercury-induced ROS activates caspase which is the key effector responsible for many of the morphological and biochemical changes of apoptosis (Shenker et al., 2002). Additionally, mercury-induced ROS increases the level of cytochrome c, the cleavage of poly (ADP-ribose) polymerase, and decreases the anti-apoptotic protein bcl-2 (Shenker et al., 2000).

Mercury and MAPKs

The mitogen-activated protein kinase (MAPK) is one of the most ancient and evolutionarily conserved signaling pathways, which is also important for many processes in immune responses (Dong, Davis, and Flavell, 2002). There are three major groups of MAPKs in mammalian cells – the extracellular signal-regulated protein kinase (ERK) (Schaeffer and Weber, 1999), the c-Jun N-terminal kinase (JNK) (Constant et al., 2000), and the p38 MAPK (Chang and Karin, 2001). These MAPKs are activated by dual phosphorylation at the tripeptide motif Thr-X-Tyr. The sequence of this tripeptide motif is different in each group of MAPKs. The dual phosphorylation of Thr and Tyr is mediated by a conserved protein kinase cascade. The ERK is activated by the MAPK kinases (MKK) MKK1 and MKK2; the JNK is activated by MKK 4 and MKK 7; the p38 MAPK is activated by MKK 3, MKK4, and MKK6. These MAPKs are activated, in turn, by several different MAPK kinase kinases (MKKK). The ERK pathway can be activated by Ras via the Raf

group of MKKK. In contrast, the p38 and JNK MAPKs are activated by Rho family GTPases.

ERK is generally associated with proliferation and growth factors. In contrast JNK and p38 MAPK are induced by stress responses and cytokines, and can mediate differentiation and cell death (Bonni et al., 1999;Seger and Krebs, 1995).

p38 MAPK activates many protein kinases and is thought to play an important role in the regulation of pro-inflammatory molecules on cellular responses, especially, TNFα, IL-1β and IL-6 (Baldassare et al., 1999;Beyaert et al., 1996). p38 exert their regulation of TNFα translation through AU-rich elements in the 3' untranslated region of TNFα transcripts (Kontoyiannis et al., 1999). On the contrary, p38 and JNK MAPKs are preferentially activated by inflammatory cytokines such as TNFα and IL-1, and they have critical functions in cellular responses to these cytokines. JNK activity can be strongly induced in multiple cell types by LPS or inflammatory cytokines such as TNFα and IL-1 (Chang and Karin, 2001).

Mercury-induced cytotoxicity is known to be mediated by MAPK signaling. Mercury inhibits Ras activation during T cell receptor-mediated signal transduction (Mattingly et al., 2001). Mercury activates JNK in LLC-PK_1 cells (Matsuoka et al., 2000). Low levels of mercury dose-dependently induced AP-1, c-jun gene expression but did not change ATF2 and ELK-1, transcription factors involved in both JNK and p38 pathways of MAPK signal transduction (Turney et al., 1999). Mercury-induced toxicity and the enhancement of toxicity by sulfur amino acid deprivation involved the JNK pathway, not p38 pathway (Son et al., 2001).

Mercury and NF-κB

Nuclear factor kappa B (NF-κB) is a mammlian transcription factor known to mediate the inducible expression of a variety of genes, particularly those involved in immune, inflammatory and acute phase responses (Sha et al., 1995). In most cell types, NF-κB exists as a heterodimer composed of 50 and 65 kDa proteins called p50 and p65, respectively. Both the p50 and p65 proteins contain a redox sensitive cysteinyl group that affects DNA binding. Current evidence indicates that the p50 subunit plays the primary role in DNA binding, whereas p65 participates principally in the transcriptional activity of the complex (Baeuerle and Baltimore, 1989). Dimer formation is also required for DNA binding of NF-κB. NF-κB mediates the inducible expression of variety of genes involved in immune, inflammatory and acute phase responses including iNOS, TNFα, IL-1 and IL-6 (Sha, Liou, Tuomanen, and Baltimore, 1995;Xie et al., 1994). In resting state, NF-κB proteins are sequestered in the cytosol through interactions with an inhibitory protein IκB. Many stimuli induce NF-κB activation including TNFα, IL-1, LPS and oxidants. These signals induce phosphorylation and degradation of inhibitory κB (IκB) protein via the ubiquitination pathway, resulting in free NF-κB that can enter the nucleus and induce gene expression (Ghosh et al., 1998;May and Ghosh, 1998). Sufficient experimental evidence exists indicating that NF-κB is a major transcription factor of iNOS gene expression (Xie, Kashiwabara, and Nathan, 1994).

It has been reported that mercury inhibits NF-κB-DNA binding in A549 cells (Shumilla et al., 1998), cell-free systems and rat kidney epithelial cells (Dieguez-Acuna and Woods, 2000;Dieguez-Acuna et al., 2001), however, the direct evidence linking mercury-induced inhibition of NF-κB-DNA binding to altered gene expression is lacking.

Strong evidence exist that the modulation of iNOS is dependent on NF-κB activation in LPS-stimulated macrophages (Xie, Kashiwabara, and Nathan, 1994). In the study described in this dissertation, we found that low doses of mercury inhibited NO and iNOS expression and blocked nuclear translocation of p65 NF-κB (Kim et al., 2002). Relatively high doses of mercury (> 0.3 mM) have been shown to inhibit NF-κB-DNA binding in TNFα-stimulated A549 cells (Shumilla, Wetterhahn, and Barchowsky, 1998). Relatively low dose of mercury inhibited NF-κB-DNA binding in cell free system and blocked LPS-induced NF-κB activation via competitive mercaptide formation in normal rat epithelial cells (Dieguez-Acuna and Woods, 2000;Dieguez-Acuna, Ellis, Kushleika, and Woods, 2001). The authors explained that mercury reduces the nuclear translocation of p65 NF-κB and inhibited IκBα phosphorylation and degradation in response to LPS. Ellis et al. (2001) recently reported that low doses of mercury (2-4 μM) promoted NO formation and enhanced LPS+IFNγ-induced iNOS mRNA and iNOS formation. In contrast, mercury did not promote NF-κB activation and attenuated LPS-induced expression of the κB-driven luciferase reporter gene. It is likely that differences in cell system, dose and exposure conditions are responsible for these different results.

Mercury and calcium

It is well established that an increase in Ca^{2+} induced by a pertubation of Ca^{2+} homeostasis, can lead to cell damage through several down stream reactions, such as ROS formation (Lipton and Nicotera, 1998). Mercury alters calcium homeostasis (Tan et al., 1993). The role of calcium in the activation of hydrolytic enzymes including protease, endonucleases, and phopholipases is well known (Farber, 1990;Nicotera et al., 1990;Reed,

1990). Mercury-induced cytotoxicity is mediated by increase of intracellular Ca^{2+} (Gasso et al., 2001;Toimela and Tahti, 2001;Close et al., 1999;InSug, Datar, Koch, Shapiro, and Shenker, 1997;Shenker et al., 1998). The rise of intracellular Ca^{2+} is due to an alteration both in intracellular Ca^{2+} stores and in Ca^{2+} influx across plasma membrane. Several reports showed that blocking of Ca^{2+} channel, Na^{+}/Ca^{2+} exchanger, and inhibition of endoplasmic reticulum intracellular Ca^{2+}-ATPase prevented mercury-induced cytotoxicity in neuronal cells (Gasso, Cristofol, Selema, Rosa, Rodriguez-Farre, and Sanfeliu, 2001;Marty and Atchison, 1998;Sakamoto et al., 1996).

Treatment with antioxidants selenium or vitamin E and the Ca^{2+} channel blocker protects against mercury neurotoxicity in rats (Sakamoto, Ikegami, and Nakano, 1996). Thus, alterations both in redox equilibrium and in Ca^{2+} homeostasis are involved in mercury toxicity.

One of the most pronounced characteristics of relation of mercury and Ca^{2+} is the effect of the thimerosal (mercury involve preservative) on the level of intracellular free calcium. The effect of thimerosal on Ca^{2+} has been reported for a variety of different cell types, such as smooth muscle cells, endothelial cells, neutrophils, and lymphocytes. The first effect of thimerosal is a release of calcium from intracelluar stores. As a consequence a calcium-induced calcium-influx of extracellular calcium occurs. The magnitude of the second process differs for different cell types. By using single cells, the thimerosal-induced increase of calcium could be demonstrated as cytosolic Ca^{2+} spikes; these were most prominent in the presence of extracellular calcium; in its absence higher thimerosal concentrations were needed to detect spikes (Thorn et al., 1992). In the absence of extracellular calcium there was only a slight increase of Ca^{2+}, representing the release of

calcium from intracellular stores. In the presence of extracellular calcium a much stronger response was observed, which is indicative of a strong calcium influx following the calcium release from stores. The release of calcium by thimerosal was dependent on the presence of mercury in the molecule: thiosalicylic acid had no effect. When thimerosal was preincubated with DTT, no effect on intracellular calcium was observed, indicating that the effect was associated with the sulfhydryl reactive properties of thimerosal.

Mercury and cytokines

Cytokines are very important regulator on immune system. Mercury induces a profound activation of the immune system marked by changes in the production of various cytokines. The modulatory effects of mercury on cytokine expressions are revealed in many cell types. Mercury increases IL-4 and IFNγ mRNA in rat spleen cells (Gillespie et al., 1996), as well as IL-12 mRNA levels in spleen and lymph nodes (Mathieson and Gillespie, 1996). Mercury increases IL-4 gene expression in T cell hybridomas (Badou et al., 1997), IL-4 and IFNγ in rat spleen cells and T cells (Prigent et al., 1995), IL-2 and IFNγ but not IL-4 in mouse spleen cells (Hu et al., 1997), increases IL-4 but decreases IFNγ in spleen cells of non-obese diabetic mice (Brenden et al., 2001), decreases IL-1 in human T lymphocytes (Shenker et al., 1992) and increases IL-2, IL-4, TNFα and IFNγ in susceptible strain of mice (Johansson et al., 1997).

Mercury can have mitogen like effects on cells and is a strong activator of the immune system (Prigent, Saoudi, Pannetier, Graber, Bonnefoy, Druet, and Hirsch, 1995). Mercury induces IL-4 in vivo and in vitro, and this IL-4 induction is mediated by protein kinase C-dependent calcium influxes. Mercury activates PKC directly and upregulates IL-4

without de novo protein synthesis (Badou, Savignac, Moreau, Leclerc, Pasquier, Druet, and Pelletier, 1997).

TNFα is a member of the TNF superfamily whose constituents are inducers of apoptosis, proliferation, NF-κB, and MAPKs (Garg and Aggarwal, 2002). Kim et al., (2002) reported that mercury increased TNFα mRNA by regulating p38 MAPK in macrophages.

Mercury and apoptosis

Studies on the effects of mercury on the apoptotic cell death are rare. Apoptosis is one of the characteristic death modes that is different from conventional necrosis. Apoptosis is initiated by Fas and TNFα family (Itoh et al., 1991), or caspases (Wilson, 1998). Caspases, a family of aspartate-specific cysteine proteases, are important in the implementation of apoptosis (Thornberry and Lazebnik, 1998). The evidence for caspase activation is downstream of ROS-induced p38 MAPK activation (Zhuang et al., 2000).

Mercury is known to induce apoptosis in human T-cells, monocytes (Shenker et al., 1997;Shenker, Guo, and Shapiro, 1998). Mercury induces apoptosis by phosphatidylserine translocation to the outer leaflet of the plasma membrane, decreases mitochondrial transmembrane potential, generates ROS, and reduced cell thiols. Mercury-induced apoptosis pathway is different depending on mercury species (Shenker, Pankoski, Zekavat, and Shapiro, 2002).

Reference

Abramson JJ, Zable AC, Favero TG, and Salama G (1995) Thimerosal interacts with the Ca(2+) release channel ryanodine receptor from skeletal muscle sarcoplasmic reticulum. *J.Biol.Chem.* **270**:29644-29647.

Badou A, Savignac M, Moreau M, Leclerc C, Pasquier R, Druet P, and Pelletier L (1997) $HgCl_2$-induced interleukin-4 gene expression in T cells involves a protein kinase C-dependent calcium influx through L-type calcium channels. *J.Biol.Chem.* **272**:32411-32418.

Baeuerle PA and Baltimore D (1989) A 65-kappaD subunit of active NF-kappaB is required for inhibition of NF-kappaB by I kappaB. *Genes Dev.* **3**:1689-1698.

Baldassare JJ, Bi Y, and Bellone CJ (1999) The role of p38 mitogen-activated protein kinase in IL-1 beta transcription. *J.Immunol.* **162**:5367-5373.

Berlin M (1986) Mercury, in Friberg L, Nordberg GF, Norman C. Handbook on the Toxicology of Metals. 2d ed. Specific Metals. Amsterdam Elsevier. 2:386-445.

Bernard S, Enayati A, Redwood L, Roger H, and Binstock T (2001) Autism: a novel form of mercury poisoning. *Med.Hypotheses* **56**:462-471.

Beyaert R, Cuenda A, Vanden Berghe W, Plaisance S, Lee JC, Haegeman G, Cohen P, and Fiers W (1996) The p38/RK mitogen-activated protein kinase pathway regulates interleukin-6 synthesis response to tumor necrosis factor. *EMBO J.* **15**:1914-1923.

Bonni A, Brunet A, West AE, Datta SR, Takasu MA, and Greenberg ME (1999) Cell survival promoted by the Ras-MAPK signaling pathway by transcription-dependent and -independent mechanisms. *Science* **286**:1358-1362.

Boveris A and Chance B (1973) The mitochondrial generation of hydrogen peroxide. General properties and effect of hyperbaric oxygen. *Biochem.J.* **134**:707-716.

Bredt DS, Hwang PM, Glatt CE, Lowenstein C, Reed RR, and Snyder SH (1991) Cloned and expressed nitric oxide synthase structurally resembles cytochrome P-450 reductase. *Nature* **351**:714-718.

Brenden N, Rabbani H, and Abedi-Valugerdi M (2001) Analysis of mercury-induced immune activation in nonobese diabetic (NOD) mice. *Clin.Exp.Immunol.* **125**:202-210.

Chang L and Karin M (2001) Mammalian MAP kinase signalling cascades. *Nature* **410**:37-40.

Christensen MM, Ellermann-Eriksen S, Rungby J, and Mogensen SC (1996) Influence of mercuric chloride on resistance to generalized infection with herpes simplex virus type 2 in mice. *Toxicology* **114**:57-66.

Clements CJ, Ball LK, Ball R, and Pratt RD (2001) Thiomersal in vaccines: is removal warranted? *Drug Saf* **24**:567-574.

Close AH, Guo TL, and Shenker BJ (1999) Activated human T lymphocytes exhibit reduced susceptibility to methylmercury chloride-induced apoptosis. *Toxicol.Sci.* **49**:68-77.

Constant SL, Dong C, Yang DD, Wysk M, Davis RJ, and Flavell RA (2000) JNK1 is required for T cell-mediated immunity against Leishmania major infection. *J.Immunol.* **165**:2671-2676.

Dieguez-Acuna FJ, Ellis ME, Kushleika J, and Woods JS (2001) Mercuric ion attenuates nuclear factor-kappaB activation and DNA binding in normal rat kidney epithelial

cells: implications for mercury-induced nephrotoxicity. *Toxicol.Appl.Pharmacol.* **173**:176-187.

Dieguez-Acuna FJ and Woods JS (2000) Inhibition of NF-kappaB-DNA binding by mercuric ion: utility of the non-thiol reductant, tris(2-carboxyethyl)phosphine hydrochloride (TCEP), on detection of impaired NF-kappaB-DNA binding by thiol-directed agents. *Toxicol.In Vitro* **14**:7-16.

Dong C, Davis RJ, and Flavell RA (2002) MAP kinases in the immune response. *Annu.Rev.Immunol.* **20**:55-72.

Eckhardt W, Bellmann K, and Kolb H (1999) Regulation of inducible nitric oxide synthase expression in beta cells by environmental factors: heavy metals. *Biochem.J.* **338 (Pt 3)**:695-700.

EPA (1999) Reducing mercury use in health care.

Eto K (1997) Pathology of Minamata disease. *Toxicol.Pathol.* **25**:614-623.

Farber JL (1990) The role of calcium in lethal cell injury. *Chem.Res.Toxicol.* **3**:503-508.

FDA (2001) Consumer advisory. The risks of mercury in fish.

Fitzgerald WF and Clarkson TW (1991) Mercury and monomethylmercury: present and future concerns. *Environ.Health Perspect.* **96**:159-166.

Garg AK and Aggarwal BB (2002) Reactive oxygen intermediates in TNF signaling. *Mol.Immunol.* **39**:509-517.

Gasso S, Cristofol RM, Selema G, Rosa R, Rodriguez-Farre E, and Sanfeliu C (2001) Antioxidant compounds and Ca(2+) pathway blockers differentially protect against methylmercury and mercuric chloride neurotoxicity. *J.Neurosci.Res.* **66**:135-145.

Gerstner HB and Huff JE (1977) Clinical toxicology of mercury. *J.Toxicol. Environ.Health* **2**:491-526.

Ghosh S, May MJ, and Kopp EB (1998) NF-kappa B and Rel proteins: evolutionarily conserved mediators of immune responses. *Annu.Rev.Immunol.* **16**:225-260.

Gillespie KM, Saoudi A, Kuhn J, Whittle CJ, Druet P, Bellon B, and Mathieson PW (1996) Th1/Th2 cytokine gene expression after mercuric chloride in susceptible and resistant rat strains. *Eur.J.Immunol.* **26**:2388-2392.

Gilman AG, Goodman LS, Gilman A (1985) Goodman and Gilman's The Pharmacological Basis of Therapeutics. 7th ed. New York: Macmillan Publishing Co., Inc.

Goodman DR, Fant ME, and Harbison RD (1983) Perturbation of alpha-aminoisobutyric acid transport in human placental membranes: direct effects by $HgCl_2$, CH_3HgCl, and $CdCl_2$. *Teratog.Carcinog.Mutagen.* **3**:89-100.

Gosselin RE, Smith RP, Hodge HC (1984) Clinical Toxicology of Commercial Products. 5th ed. Baltimore: Williams and Wilkins. 3-270.

Grant WM (1986) Toxicology of the Eye. 3rd ed. Springfield, IL: Charles C. Thomas Publisher.

Greated A, Lambertsen L, Williams PA, and Thomas CM (2002) Complete sequence of the IncP-9 TOL plasmid pWW0 from Pseudomonas putida. *Environ.Microbiol.* **4**:856-871.

Gregory SH and Wing EJ (2002) Neutrophil-Kupffer cell interaction: a critical component of host defenses to systemic bacterial infections. *J.Leukoc.Biol.* **72**:239-248.

Gritzka TL and Trump BF (1968) Renal tubular lesions caused by mercuric chloride. Electron microscopic observations: degeneration of the pars recta. *Am.J.Pathol.* **52**:1225-1277.

Gstraunthaler G, Pfaller W, and Kotanko P (1983) Glutathione depletion and in vitro lipid peroxidation in mercury or maleate induced acute renal failure. *Biochem.Pharmacol.* **32**:2969-2972.

Hu H, Moller G, and Abedi-Valugerdi M (1997) Major histocompatibility complex class II antigens are required for both cytokine production and proliferation induced by mercuric chloride in vitro. *J.Autoimmun.* **10**:441-446.

InSug O, Datar S, Koch CJ, Shapiro IM, and Shenker BJ (1997) Mercuric compounds inhibit human monocyte function by inducing apoptosis: evidence for formation of reactive oxygen species, development of mitochondrial membrane permeability transition and loss of reductive reserve. *Toxicology* **124**:211-224.

Itoh N, Yonehara S, Ishii A, Yonehara M, Mizushima S, Sameshima M, Hase A, Seto Y, and Nagata S (1991) The polypeptide encoded by the cDNA for human cell surface antigen Fas can mediate apoptosis. *Cell* **66**:233-243.

Johansson U, Sander B, and Hultman P (1997) Effects of the murine genotype on T cell activation and cytokine production in murine mercury-induced autoimmunity. *J.Autoimmun.* **10**:347-355.

Jouany JM (1998) Environment and Human Health. *Ecotoxicol.Environ.Saf* **40**:2-3.

Kim SH, Johnson VJ, and Sharma RP (2002) Mercury inhibits nitric oxide production but activates proinflammatory cytokine expression in murine macrophage: differential modulation of NF-kappaB and p38 MAPK signaling pathways. *Nitric.Oxide.* **7**:67-74.

Klaassen CD (1996) Toxic effects of metals. In Casarett and Doull's toxicology: The basic science of poisons.

Koller LD (1975) Methylmercury: effect on oncogenic and nononcogenic viruses in mice. *Am.J.Vet.Res.* **36**:1501-1504.

Kontoyiannis D, Pasparakis M, Pizarro TT, Cominelli F, and Kollias G (1999) Impaired on/off regulation of TNF biosynthesis in mice lacking TNF AU-rich elements: implications for joint and gut-associated immunopathologies. *Immunity.* **10**:387-398.

Lipton SA and Nicotera P (1998) Calcium, free radicals and excitotoxins in neuronal apoptosis. *Cell Calcium* **23**:165-171.

Lorscheider FL, Vimy MJ, and Summers AO (1995a) Mercury exposure from "silver" tooth fillings: emerging evidence questions a traditional dental paradigm. *FASEB J.* **9**:504-508.

Lorscheider FL, Vimy MJ, Summers AO, and Zwiers H (1995b) The dental amalgam mercury controversy--inorganic mercury and the CNS; genetic linkage of mercury and antibiotic resistances in intestinal bacteria. *Toxicology* **97**:19-22.

Lund BO, Miller DM, and Woods JS (1993) Studies on Hg(II)-induced H2O2 formation and oxidative stress in vivo and in vitro in rat kidney mitochondria. *Biochem.Pharmacol.* **45**:2017-2024.

Lynch AM and Parry JM (1993) The cytochalasin-B micronucleus/kinetochore assay in vitro: studies with 10 suspected aneugens. *Mutat.Res.* **287**:71-86.

Marrazzini A, Betti C, Bernacchi F, Barrai I, and Barale R (1994) Micronucleus test and metaphase analyses in mice exposed to known and suspected spindle poisons. *Mutagenesis* **9**:505-515.

Marty MS and Atchison WD (1998) Elevations of intracellular Ca(2+) as a probable contributor to decreased viability in cerebellar granule cells following acute exposure to methylmercury. *Toxicol.Appl.Pharmacol.* **150**:98-105.

Mathieson PW and Gillespie KM (1996) Cloning of a partial cDNA for rat interleukin-12 (IL-12) and analysis of IL-12 expression in vivo. *Scand.J.Immunol.* **44**:11-14.

Matsuoka M, Wispriyono B, Iryo Y, and Igisu H (2000) Mercury chloride activates c-Jun N-terminal kinase and induces c-jun expression in LLC-PK_1 cells. *Toxicol.Sci.* **53**:361-368.

Mattingly RR, Felczak A, Chen CC, McCabe MJ, Jr., and Rosenspire AJ (2001) Low concentrations of inorganic mercury inhibit Ras activation during T cell receptor-mediated signal transduction. *Toxicol.Appl.Pharmacol.* **176**:162-168.

May MJ and Ghosh S (1998) Signal transduction through NF-kappa B. *Immunol.Today* **19**:80-88.

Mezna M and Michelangeli F (1997) Effects of thimerosal on the transient kinetics of inositol 1,4,5-trisphosphate-induced Ca(2+) release from cerebellar microsomes. *Biochem.J.* **325 (Pt 1)**:177-182.

Montero M, Barrero MJ, Torrecilla F, Lobaton CD, Moreno A, and Alvarez J (2001) Stimulation by thimerosal of histamine-induced Ca(2+) release in intact HeLa cells seen with aequorin targeted to the endoplasmic reticulum. *Cell Calcium* **30**:181-190.

Nakamura H, Nakamura K, and Yodoi J (1997) Redox regulation of cellular activation. *Annu.Rev.Immunol.* **15**:351-369.

Nicotera P, Bellomo G, and Orrenius S (1990) The role of Ca2+ in cell killing. *Chem.Res.Toxicol.* **3**:484-494.

Perlingeiro RC and Queiroz ML (1994) Polymorphonuclear phagocytosis and killing in workers exposed to inorganic mercury. *Int.J.Immunopharmacol.* **16**:1011-1017.

Pietsch P, Vohr HW, Degitz K, and Gleichmann E (1989) Immunological alterations inducible by mercury compounds. II. $HgCl_2$ and gold sodium thiomalate enhance serum IgE and IgG concentrations in susceptible mouse strains. *Int.Arch.Allergy Appl.Immunol.* **90**:47-53.

Prigent P, Saoudi A, Pannetier C, Graber P, Bonnefoy JY, Druet P, and Hirsch F (1995) Mercuric chloride, a chemical responsible for T helper cell (Th)2-mediated autoimmunity in brown Norway rats, directly triggers T cells to produce interleukin-4. *J.Clin.Invest* **96**:1484-1489.

Prouvost-Danon A, Abadie A, Sapin C, Bazin H, and Druet P (1981) Induction of IgE synthesis and potentiation of anti-ovalbumin IgE antibody response by $HgCl_2$ in the rat. *J.Immunol.* **126**:699-792.

Reed DJ (1990) Review of the current status of calcium and thiols in cellular injury. *Chem.Res.Toxicol.* **3**:495-502.

Roberts MC (1998a) Antibiotic resistance in oral/respiratory bacteria. *Crit Rev.Oral Biol.Med.* **9**:522-540.

Roberts MC (1998b) Antibiotic resistance mechanisms in bacteria of oral and upper respiratory origin. *Int.J.Antimicrob.Agents* **9**:255-267.

Sabry SA, Ghozlan HA, and Abou-Zeid DM (1997) Metal tolerance and antibiotic resistance patterns of a bacterial population isolated from sea water. *J.Appl.Microbiol.* **82**:245-252.

Sakamoto M, Ikegami N, and Nakano A (1996) Protective effects of Ca(2+) channel blockers against methyl mercury toxicity. *Pharmacol.Toxicol.* **78**:193-199.

Sapin C, Druet E, and Druet P (1977) Induction of anti-glomerular basement membrane antibodies in the Brown-Norway rat by mercuric chloride. *Clin.Exp.Immunol.* **28**:173-179.

Sayers LG, Brown GR, Michell RH, and Michelangeli F (1993) The effects of thimerosal on calcium uptake and inositol 1,4,5-trisphosphate-induced calcium release in cerebellar microsomes. *Biochem.J.* **289 (Pt 3)**:883-887.

Sbrana I, Di Sibio A, Lomi A, and Scarcelli V (1993) C-mitosis and numerical chromosome aberration analyses in human lymphocytes: 10 known or suspected spindle poisons. *Mutat.Res.* **287**:57-70.

Schaeffer HJ and Weber MJ (1999) Mitogen-activated protein kinases: specific messages from ubiquitous messengers. *Mol.Cell Biol.* **19**:2435-2444.

Seger R and Krebs EG (1995) The MAPK signaling cascade. *FASEB J.* **9**:726-735.

Sha WC, Liou HC, Tuomanen EI, and Baltimore D (1995) Targeted disruption of the p50 subunit of NF-kappa B leads to multifocal defects in immune responses. *Cell* **80**:321-330.

Shenker BJ, Datar S, Mansfield K, and Shapiro IM (1997) Induction of apoptosis in human T-cells by organomercuric compounds: a flow cytometric analysis. *Toxicol.Appl.Pharmacol.* **143**:397-406.

Shenker BJ, Guo TL, and Shapiro IM (1998) Low-level methylmercury exposure causes human T-cells to undergo apoptosis: evidence of mitochondrial dysfunction. *Environ.Res.* **77**:149-159.

Shenker BJ, Guo TL, and Shapiro IM (2000) Mercury-induced apoptosis in human lymphoid cells: evidence that the apoptotic pathway is mercurial species dependent. *Environ.Res.* **84**:89-99.

Shenker BJ, Pankoski L, Zekavat A, and Shapiro IM (2002) Mercury-induced apoptosis in human lymphocytes: caspase activation is linked to redox status. *Antioxid.Redox.Signal.* **4**:379-389.

Shenker BJ, Rooney C, Vitale L, and Shapiro IM (1992) Immunotoxic effects of mercuric compounds on human lymphocytes and monocytes. I. Suppression of T-cell activation. *Immunopharmacol.Immunotoxicol.* **14**:539-553.

Shumilla JA, Wetterhahn KE, and Barchowsky A (1998) Inhibition of NF-kappa B binding to DNA by chromium, cadmium, mercury, zinc, and arsenite in vitro: evidence of a thiol mechanism. *Arch.Biochem.Biophys.* **349**:356-362.

Silbergeld EK, Sacci JB, Jr., and Azad AF (2000) Mercury exposure and murine response to Plasmodium yoelii infection and immunization. *Immunopharmacol. Immunotoxicol.* **22**:685-695.

Son MH, Kang KW, Lee CH, and Kim SG (2001) Potentiation of cadmium-induced cytotoxicity by sulfur amino acid deprivation through activation of extracellular signal-regulated kinase1/2 (ERK1/2) in conjunction with p38 kinase or c-jun N-terminal kinase (JNK). Complete inhibition of the potentiated toxicity by U0126 an ERK1/2 and p38 kinase inhibitor. *Biochem.Pharmacol.* **62**:1379-1390.

Song J, Jang YY, Shin YK, Lee MY, and Lee C (2000) Inhibitory action of thimerosal, a sulfhydryl oxidant, on sodium channels in rat sensory neurons. *Brain Res.* **864**:105-113.

Tan XX, Tang C, Castoldi AF, Manzo L, and Costa LG (1993) Effects of inorganic and organic mercury on intracellular calcium levels in rat T lymphocytes. *J.Toxicol.Environ.Health* **38**:159-170.

Thorn P, Brady P, Llopis J, Gallacher DV, and Petersen OH (1992) Cytosolic Ca(2+) spikes evoked by the thiol reagent thimerosal in both intact and internally perfused single pancreatic acinar cells. *Pflugers Arch.* **422**:173-178.

Thornberry NA and Lazebnik Y (1998) Caspases: enemies within. *Science* **281**:1312-1316.

Tian L and Lawrence DA (1996) Metal-induced modulation of nitric oxide production in vitro by murine macrophages: lead, nickel, and cobalt utilize different mechanisms. *Toxicol.Appl.Pharmacol.* **141**:540-547.

Toimela TA and Tahti H (2001) Effects of mercuric chloride exposure on the glutamate uptake by cultured retinal pigment epithelial cells. *Toxicol.In Vitro* **15**:7-12.

Turney KD, Parrish AR, Orozco J, and Gandolfi AJ (1999) Selective activation in the MAPK pathway by Hg(II) in precision-cut rabbit renal cortical slices. *Toxicol.Appl.Pharmacol.* **160**:262-270.

Ueda S, Masutani H, Nakamura H, Tanaka T, Ueno M, and Yodoi J (2002) Redox control of cell death. *Antioxid.Redox.Signal.* **4**:405-414.

Verschaeve L, Kirsch-Volders M, and Susanne C (1984) Mercury-induced segregational errors of chromosomes in human lymphocytes and in Indian muntjac cells. *Toxicol.Lett.* **21**:247-253.

WHO (1990) IPCS Environmental Health Criteria 101, Methyl mercury. Geneva: World Health Organization.

WHO (1991) IPCS Environmental Health Criteria 118, Inorganic mercury. Geneva: World Health Organization.

Wild LG, Ortega HG, Lopez M, and Salvaggio JE (1997) Immune system alteration in the rat after indirect exposure to methyl mercury chloride or methyl mercury sulfide. *Environ.Res.* **74**:34-42.

Wilson MR (1998) Apoptotic signal transduction: emerging pathways. *Biochem.Cell Biol.* **76**:573-582.

Xie QW, Cho HJ, Calaycay J, Mumford RA, Swiderek KM, Lee TD, Ding A, Troso T, and Nathan C (1992) Cloning and characterization of inducible nitric oxide synthase from mouse macrophages. *Science* **256**:225-228.

Xie QW, Kashiwabara Y, and Nathan C (1994) Role of transcription factor NF-kappa B/Rel in induction of nitric oxide synthase. *J.Biol.Chem.* **269**:4705-4708.

Zhuang S, Demirs JT, and Kochevar IE (2000) p38 mitogen-activated protein kinase mediates bid cleavage, mitochondrial dysfunction, and caspase-3 activation during apoptosis induced by singlet oxygen but not by hydrogen peroxide. *J.Biol.Chem.* **275**:25939-25948.

CHAPTER 3

MERCURY INHIBITS NITRIC OXIDE PRODUCTION BUT ACTIVATES PRO-INFLAMMATORY CYTOKINE EXPRESSION IN MURINE MACROPHAGE: DIFFERENTIAL MODULATION OF NF-κB AND p38 MAPK SIGNALING PATHWAYS[1]

---

[1]Kim, S.H., Victor J. Johnson, Raghubir P. Sharma. *Nitric Oxide* 2002 7:67-74.

Abstract

Mercury is well known to adversely affect the immune system; however, little is known regarding its molecular mechanisms. Macrophages are major producers of nitric oxide (NO) and this signaling molecule is important in the regulation of immune responses. The present study was designed to determine the impact of mercury on NO and cytokine production and investigate the signaling pathways involved. The murine macrophage cell line, J774A.1, was used to study the effects of low dose inorganic mercury on the production of NO and pro-inflammatory cytokines. Cells were treated with mercury in the presence or absence of lipopolysaccharide (LPS). Mercury (5-20 μM) dose-dependently decreased the production of NO in LPS-stimulated cells. Concomitant decreases in the expression of inducible nitric oxide synthase (iNOS) mRNA and protein were detected. Treatment of J774A.1 cells with mercury alone did not affect the production of NO nor the expression of iNOS mRNA or protein. Interestingly, mercury alone stimulated the expression of tumor necrosis factor α (TNFα), and increased LPS-induced TNFα and interleukin-6 mRNA expression. Mercury inhibited LPS-induced nuclear translocation of NF-κB but had no effect alone. In contrast, mercury activated p38 MAPK and additively increased LPS-induced p38 MAPK phosphorylation. These results indicate that mercury suppresses NO synthesis by inhibition of the NF-κB pathway and modulates cytokine expression by p38 MAPK activation in J774A.1 macrophage cells.

Keywords: mercury, NO, nitric oxide, NF-κB, p38 MAPK, pro-inflammatory cytokine, TNFα, LPS.

Abbreviations used: NO, nitric oxide; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; NF-κB, nuclear factor κB; p38 MAPK, p38 mitogen-activated protein kinase; TNFα, tumor necrosis factor α; IL-6, interleukin-6; IL-1β, interleukin-1β.

1. Introduction

Nitric oxide (NO) plays important roles in many physiological and pathophysiological processes as well as in the maintenance of neuronal communication, vascular regulation and immune systems (1). NO produced by macrophage is an important component of host defense against pathogens and tumor cells (2, 3). Stimulation of macrophages by lipopolysaccharide (LPS) results in the expression of inducible NO synthase (iNOS) that catalyze the production of NO (4).

Mercury is toxic to most mammalian organ systems, especially the immune system, and is known to decrease host resistance to viral infections in mice (5). NO is important to defense against infection and mercury-induced alterations in NO production are detrimental to host defense. It has been reported that inorganic mercury decreased NO production stimulated by interferon γ (IFNγ) and tumor necrosis factor α (TNFα) in murine macrophages (6), and inhibited NO and iNOS expression induced by interleukin-1β (IL-1β) in pancreatic islet β cells (7). Also, mercury impaired host resistance to malaria infection via decreasing NO production in mice (8). Production of NO and expression of iNOS gene is regulated by various inflammatory agents including LPS and several cytokines (9, 10). LPS potentiated mercury-induced nephrotoxicity in mice (11) and mercury inhibited LPS-induced human monocyte activation (12). Because LPS is a toxic component of bacteria and naturally present in the environment and foods, the interaction between mercury and LPS may present important implication for host defense specifically through NO production.

Nuclear factor kappa B (NF-κB) is a transcription factor that mediates the inducible expression of variety of genes involved in immune, inflammatory and acute phase responses including iNOS, TNFα, IL-1 and IL-6, (4, 13, 14). In resting state, NF-κB proteins are

sequestered in the cytosol through interactions with an inhibitory protein IκB. Several stimuli, including TNFα, IL-1, LPS and oxidants, induce NF-κB activation. These signals induce phosphorylation and degradation of IκB protein, resulting in nuclear translocation of NF-κB and increased gene expression (15, 16). There is sufficient experimental evidence indicating that NF-κB is a major transcription factor of iNOS gene expression (4). Mercury inhibits NF-κB-DNA binding in A549 cells, cell-free systems, and rat kidney epithelial cells (17-19); however, a direct evidence linking mercury-induced inhibition of NF-κB-DNA binding to altered gene expression is lacking.

Mitogen-activated protein kinases (MAPKs) have important activities as mediators of cellular responses to extracellular signals. Some types of MAPKs important to mammalian cells include extracellular signal-regulated kinase (ERK), c-jun N-terminal kinase (JNK), and p38 kinase (20). p38 MAPK is thought to play an important role in the regulation of pro-inflammatory molecules, especially, TNFα, IL-1β and IL-6 (21, 22). However, there is little information about direct effects of mercury on p38 MAPK signaling pathway.

The objective of the present study was to examine the effect of mercury on NO production, cytokine gene expression and associated signaling pathways in murine macrophages. The results of this study reveal that mercury inhibits NO production through decreases in iNOS mRNA and protein. The data suggest that NF-κB is important in this effect. Mercury increased TNFα expression and potentiated LPS-induced TNFα and IL-6 expression. Results indicated that mercury directly stimulated p38 MAPK, and that the p38 MAPK signaling pathway may be dominant in controlling the expression of cytokines in the present system.

2. Materials and Method

2.1. Reagents

LPS (from *E.coli* 026:B6, Sigma, St. Louis, MO) was dissolved in the culture medium. The stocks were aliquoted and stored at –20^{o}C until use. $HgCl_2$ (Sigma) was prepared in distilled water, filter sterilized (0.22 μM filter), and added into cultures at the indicated concentrations.

2.2. Cell cultures

BALB/c murine macrophage cells (J774A.1) from American Type Culture Collection (ATCC, Rockville, MD) were grown in DMEM medium (2 mM glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin) with 10% non-heat-inactivated fetal bovine serum (Atlanta Biologicals, Atlanta, GA) in 5% CO_2 at 37^{o}C.

2.3. Bioassay for cytotoxicity of mercury

The 3(4,5-dimethyl thiazolyl-2)2,5 diphenyl tetrazolium bromide (MTT, Sigma) cytotoxicity assay was used to measure cytotoxic response to mercury as previously described (23). J774A.1 cells were seeded at 2 x 10^4 cells/well in 96-well microplates (Falcon, Becton Dickinson, Franklin Lakes, NJ). After 24 h of incubation with mercury, 20 μl of MTT (5 mg/ml) was added per well and incubated for 4 h. The formazan crystals dissolved in isopropyl alcohol with 0.04 N HCl and absorbance was read at 570 nm using an SLT microplate reader (Quest Scientific, Duluth, GA). Cytotoxicity was calculated as relative absorbance compared by control.

2.4. LPS and mercury induced proliferation

DNA synthesis was used as an index of proliferation in macrophages exposed to LPS and mercury as described previously (24). J774A.1 cells were seeded at 2 x 10^4 cells/well in

96-well microplates and treated with mercury (1-20 μM) with or without LPS. After 24 h of treatment, each well was pulsed with 20 μl of [methyl-^{3}H]thymidine (25 μCi/ml, 6.7 Ci/mmol, DuPont NEN Products, Boston, MA) and incubated for an additional 18 h. Cells were harvested onto glass fiber filter paper (Cambridge Technology, Watertown, MA) using a cell harvester (PHD, Cambridge Technology). The harvested cells were lysed with deionized water and the filters were dehydrated with 95% ethanol. Filter papers were placed in scintillation vials containing 2 ml of liquid scintillation cocktail (Ready-Solv, Beckman, Fullerton, CA) and counted in a liquid scintillation counter (Pharmacia, Turku, Finland). Proliferative response (uptake of [^{3}H]thymidine) were expressed as net disintegrations per min (DPM).

2.5. Nitric oxide assay

The amount of stable nitrite, the end product of NO generation by activated macrophages was determined by a colorimetric assay as previously described (23). Briefly, 50 μl of culture supernatant was mixed with an equal volume of Griess reagent (1% sulfanilamide, 0.1% naphthylethylenediamine dihydrocholoride, 2.5% H_3PO_4), and incubated at room temperature for 10 min. The absorbance at 540 nm was read and concentration determined by extrapolation from a sodium nitrite standard curve.

2.6. Analysis of mRNA expression

Total cellular RNA was isolated from macrophages following 6 h and 24 h of treatment with mercury using TRI reagent LS (Molecular Research Center, Cincinnati, OH) according to manufacturer's protocol. The first strand complimentary DNA (cDNA) was synthesized using Superscript II reverse transcriptase enzyme (Life Technologies, Grand Island, NY). Reverse-transcriptase polymerase chain reaction (RT-PCR) was used to analyze

the expression of mRNA for iNOS, TNFα, IL-6, IL-1β and β-actin (internal control). The condition for reverse transcription and PCR steps were performed as previously reported (25) with the exception of primer sets. Optimization of cycle number was performed to ensure that product accumulation was in the linear range. Amplified products were separated by electrophoresis on 2% agarose gel containing ethidium bromide. The gels were documented using a Kodak DC 290 digital camera and digitized using UN-SCAN-IT software (Silk Scientific, Orem, UT). Band intensities for the genes of interest were normalized to that of β-actin in the same sample.

2.7. Western blot analysis

Cell lysates were prepared as described for iNOS, phospho-p38 (22) and for NF-κB (26). Protein concentrations in the cell lysates were determined by Bio-Rad (Richmond, CA) protein assay. Western blot analyses were performed on 2-25 μg of proteins as previously described (26). Briefly, samples were mixed with 2X sample buffer, heated to 95^{o}C for 5min, separated electrophoretically using 8-12% SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) and then transferred to nitrocellulose paper. The iNOS and p65 NF-κB were assayed by anti-iNOS antibody and anti-p65 NF-κB antibody (Santa Cruz Biotech, Santa Cruz, CA). The p38 activation was assayed using anti-phospho-p38 antibody (New England Biolabs, Beverly, MA). Immunodetection was performed using enhanced chemiluminescence (ECL) detection kit (Amersham Pharmacia, Piscataway, NJ).

2.8. Data presentation and statistical analysis

All experiments were repeated 2 to 3 times with similar trends; however, data from a representative trial are depicted in the results. Statistical analyses were performed using SAS statistical software (SAS Institute, Cary, NC). Treatment effects were analyzed using one

way analysis of variance (ANOVA) followed by Duncan's Multiple Range test. A value of $p < 0.05$ was used to indicate significant differences.

3. Results

3.1. Cell viability and proliferation

Exposure to ≤ 20 μM of mercury for 24 h did not affect the viability of J774A.1 macrophages (Fig. 3.1A). The viability of cells exposed to 50 μM of mercury for 24 h was reduced to 75% of control cultures. Macrophages treated with mercury (5-20 μM) with or without LPS showed no changes in DNA synthesis as indicated by [^{3}H]thymidine incorporation (Fig. 3.1B). These data indicate that concentrations of mercury up to 20 μM, did not affect viability or proliferation of J774A.1 macrophages.

3.2. Nitric oxide production

The effect of various concentrations of mercury on LPS-induced NO production in macrophages is shown in Fig. 3.2. Mercury by itself had no effect on NO production. Treatment of macrophages with mercury (5-20 μM) dose-dependently decreased their ability to produce NO in response to LPS. LPS stimulated the production of NO up to 96 h following treatment as shown in Fig. 3.3. Treatment with mercury consistently inhibited NO production in LPS-stimulated macrophages at all times examined with the maximum inhibition seen at 24 h.

3.3. Effect of mercury on expression of iNOS

The correlation between decreased NO production and the effects of mercury on iNOS mRNA expression and protein production were determined by RT-PCR and western blotting, respectively. iNOS mRNA (Fig. 3.4) and protein (Fig. 3.5) were increased

following 6 h and 24 h treatment with LPS, respectively. Treatment with mercury significantly attenuated LPS-induced changes in iNOS mRNA and protein.

3.4. Cytokine gene expression

Treatment with mercury for 24 h increased TNFα mRNA and potentiated LPS-induced TNFα mRNA production measured by RT-PCR (Fig. 3.6). Mercury itself did not stimulate IL-6 mRNA production, but increased LPS-induced IL-6 mRNA. LPS also stimulated IL-1β mRNA in macrophages; however, mercury showed no effect on IL-1β mRNA expression.

3.5. Effect of mercury on translocation of NF-κB

LPS treatment induced the nuclear translocation of p65 NF-κB at 4 h incubation as determined by p65 NF-κB western blot. Mercury (20 μM) inhibited the LPS-induced nuclear translocation of p65 NF-κB but had no effect alone. Cytoplasmic p65 NF-κB showed no change in each treatment (Fig. 3.7).

3.6. Effect of mercury on activation of p38 MAPK

Stimulation of macrophages with LPS resulted in phosphorylation of p38 MAPK and this phosphorylation peaked at 15 min and decreased after 30 min (data not shown). Mercury (20 μM) alone activated the dual phosphorylation of p38 MAPK to same extent as LPS, and additively increased LPS-induced phosphorylation of p38 MAPK (Fig. 3.8).

4. Discussion

The objectives of this study were to determine if mercury alters NO and cytokine production in macrophages and to elucidate the underlying signaling pathways. Mercury is a known immunotoxic agent and immunologically mediated manifestations; for example, lymphocyte hyperplasia, increase of Ig level in serum, and increased autoantibodies have been demonstrated following treatment with $HgCl_2$ (27, 28). In the present study, we selected a non-cytotoxic concentration of mercury (≥ 20 μM) to exclude mercury-induced toxicity as indicated by cell viability.

Lead, cadmium, copper and mercury modulate the cellular inflammatory response through inhibition of iNOS-dependent production of NO in murine macrophages in response to TNFα (29). In murine macrophages, iNOS is readily induced by LPS or synergistically by LPS and IFNγ, resulting in NO formation. It has been reported that mercury inhibited TNFα and IFNγ-induced NO expression but not iNOS activity in murine splenic macrophages (6) and IL-1β-induced NO and iNOS mRNA and protein expression in pancreatic islet β cells (7). As previously reported, LPS (30 ng/ml) stimulated NO production in J774A.1 macrophages (23). In the present study, LPS-induced NO production was inhibited dose-dependently by $HgCl_2$ (5-20 μM) in J774A.1 macrophages. The decrease in NO production was shown to be due to mercury-induced inhibition of iNOS mRNA expression and protein synthesis.

Evidence exists that the modulation of iNOS is dependent on NF-κB activation in LPS-stimulated macrophages (4, 14). In this study, we found that low doses of mercury blocked nuclear translocation of p65 NF-κB. Inhibition of NF-κB translocation is likely responsible for the observed inhibitory effects on iNOS expression and the production of NO.

Relatively high doses of mercury (> 0.3 mM) have been shown to inhibit NF-κB-DNA binding in TNFα-stimulated A549 cells (17). Relatively low doses of mercury inhibited NF-κB-DNA binding in cell free system and blocked LPS-induced NF-κB activation via competitive mercaptide formation in normal rat epithelial cells (18, 19). The authors (18, 19) explained that mercury reduced the nuclear translocation of p65 NF-κB and inhibited IκBα phosphorylation and degradation in response to LPS. In contrast, it was recently reported that low doses of mercury (2-4 μM) promoted NO formation and enhanced LPS and IFNγ-induced iNOS mRNA and iNOS formation in normal rat epithelial cells (30). These authors observed that these effects were not dependent on NF-κB activation since mercury attenuated LPS-induced expression of the κB-driven luciferase reporter gene. It is likely that differences in cell system, dose, and exposure conditions are responsible for the desperate results.

To further investigate the effects of mercury on macrophages, we examined cytokine expression. Mercury stimulated the expression of TNFα mRNA and increased LPS-induced TNFα and IL-6 mRNA but did not affect IL-1β mRNA expression as detected by RT-PCR. There are several reports showing that mercury altered cytokine expression in various cell types. Low doses of mercury increased IL-2 and IFNγ in mouse spleen cells (31), increased IL-4, and IFNγ in rat spleen cells (32), and IL-1 in macrophages (33). The inhibitory activity of mercury on LPS-induced NF-κB activation appears to contradict the observed increases in pro-inflammatory cytokine expressions. To pursue these paradoxical results, we investigated the impact of mercury on MAPK signaling. The p38 MAPK protein is an important mediator of stress-induced gene expression. p38 is known to play a key role in LPS-induced signal transduction pathways leading to cytokine synthesis (22, 34). However, there are few reports

regarding the effects of mercury on p38 MAPK. Low levels of mercury dose-dependently induced AP-1 and c-jun gene expression but did not change ATF2 and ELK-1, transcription factors involved in both JNK and p38 pathways of MAPK signal transduction (35). Mercury-induced toxicity and the enhancement of toxicity by sulfur amino acid deprivation involved the JNK pathway, not p38 pathway (36).

The interplay between NF-κB and MAPK is important in determining the signal pathways. The transcription factors of ATF-2, c-jun, Egr-1 and Sp1 are recruited to the TNFα promoter and the binding of each of these activators is required for LPS-stimulated TNFα gene expression (37). In recent studies, it has been shown that within a specific cell type, different stimuli result in the formation of a distinct set of protein complexes at the TNFα promoter (38). Furthermore, inhibition of NF-κB, using dominant-negative NF-κB p65 or IκBα, did not completely abolish LPS-induced TNFα expression (39). These reports may help explain our results showing increased expression of TNFα gene by mercury despite decreased NF-κB activation. Mercury-induced activation of p38 signaling may drive the expression of TNFα mRNA in our system, and this thought is supported by the pattern of mercury stimulation to TNFα and p38 MAPK. Mercury alone increased TNFα gene and p38 MAPK, and mercury plus LPS treatment additively increased TNFα gene and p38 MAPK to a similar extent. In the present report, we revealed that mercury directly activated p38 MAPK and increased LPS-induced p38 MAPK activation. Such increased p38 MAPK activation may be responsible for the increased TNFα gene expression.

The mechanism by which the p38 MAPK regulates NF-κB-dependent gene expressions has not been clearly shown. There are reports that p38 activation by stress stimulation inhibited TNF-induced IκB phosphorylation and NF-κB activation, and transient

expression of MAPK kinase (MKK) 6b, a sensitive activator of p38, reduced TNF-induced IκB phosphorylation and NF-κB dependent reporter activity (40). This suggests that the target of p38 lies upstream of NF-κB, therefore, p38 might regulate NF-κB dependent gene regulation. To reveal more detailed signal transduction in our system, experiment using specific inhibitors of p38 and NF-κB are under investigation.

Mercury is one of the most potent thiol-binding agents. Mercury has a great predilection to bond to reduced sulfur atoms, especially those on endogenous thiol-containing molecules, such as glutathione, cysteine, homocysteine, metallothionein, and albumin (41). The thiol-reactive gold compounds have an inhibitory effect to LPS-stimulated NF-κB activation in murine macrophages (42). Competitive thiols reverse the inhibition of NF-κB-DNA binding by mercury (19). Therefore, the thiol binding properties of mercury may be responsible for the inhibitory effects of mercury on NF-κB activation and downstream NO production. The effects observed here may indicate sensitive biomacromolecules that react with mercury at relatively low concentrations, while higher concentrations are cotytoxic.

NO is an important element of host resistance and acquisition of immunity (2, 3). Mercury is known to impair host defense to viral and bacteria infection. Therefore inhibitory effect of mercury on NO production may be involved in interference with response to infection through inhibition of LPS signaling. According to our results, low doses of mercury decreased LPS-induced NO production in murine macrophages, suggesting that even low dose exposure to mercury may impair the immune response to infectious diseases by lowering host defense elements. More studies are required to further our understanding of mercury effects on the immune system.

References

1. Gross, S. S., and Wolin, M. S. (1995). Nitric oxide: pathophysiological mechanisms. *Annu. Rev. Physiol.* **57,** 737-769.
2. Nussler, A. K., and Billiar, T. R. (1993). Inflmmation immunoregulation and inducible nitric oxide synthase. *J. Leukoc. Biol.* **54,** 171-178.
3. Green, S. J., Scheller, L. F., Marletta, M. A., Seguin, M. C., Klotz, F. W., Slayter, M., Nelson, B. J., and Nacy, C. A. (1994). Nitric oxide: cytokine-regulation of nitric oxide in host resistance to intracellular pathogens. *Immunol. Lett.* **43,** 87-94.
4. Xie, Q. W., Kashiwabara, Y., and Nathan, C. (1994). Role of transcription factor NF-kappa B/Rel in induction of nitric oxide synthase. *J. Biol. Chem.* **269,** 4705-4708.
5. Christensen, M. M., Ellermann-Eriksen, S., Rungby, J., and Mogensen, S. C. (1996). Influence of mercuric chloride on resistance to generalized infection with herpes simplex virus type 2 in mice. *Toxicology* **15,** 57-66.
6. Tian, L., and Lawrence, D. A. (1996). Metal-induced modulation of nitric oxide production in vitro by murine macrophage: lead, nickel, and cobalt utilize different mechanisms. *Toxicol. Appl. Pharmacol.* **141,** 540-547.
7. Eckhardt, W., Bellmann, K., and Kolb, H. (1999). Regulation of inducible nitric oxide synthase expression in beta cells by environmental factors: heavy metals. *Biochem. J.* **338,** 695-700.
8. Silbergeld, E. K., Sacci, J. B. Jr., and Azad, A. F. (2000). Mercury exposure and murine response to *Plasmodium yoelii* infection and immunization. *Immunopharmacol. Immunotoxicol*. **22,** 685-695.

9. Owens, M. W., and Grisham, M. B. (1993). Nitric oxide synthesis by rat pleural mesothelial cells: induction by cytokines and lipopolysaccharide. *Am. J. Physiol.* **265,** L110-116.

10. Williams, G., Brown, T., Becker, L., Prager, M., and Giroir, B. P. (1994). Cytokine-induced expression of nitric oxide synthase in C2C12 skeletal muscle myocytes. *Am. J. Physiol.* **267,** R1020-1025.

11. Rumbeiha, W. K., Fitzgerald, S. D., Braselton, W. E., Roth, R. A., Pestka, J. J., and Kaneene, J. B. (2000). Augmentation of mercury-induced nephrotoxicity by endotoxin in the mouse. *Toxicology* **26,** 103-116.

12. Koropatnick, J., and Zalups, R. K. (1997). Effect of non-toxic mercury, zinc or cadmium pretreatment on the capacity of human monocytes to undergo lipopolysaccharide-induced activation. *Br. J. Pharmacol*. **120,** 797-806.

13. Sha, W. C., Liou, H. C., Tuomanen, E. I., and Baltimore, D. (1995). Targeted disruption of the p50 subunit of NF-kappa B leads to multifocal defects in immune responses. *Cell* **80,** 321-330.

14. Goldring, C. E., Narayanan, R., Lagadec, P., and Jeannin, J. F. (1995). Transcriptional inhibition of the inducible nitric oxide synthase gene by competitive binding of NF-kappa B/Rel proteins. *Biochem. Biophys. Res. Commun.* **209,** 73-79.

15. May, M. J., and Ghosh, S. (1998). Signal transduction through NF-kappa B. *Immunol. Today* **19,** 80-88.

16. Ghosh, S., May, M. J., and Kopp, E. B. (1998). NF-kappa B and Rel proteins: evolutionarily conserved mediators of immune responses. *Annu. Rev. Immunol.* **16,** 225-260.

17. Shumilla, J. A., Wetterhahn, K. E., and Barchowsky, A. (1998). Inhibition of NF-kappa B binding to DNA by chromium, cadmium, mercury, zinc, and arsenite in vitro: evidence of a thiol mechanism. *Arch. Biochem. Biophys.* **349,** 356-362.

18. Dieguez-Acuna, F. J., and Woods, J. S. (2000). Inhibition of NF-kappaB-DNA binding by mercuric ion: utility of the non-thiol reductant, tris(2-carboxyethyl)phosphine hydrochloride (TCEP), on detection of impaired NF-kappaB-DNA binding by thiol-directed agents. *Toxicol. In Vitro* **14,** 7-16.

19. Dieguez-Acuna, F. J., Ellis, M. E., Kushleika, J., and Woods, J. S. (2001). Mercuric ion attenuates nuclear factor-kappaB activation and DNA binding in normal rat kidney epithelial cells: implications for mercury-induced nephrotoxicity. *Toxicol. Appl. Pharmacol.* **173,** 176-187.

20. Seger, R., and Krebs, E. G. (1995). The MAPK signaling cascade. *FASEB J.* **9,** 726-735.

21. Beyaert, R., Cuenda, A., Vanden Berghe, W., Plaisance, S., Lee, J. C., Haegeman, G., Cohen, P., and Fiers, W. (1996). The p38/RK mitogen-activated protein kinase pathway regulates interleukin-6 synthesis response to tumor necrosis factor. *EMBO J.* **15,** 1914-1923.

22. Baldassare, J. J., Bi, Y., and Bellone, C. J. (1999). The role of p38 mitogen-activated protein kinase in IL-1 beta transcription. *J. Immunol.* **162,** 5367-5373.

23. Mathew, J. S., and Sharma, R. P. (2000). Effect of all-trans-retinoic acid on cytokine production in a murine macrophage cell line. *Int. J. Immunopharmacol.* **22,** 693-706.

24. Johnson, V. J., Tsunoda, M., and Sharma, R. P. (2000). Increased production of proinflammatory cytokines by murine macrophages following oral exposure to

sodium selenite but not to seleno-L-methionine. *Arch. Environ. Contam. Toxicol.* **39,** 243-250.

25. Tsunoda, M., and Sharma, R. P. (1999). Modulation of tumor necrosis factor alpha expression in mouse brain after exposure to aluminum in drinking water. *Arch. Toxicol.* **73,** 419-426.
26. Sharma, R. P., Bhandari, N., Tsunoda, M., Riley, R. T., and Voss, K. A. (2000). Fumonisin hepatotoxicity is reduced in mice carrying the human tumour necrosis factor alpha transgene. *Arch. Toxicol*. **74,** 238-248.
27. Jiang, Y., and Moller, G. (1995). In vitro effect of $HgCl_2$ on murine lymphocytes. *J. Immunol.* **154,** 3138-3146.
28. Hultman, P., and Hansson-Georgiadis, H. (1999). Methyl mercury-induced autoimmunity in mice. *Toxicol. Appl. Pharmacol.* **154,** 203-211.
29. Buzard, G. S., and Kasprzak, K. S. (2000). Possible roles of nitric oxide and redox cell signaling in metal-induced toxicity and carcinogenesis. *J. Environ. Pathol. Toxicol. Oncol.* **19,** 179-199.
30. Ellis, M. E., Corral, J., Kushleika, J., Simmonds, P. L., Dieguez-Acuna, F. J., and Woods, J. S. (2001). Mercuric ion induces nitric oxide synthetase (NOS) independently of NF-κB activation in rat kidney (NRK52E) cells. *Toxicologist* **60**, 211.
31. Hu, H., Abedi-Valugerdi, M., and Moller, G. (1997). Pretreatment of lymphocytes with mercury in vitro induces a response in T cells from genetically determined low-responders and a shift of the interleukin profile. *Immunology* **90,** 198-204.

32. Prigent, P., Saoudi, A., Pannetier, C., Graber, P., Bonnefoy, J. Y., Druet, P., and Hirsch, F. (1995). Mercuric chloride, a chemical responsible for T helper cell (Th)2-mediated autoimmunity in brown Norway rats, directly triggers T cells to produce interleukin-4. *J. Clin. Invest*. **96,** 1484-1489.

33. Zdolsek, J. M., Soder, O., and Hultman, P. (1994). Mercury induces in vivo and in vitro secretion of interleukin-1 in mice. *Immunopharmacology* **28,** 201-208.

34. Lee, J. C., and Young, P. R. (1996). Role of CSB/p38/RK stress response kinase in LPS and cytokine signaling mechanisms. *J. Leukoc. Biol.* **59,** 152-157.

35. Turney, K. D., Parrish, A. R., Orozco, J., and Gandolfi, A. J. (1999). Selective activation in the MAPK pathway by Hg(II) in precision-cut rabbit renal cortical slices. *Toxicol. Appl. Pharmacol.* **160,** 262-270.

36. Son, M. H., Kang, K. W., Lee, C. H., and Kim, S. G. (2001). Potentiation of arsenic-induced cytotoxicity by sulfur amino acid deprivation (SAAD) through activation of ERK1/2, p38 kinase and JNF1: the distinct role of JNK1 in SAAD-potentiated mercury toxicity. *Toxicol. Lett.* **121,** 45-55.

37. Tsai, E. Y., Falvo, J. V., Tsytsykova, A. V., Barczak, A. K., Reimold, A. M., Glimcher, L. H., Fenton, M. J., Gordon, D. C., Dunn, I. F., and Goldfeld, A. E. (2000). A lipopolysaccharide-specific enhancer complex involving Ets, Elk-1, Sp1, and CREB binding protein and p300 is recruited to the tumor necrosis factor alpha promoter in vivo. *Mol. Cell. Biol.* **20,** 6084-6094.

38. Falvo, J. V., Uglialoro, A. M., Brinkman, B. M, Merika, M., Parekh, B. S., Tsai, E. Y., King, H. C., Morielli, A. D., Peralta, E. G., Maniatis, T., Thanos, D., and

Goldfeld, A. E. (2000). Stimulus-specific assembly of enhancer complexes on the tumor necrosis factor alpha gene promoter. *Mol. Cell. Biol.* **20,** 2239-2247.

39. Liu, H., Sidiropoulos, P., Song, G., Pagliari, L. J., Birrer, M. J., Stein, B., Anrather, J., and Pope, R. M. (2000). TNF-alpha gene expression in macrophages: regulation by NF-kappa B is independent of c-Jun or C/EBP beta. *J. Immunol.* **164,** 4277-4285.
40. Alpert, D., Schwenger, P., Han, J., and Vilcek, J. (1999). Cell stress and MKK6b-mediated p38 MAP kinase activation inhibit tumor necrosis factor-induced IkappaB phosphorylation and NF-kappaB activation. *J. Biol. Chem.* **274,** 22176-22183.
41. Zalups, R. K. (2000). Molecular interactions with mercury in the kidney. *Pharmacol. Rev.* **52,** 113-143.
42. Jeon, K. I., Jeong, J. Y., and Jue, D. M. (2000). Thiol-reactive metal compounds inhibit NF-kappa B activation by blocking I kappa B kinase. *J. Immunol.* **164,** 5981-5989.

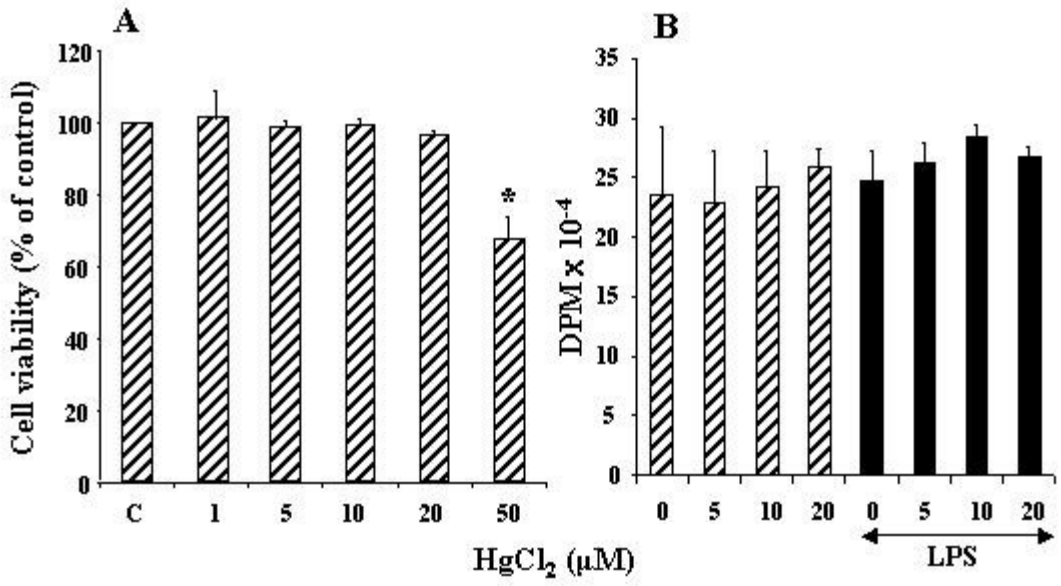


Fig. 3.1. Effects of $HgCl_2$ on cell viability and proliferation. (A) J774A.1 cell were incubated with various concentration of $HgCl_2$ for 24 h; MTT (0.5 mg/ml) was added and incubated for 4 h. After incubation the absorbance was read at 570 nm. (B) Proliferation of J774A.1 cells was measured by [^{3}H]thymidine incorporation. Cells were activated with LPS (30 ng/ml) and $HgCl_2$ at the indicated concentrations. Results are expressed as mean ± SE (n=3). *Significantly different than the control group at $p < 0.05$.

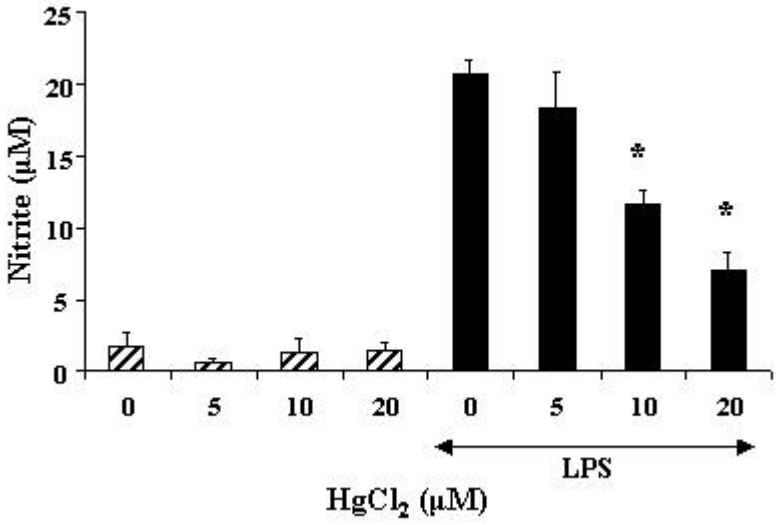


Fig. 3.2. Nitrite production in J774A.1 cells following treatment with $HgCl_2$ in the presence or absence of LPS. Cells were activated with LPS (30 ng/ml) and $HgCl_2$ at the indicated concentrations. Culture supernatants were collected after 24 h of treatment. Results are expressed as mean ± SE (n=3). *Significantly different than the LPS alone group at $p < 0.05$.

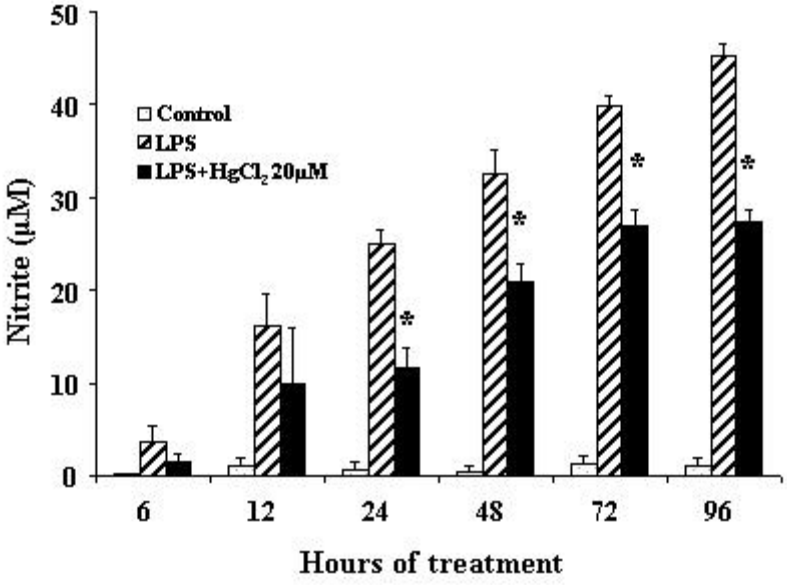


Fig. 3.3. Time course of nitrite production in J774A.1 cells treated with $HgCl_2$ (20 µM) and LPS (30 ng/ml). Supernatants were removed at 6, 12, 24, 48, 72 and 96 h following treatment and nitrite was determined using Griess reagent. Results are expressed as mean ± SE (n=3). *Significantly different than the LPS alone group at $p < 0.05$.

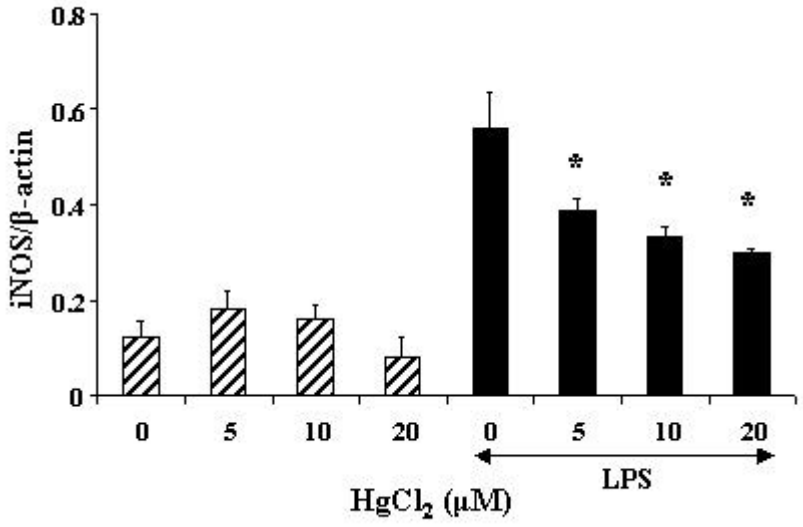


Fig. 3.4. The relative mRNA expression of iNOS in J774A.1 cells activated 6 h with varying concentration of $HgCl_2$ with or without LPS (30 ng/ml). Extraction and analysis of mRNA performed as described in Materials and Methods, and iNOS mRNA levels were quantified by RT-PCR. The iNOS expression was normalized against β-actin. Results are expressed as mean ± SE (n=3). *Significantly different than the LPS alone group at $p < 0.05$.

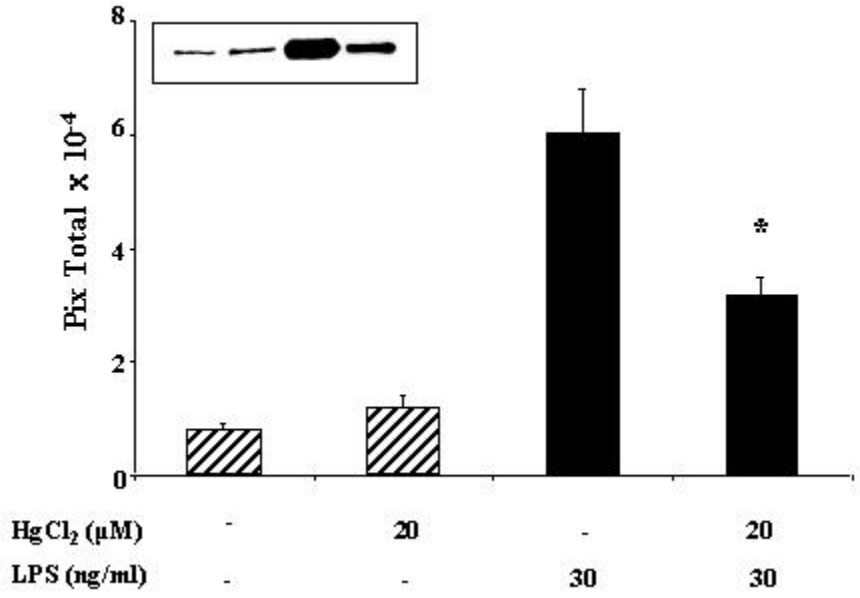


Fig. 3.5. Effect of $HgCl_2$ on iNOS protein expression. J774A.1 cells activated 24 h with $HgCl_2$ (20 µM) with or without LPS (30 ng/ml). The cell lysates (5 µg) were analyzed by 8% SDS-PAGE, and iNOS was visualized by western blot analysis. Results are expressed as mean ± SE (n=3). *Significantly different than the LPS alone group at $p < 0.05$. Insert, a representative western blot in the same order.

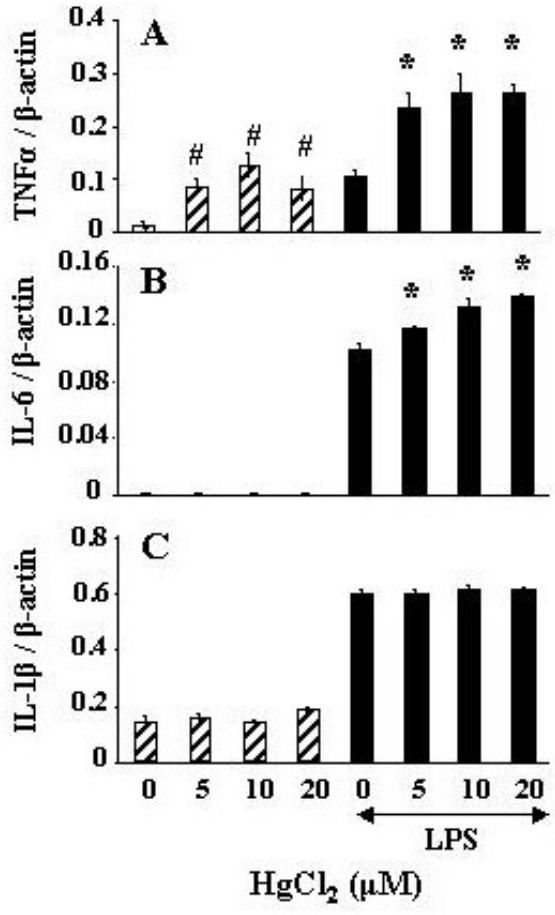


Fig. 3.6. The relative mRNA expression of TNFα (A), IL-6 (B) and IL-1β (C) in J774A.1 cells activated 24 h with varying concentration of $HgCl_2$ with or without LPS (30 ng/ml). Extraction and analysis of mRNA performed as described in Materials and Methods, and TNFα, IL-6 and IL-1β mRNA levels quantified by RT-PCR. Each cytokine expression was normalized against β-actin. Results are expressed as mean ± SE (n=3). *Significantly different than the LPS alone group at $p < 0.05$. #Significantly different than the control group at $p < 0.05$.

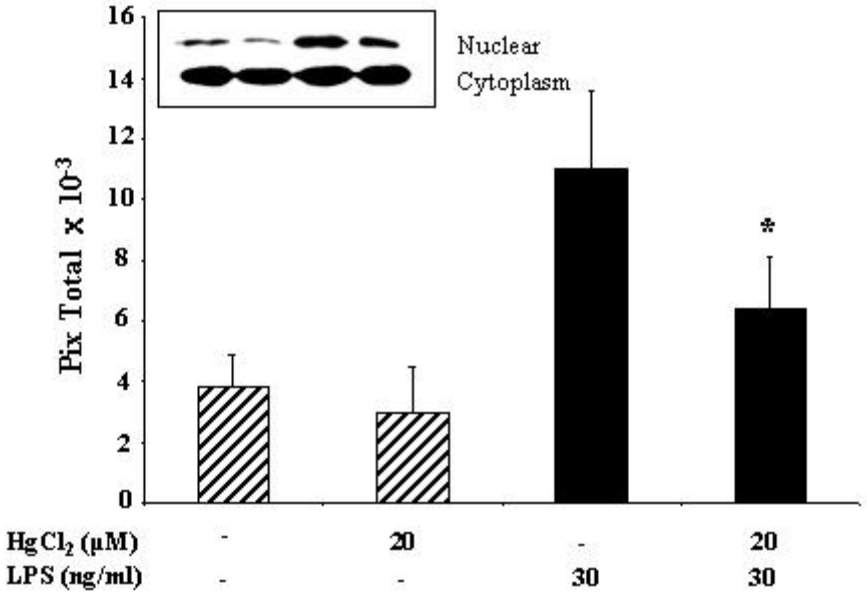


Fig. 3.7. Effect of $HgCl_2$ on p65 NF-κB levels in nuclear and cytoplasm extracts in J774A.1 cells activated 4 h with $HgCl_2$ (20 μM) with or without LPS (30 ng/ml). Nuclear extract (5 μg) and cytoplasmic extract (2 μg) were analyzed by 8% SDS-PAGE, and p65 NF-κB was visualized by western blot analysis. Results are expressed as mean ± SE (n=3). *Significantly different than the LPS alone group at $p < 0.05$. Insert, a representative western blot in the same order.

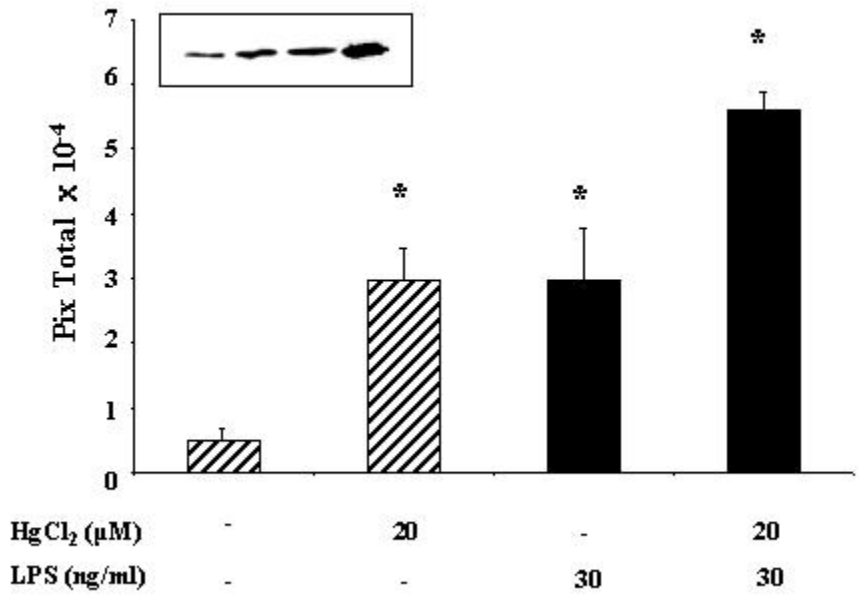


Fig. 3.8. Effect of $HgCl_2$ on p38 MAPK activation. J774A.1 cells activated 15 min with $HgCl_2$ (20 μM) with or without LPS (30 ng/ml). Cell extracts (25 μg) were analyzed by 12% SDS-PAGE, and p38 MAPK was visualized by western blot analysis using an antibody specific for phosphorylated p38 (activated state). Results are expressed as mean ± SE (n=3). *Significantly different than the LPS alone group at $p < 0.05$. Insert, a representative western blot in the same order.

CHAPTER 4

MERCURY INDUCED APOPTOSIS AND NECORSIS IN MACROPHAGES: CRITICAL ROLE OF REDOX STATUS AND p38 MITOGEN-ACTIVATED PROTEIN KINASE[1]

[1]Kim S.H., Raghubir P. Sharma. Submitted to Journal of Leukocyte Biology.

Abstract

The objective of our study was to define the mechanism by which mercury, a known immunotoxic agent, induces cell death. Macrophages play a critical role in immune response by producing reactive oxygen species (ROS) and inflammatory cytokines. The EC_{50} of mercury was in the range of 62.7 to 102.8 μM by various cytotoxicity assays; accordingly we used 70 μM of mercury in this study. Mercury increased ROS production. Mercury stimulated mitogen-activated protein kinases (MAPKs), p38 and extracellular signal-regulated kinase, but not c-jun N-terminal kinase; moreover, mercury additively activated lipopolysaccharide-stimulated p38. Mercury-activated p38 was decreased by pretreatment of antioxidant *N*-acetylcysteine and a flavonoid silymarin. Mercury increased expression of tumor necrosis factor α (TNFα), the antioxidants and specific p38 inhibitor decreased this effect. Mercury induced both apoptosis and necrosis as indicated by annexin V binding and caspase-3 activity, and propidium iodide binding. Pretreatment with antioxidants, p38 inhibitor, and anti-TNFα antibody decreased mercury-induced necrosis; however, only antioxidants and p38 inhibitor decreased mercury-induced apoptosis. Mercury increased intracellular calcium whereas pretreatment of various intracellular calcium antagonists blocked mercury-induced ROS production. These data suggest that mercury-induced cell death is a mix of apoptosis and necrosis with different pathways. Mercury-induced ROS and downstream p38 regulate both apoptosis and necrosis. p38-mediated caspase activation regulates mercury-induced apoptosis and p38-mediated TNFα regulates necrosis. Intracellular calcium is upstream of ROS and calcium flux is important for these effects.

Keywords: Mercury, ROS, calcium, mitogen-activated protein kinase, TNFα, caspase

Abbreviations used: ROS, reactive oxygen species; p38 MAPK, p38 mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; JNK, c-jun N-terminal kinase; TNFα, tumor necrosis factor α; NAC, *N*-acetylcysteine; PI, propidium iodide; DHR, dihydrorhodamine; CM-H_2DCFDA, 5-chloromethyl-2`,7`-dichlorodihydrofluorescein diacetate

Introduction

Mercury (Hg) is a well known immunotoxic agent and immune-mediated manifestations, such as autoantibody synthesis, apoptosis, and necrosis have been demonstrated following treatment with $HgCl_2$ [1-3]. Mercury has toxic effect in mammalian organ systems and is known to decrease host resistance to viral infections [4,5]. There has been growing concern regarding the human health effects of low level of environmental mercury contamination [6].

Macrophages play an important role in various aspects of inflammatory responses, immunity, host defense, and tissue repair [7]. Macrophages produce reactive oxygen species (ROS) which play an important role in the ability of macrophages to kill pathogens and infected cells [8]. Oxidative stress constitutes a major threat to organism living in an aerobic environment, and in humans it may be etiology for many disease processes. ROS induce undesirable biological reactions, including apoptosis [9]. Although mercury is broadly sulfhydryl reactive and modulates ROS, little is understood as to the specific cellular processes and biochemical pathways targeted by this metal. It has been reported that mercury regulates redox status and resulted in induction of apoptosis in human T cells and monocytes [2,10,11].

Mitogen-activated protein kinases (MAPKs) mediate signal transduction in mammalian cells and include the extracellular signal-regulated kinase (ERK), c-jun N-terminal kinase (JNK) and p38 MAPK. ERK is generally associated with proliferation and growth. In contrast JNK and p38 MAPK are induced by stress responses and cytokines, and mediate differentiation and cell death [12,13]. Oxidative stress induces activation of JNK and p38 MAPK, which induce stress-mediated apoptosis signal [14]. Although many

JNK/p38-activating stimuli are proapoptotic, the outcome of JNK or p38 activation strongly depends on the cell type and the cellular context [15]. We previously reported that in vitro, non-cytotoxic concentrations of inorganic mercury increased TNFα expression by regulating p38 pathway in murine macrophages [16]; p38 is an important mediator of stress-induced gene expression and related cytokine synthesis [17]. However, direct effects of mercury on p38 signaling pathway have not been fully characterized.

Caspases, a family of aspartate-specific cysteine proteases, are the key effectors responsible for many morphological and biochemical changes in apoptosis [18]. Apoptotic stimuli recruit procaspase and release cytochrome *c* from mitochondria and induce early event of apoptosis. Mercury decreases mitochondrial membrane potential and alteration of mitochondria membrane potential is involved in apoptosis [11,19]. Activation of caspases was observed following ROS-induced p38 MAPK activation in human leukemia cells [20].

In this study, we hypothesized that the initial effect of mercury in macrophages is the mobilization of Ca^{2+} and generation of ROS, these effects in turn activate pathways leading to apoptosis or necrosis. We therefore investigated the possibility that intracellular Ca^{2+}, ROS, p38, TNFα, and caspases are implicated in mercury-induced apoptosis and necrosis in macrophages.

Materials and Method

Materials

Solution of $HgCl_2$ (Sigma, St. Louis, MO) was freshly prepared in distilled water, filter sterilized (0.22 μM filter), and added to cultures at the indicated concentrations. *N*-acetylcysteine (NAC), silymarin, ethylene glycol-bis(β-aminoethyl ether)-tetra acetic acid

(EGTA), thapsigargin and (Tha) , and benzamil (Ben) were purchased form Sigma Chemical Company (St. Louis, MO). Specific p38 inhibitor SB 203580 was purchased from Calbiochem (La Jolla, CA). Monoclonal anti-TNFα antibody, and fluorescent probes propidium iodide (PI), Hoechst 33258 (H33258), and annexin V were procured from Molecular probes (Eugene, OR).

Cell cultures

BALB/c macrophage cells (J774A.1, ATCC TIB-67) were grown in Dulbecco's minimum essential medium (DMEM, 2 mM glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin) with 10% fetal bovine serum (Atlanta Biologicals, Atlanta, GA) in 5% CO_2 at 37^oC. Solution of $HgCl_2$ was added to cultures to provide 70 μM of $HgCl_2$, controls were treated with equal amount of saline. The concentrations used for various reagents, added 30 min prior to $HgCl_2$ treatment, were 1 mM for NAC, 5 μM for silymarin, 2 μM for SB 203580, 1 μg/ml for anti-TNFα antibody, 1 mM for EGTA, 1 μM for thapsigargin, and 50 μM for benzamil.

ROS generation

The production of ROS was measured by detecting the fluorescent intensity of oxidant-sensitive probes dihydrorhodamine (DHR, Molecular Probes) or 5-chloromethyl-2`,7`-dichlorodihydrofluorescein diacetate (CM-H_2DCFDA, Molecular Probes) [21]. Cells (1×10^5/well in 96-well plates) were washed with Lock' buffer then supplemented with 10 μM DHR or 5 μM CM-H_2DCFDA, and the intracellular and extracellular dye concentrations were allowed to equilibrate at 37^oC/5% CO_2 for 30 min. The cells were incubated with mercury and the kinetics of fluorescent intensity for DHR were recorded using fluorescent

plate reader at excitation of 507 nm, and emission of 529 nm. For CM-H_2DCFDA, fluorescence was detected at excitation of 485 nm, and emission of 530 nm. The fluorescent readings were digitized using SoftMax Pro^{TM}. The results were similar in three independent replications and data from a representative experiment (n = 5 wells) have been illustrated.

MAPK phosphorylation

Phosphorylation of p38, ERK, and JNK were analyzed using anti-phospho-p38, ERK, and JNK antibody (Cell Signaling, Beverly, MA). Cells (1×10^6/well in 6-well plate) were treated with mercury for 15 min. Cells were washed with ice-cold PBS and harvested in a lysis buffer, and cell lysates (25 μg) were subjected to western blot analysis, as described earlier [16]. Immunodetection was performed using enhanced chemiluminescence (ECL) detection kit (Amersham Pharmacia, Piscataway, NJ).

Semiquantitative analysis of TNFα mRNA expression

Total cellular RNA was isolated from cells (5×10^5/well in 24-well plate) following 6 hr treatment with mercury using TRI reagent LS (Molecular Research Center, Cincinnati, OH) according to manufacturer's protocol. The first strand complimentary DNA (cDNA) was synthesized using Superscript II reverse transcriptase enzyme (Life Technologies, Grand Island, NY). Reverse-transcriptase polymerase chain reaction (RT-PCR) was used to analyze the expression of mRNA for TNFα and β-actin (internal control). The condition for reverse transcription and PCR steps were performed as previously reported [16], the primer sets were chosen by Primer3 program (Whithead Institute, Cambridge, MA). Cycle number was optimized to ensure product accumulation in exponential range. Amplified products were separated by electrophoresis on 2% agarose gel and documented using a Kodak DC 290

digital camera and digitized using UN-SCAN-IT software (Silk Scientific, Orem, UT). Band intensities for the TNFα were normalized to that of β-actin in the same sample.

Detection of apoptosis and necrosis

The fluorescent probes PI, H33258, and annexin V were used to assay the necrotic and apoptotic cells following the manufacturer's protocol. Cells (1×10^5/well in 96-well plates) were incubated with mercury for 24 hr. Following incubation, cells were stained with PI (10 μg/ml in PBS), H33258 (10 μg/ml in PBS), or annexin V (5 μl/100 μl in annexin binding buffer) for 15 min and fluorescent intensity was recorded using fluorescent plate reader. PI fluorescent intensity was detected at excitation of 535 nm, emission of 617 nm, H33258 was detected at excitation of 350 nm, emission of 450 nm, and annexin V was detected at excitation of 495 nm, emission of 520 nm. Morphological detection of apoptotic and necrotic bodies were determined using microscope as indicated above.

Determination of caspase-3 activation

Caspase-3 activity was determined using the CaspACE^{TM} fluorometric activity assay (Promega, Madison, WI) with modifications as follows. Briefly, cells were treated in 24 wells following which Triton X-100 was added and repeatedly pipetted to lyse the cells. The homogenates were centrifuged at 10,000×g for 10 min to remove cell debris. The supernatant was assayed for caspase-3 activities using the CaspACE^{TM} system according to the manufacturer's instructions. The fluorescence of cleaved substrate was determined using a fluorescent plate reader. The fluorescence signal was digitized and analyzed using SoftMax Pro^{TM}.

Determination of intracellular Ca^{2+}

Morphological detection of cells was determined using Olympus IX71 inverted microscope (Olympus America, Melville, NY). Fluo-3/AM (Molecular probes) was used to determine intracellular Ca^{2+}. Digital images were acquired using the Magnafire SP (Olympus) digital camera.

Replication, data presentation and statistical Analysis

All experiments were repeated at least 3 times with similar trends; however, data from a representative trial are depicted in the results. Statistical analyses were performed using SAS statistical software (SAS Institute, Cary, NC). Treatment effects were analyzed using one way analysis of variance (ANOVA) followed by Duncan's Multiple Range test. A value of $p < 0.05$ was used to indicate significant differences.

Results

Cytotoxicity of mercury in macrophages

The cytotoxicity of $HgCl_2$ was obtained after 24 hr exposure in J774A.1 macrophages using various measuring parameters (Fig. 1). The EC_{50} was calculated using membrane impermeable DNA binding dye PI, nuclear condensation detecting dye H33258, externalized phosphatidylserine binding dye annexin V, caspase-3 activity, and MTT assay (indirect measure of viable respiring cell). Different assays indicated the range of toxic concentrations (EC_{50}) from 62.7 to 102.8 μM of mercury. We previously reported that mercury at 20 μM for 24 hr did not show any cytotoxicity [16], or caused any necrosis and apoptosis up to 96 hr. In the following experiments, we used 70 μM of mercury for investigating the mechanisms of toxicity for mercury and its signal transduction.

Effect of mercury on ROS

The generation of ROS increased in a time-dependent manner in macrophages by oxidant-sensitive probe DHR in a kinetic assay (Fig. 2A). Iron ($FeSO_4$, 30 μM) was used as a positive control. Mercury-induced generation of ROS was indicated by another oxidant-sensitive probe CM-H_2DCFDA (Fig. 2B). Pretreatment of NAC, an antioxidant and a glutathione (GSH) precursor [22], and silymarin, a scavenger and GSH regulator as well as cell membrane stabilizer [23] reduced mercury-induced ROS generation. Pretreatment of cells with specific p38 inhibitor SB 203580 did not reduce mercury-induced ROS production.

Effect of mercury on MAPKs

To examine the effect of mercury on activation of MAPKs, we assayed the phosphorylation of three types of MAPK, i.e., p38, ERK, and JNK (Fig. 3). Stimulation of macrophages with LPS (as a positive control) activated all three types of MAPKs (Fig. 3A); this activation peaked at 15 min and decreased after 30 min (data not shown). The non-cytotoxic concentration of mercury (20 μM) activated p38 and ERK but not JNK. Mercury activated p38 as much as LPS; moreover, mercury additively activated LPS-induced p38 but not ERK. To examine the relation of mercury-induced cytotoxic signal transduction in macrophages between ROS production and p38 activation, we pretreated the cells with antioxidants (Fig. 3B). Mercury at 70 μM activated p38, but antioxidants NAC and silymarin, and the specific p38 inhibitor, SB 203580, decreased mercury-induced p38 activation.

Effect of mercury on TNFα expression

The induction of TNFα is mediated by the activation of different members of the MAPKs [24,25]. p38 is a known regulator of TNFα expression [26]. Expression of TNFα in

macrophages was measured by RT-PCR (Fig. 4). Treatment of cells with mercury for 6 hr increased TNFα mRNA and pretreatment of cultures with antioxidant NAC and silymarin, and specific p38 inhibitor, SB 203580, reduced mercury-induced TNFα. However, anti-TNFα antibody had no effect on mercury induced TNFα expression.

Effect of mercury on apoptosis and necrosis in macrophage cells

Mercury-induced apoptosis and necrosis, and effects of pharmacological agents were assayed (Fig. 5). The binding of cell membrane-impermeable nuclear stain dye (PI) recognized the necrotic cells [27]. Treatment of cells with non-cytotoxic level of mercury (20 μM) did not induce necrosis up to 96 hr (data not shown). Treatment of cells with mercury at 70 μM induced necrosis at 24 hr (Fig. 5A). Pretreatment of cells with antioxidants, p38 inhibitor, and anti-TNFα antibody decreased mercury-induced necrosis.

The externalization of phosphatidyl serine (PS) is an early event in apoptosis and represents a marker to induce phagocytosis by mononuclear cells [27]. This event as apoptosis was determined using the annexin V binding assay. The activation of caspase-3 is an integral step in the majority of apoptotic events. This enzyme belongs to the cysteine protease family and is responsible for cleaving substrates such as DNA fragmentation factor that can damage DNA [18]. Treatment of cells with 20 μM of mercury did not induce apoptosis up to 96 hr as indicated by annexin V binding and caspase-3 activity (data not shown). Treatment of cells with mercury at 70 μM induced apoptosis at 24 hr (Fig. 5B, C). Pretreatment of antioxidants and p38 inhibitor decreased mercury-induced annexin V binding and caspase-3 activity.

The presence of both apoptosis and necrosis was further confirmed by cell morphology in a microscope. The nuclear morphology of cells exposed to mercury displayed

increased number of stained cells with fragmented nuclei, a hallmark of apoptosis (Fig. 6A). Treatment of macrophage cell lines with 70 μM mercury caused a mixture of apoptosis and necrosis. In Fig. 6B, some cells were stained by annexin V, whereas others were stained by PI. The two cell populations, apoptotic and necrotic are also indicated by overlay of the two images, (Fig. 6B, upper right), along with a phase contrast of the same field (Fig. 6B, upper left) for orientation of cells.

Role of intracellular Ca^{2+} on mercury-induced signaling

To examine the effect of mercury on the intracellular Ca^{2+}, we measured the intracellular Ca^{2+} as fluorescence microscopic assay using Fluo-3/AM (Fig. 7A). Mercury increased intracellular Ca^{2+} and pretreatment of Ca^{2+} chelator EGTA reduced mercury-induced intracellular Ca^{2+}. To assay the relation between intracellular Ca^{2+} and ROS, we treated the various types of intracellular Ca^{2+} inhibitors and measured ROS production at 30 min (Fig. 7B). EGTA is chelator of extracellular Ca^{2+}, thapsigargin is endoplasmic reticulum (ER) Ca^{2+}-ATPase inhibitors, and benzamil is Na^{+}/Ca^{2+} exchanger blocker. All three types of intracellular Ca^{2+} inhibitors significantly decreased mercury-induced ROS production and combine of all inhibitors completely blocked ROS generation.

Discussion

It is well known that induction of apoptosis plays a key role in maintaining homeostasis in the immune system; however, the mechanisms of the effects of toxicants such as mercury on immune cell death are not established. The objectives of this study were to determine the nature of mercury-induced cell death, to discriminate between mercury-

induced apoptosis and necrosis, and to elucidate the underlying signaling pathways involved in these processes in macrophages.

The dose-response toxicity of mercury showed relatively steep curves. Up to 20 μM mercury did not induce any cytotoxicity in 96 hr treatment, however, over 150 μM of mercury killed majority of cells. When we determined the EC_{50} of mercury using various types of indictors, we found an EC_{50} of approximately 70 μM of mercury. Treatment with mercury initiated both apoptosis and necrosis in murine macrophages. The results of this study provide convincing evidence that mercury is immunotoxic, at least in part, due to its ability to induce apoptosis.

Mitochondria appear to be a target organelle for mercury-induced cell death. Mercury decreased mitochondrial transmembrane potential and increased ROS generation, and consequent depletion of GSH and lipid peroxidation and caspase activation in human lymphocytes [2,11,28]. It has been suggested that ROS act as a second messenger in cell death signaling and regulate both apoptosis and necrosis. There is cross talk between the cellular signaling system and the cellular redox state. Death signals induce apoptosis through the activation of caspases in cells [29]. The enzymatic activity of caspase is regulated by the redox conditions of the cysteine residues. There are two main caspase pathways. One is mitochondria-mediated caspase-9 cascade and another is Fas-mediated caspase-8 cascade, and both pathways converge on caspase-3 activation. The changes in mitochondrial membrane potential open the megapore channels and release variety of mitochondrial associated proteins include cytochrome *c* and Apaf-1, component of apoptosome, and activates caspase cascade. Activation of downstream caspase-3 is regulated by cytochrome c and caspase-9. It has been reported that mercury induces caspase-9 dependent apoptosis in

lymphocytes and leukemia cell, and in part Fas-mediated caspase-8 also regulates mercury-induced apoptosis in lymphocytes [19,30].

In our current study, mercury increased ROS, p38, and caspase-3 activation; moreover, pretreatment of antioxidants decreased both mercury-induced apoptosis and necrosis. These data suggest that ROS are the major mediator and regulator of mercury-induced cell death and control both apoptosis and necrosis. Ueda et al., [31] proposed the mechanism of ROS regulation on apoptosis and necrosis. When apoptosis was induced in cells with apoptotic agent, the intracellular ROS level was maintained by buffering of reducing environment and result in csapase-3 activation. In contrast, in some case, the intracellular ROS was increased and process of caspase-3 was not detected, despite cytochrome *c* release resulting in necrosis in lymphocytes.

Oxidative stress induces activation of MAPKs and results in caspase activation [31,32]. MAPKs have important activities as mediators of cellular responses to extracellular signals. There are only a few reports about MAPKs related to mercury effects. Our laboratory, for the first time, reported that mercury activated p38 and additively increased LPS (as MAPKs stimulator)-induced p38, and this p38 regulated expression of TNFα in macrophages [16]. Our present study shows that antioxidants blocked mercury-induced activation of p38 but specific p38 inhibitor did not block mercury-induced ROS production, which indicates ROS play a role as upstream regulator of p38 in macrophages. Pretreatment of antioxidants and p38 inhibitor blocked mercury-induced cell death. Taken together, the results suggest that mercury-induced ROS initiated cell damage by p38 mediated caspase-3 death signaling. Oxidative stress activates apoptosis signal-regulating kinase 1 (ASK1) [31]. ASK1 is upstream of p38 and is involved in the mechanism of stress and cytokine-induced

apoptosis, and induces cytochrome *c* and activation of caspase-3. A more detailed study to mercury-induced cell death signaling, especially p38 MAPK related signaling cascade such as ASK1 and MKK3/6 is necessary. In addition to p38, ERK also regulates TNFα production in macrophages [33]. Our data show that treatment of mercury at 20 μM activated ERK (Fig. 4A). However, we predict that ERK is not a major pathway for mercury-induced cytotoxicity because 1) ERK is thought to act as mediator of proliferation and differentiation whereas p38 and JNK are classified as stress-induced MAPK [24]. 2) treatment of mercury plus LPS in macrophages did not exceed ERK activation than mercury alone, and 3) specific ERK inhibitor, PD 98059, did not alter LPS-induced TNFα expression in macrophages (data not shown).

TNFα is the most potent inducer of several intracellular signals including apoptosis, and is produced abundantly by macrophages and regulated by p38 [34]. We here show that mercury increased the expression of TNFα, and pretreatment with antioxidants and specific p38 inhibitor prevented this effect. Interestingly, mercury-induced necrosis was decreased by pretreatment with anti-TNFα antibody but apoptosis was not affected. It may suggest that TNFα produced by mercury acts as inducer of necrotic cell death but not apoptotic cell death.

Mercury-induced cytotoxicity was mediated by increase of intracellular Ca^{2+} and ROS in many cell types [2,10,11]. Increase in intracellular Ca^{2+} leads to cell death through several downstream reactions, such as inducing ROS [11,35]. The rise of intracellular Ca^{2+} may be due to an alteration both in intracellular Ca^{2+} stores and in Ca^{2+} influx across plasma membrane. Blocking of Ca^{2+} channel, Na^{+}/Ca^{2+} exchanger, and inhibition of endoplasmic reticulum (ER) intracellular Ca^{2+}-ATPase prevented mercury-induced cytotoxicity in neuronal cells [36-38]. Our data elucidated that intracellular Ca^{2+} is upstream of mercury-

induced ROS production, evidenced by the decrease of ROS by Ca^{2+} antagonists. Entry of Ca^{2+} from extracellular, ER, and Na^{+}/Ca^{2+} channel contributes to mercury-induced cell death in macrophages.

Extracellular stimuli elicit a variety of responses through the cellular signaling systems such as Ca^{2+} and ROS, which in turn lead to the activation of protein tyrosine kinases. These biochemical reactions finally reach the nucleus, resulting in expression of inflammatory cytokines, TNFα, mediated by the activation of several factors such as MAPKs and NF-κB [29,39]. NF-κB, a pleiotropic transcriptional factor, promotes cell survival and protects cells from apoptosis, and requires reduced thiols at critical steps in its activation pathway. Mercury, one of the strongest thiol-binding agents known, impairs NF-κB activation and transcriptional activity via competitive mercaptide formation, and this inhibition is reversed by the addition of competitive thiols [40]. However, our previous study revealed that mercury itself did not modulate NF-κB, instead it decreased LPS-stimulated NF-κB activation and downstream expression of iNOS but not of TNFα in macrophages [16]. Additionally, it has been reported that activation of NF-κB in epithelial cells is mediated via a ROS-insensitive, calcium-dependent pathway [41]. This may suggest that the ROS-induced cell death/survival signaling pathways differ from cell to cell, and p38 but not NF-κB is dominant signaling pathway in mercury-induced cell death in macrophages.

In summary, this report is the first to show that mercury-induced apoptosis is mediated by ROS-activated p38 MAPK followed by caspase activation in macrophages. A schematic diagram of mercury-induced cell apoptosis vs necrosis is depicted in Fig. 8; mercury-induced cell death was a mix of apoptosis and necrosis. Mercury-induced ROS and downstream p38 MAPK regulate both apoptosis and necrosis signaling pathways. p38

medicated caspase-3 activation is responsible for mercury-induced apoptosis; p38 mediated TNFα is responsible for mercury-induced necrosis in macrophages. Ca^{2+} is upstream of mercury-induced ROS production.

Reference

[1] Guo, T. L.; Miller, M. A.; Shapiro, I. M.; Shenker, B. J. Mercuric chloride induces apoptosis in human T lymphocytes: evidence of mitochondrial dysfunction. *Toxicol. Appl. Pharmacol.* **153**:250-257; 1998.

[2] InSug, O.; Datar, S.; Koch, C. J.; Shapiro, I. M.; Shenker, B. J. Mercuric compounds inhibit human monocyte function by inducing apoptosis: evidence for formation of reactive oxygen species, development of mitochondrial membrane permeability transition and loss of reductive reserve. *Toxicology* **124**:211-224; 1997.

[3] Jiang, Y.; Moller, G. In vitro effects of HgCl2 on murine lymphocytes. I. Preferable activation of CD4+ T cells in a responder strain. *J. Immunol.* **154**:3138-3146; 1995.

[4] Christensen, M. M.; Ellermann-Eriksen, S.; Rungby, J.; Mogensen, S. C. Influence of mercuric chloride on resistance to generalized infection with herpes simplex virus type 2 in mice. *Toxicology* **114**:57-66; 1996.

[5] Silbergeld, E. K.; Sacci, J. B., Jr.; Azad, A. F. Mercury exposure and murine response to Plasmodium yoelii infection and immunization. *Immunopharmacol. Immunotoxicol.* **22**:685-695; 2000.

[6] Kaiser, J. Mercury report backs strict rules. *Science* **289**: 371-372; 2000.

[7] Morrissette, N.; Gold, E.; Aderem, A. The macrophage--a cell for all seasons. *Trends Cell Biol.* **9**:199-201; 1999.

[8] Gregory, S. H.; Wing, E. J. Neutrophil-Kupffer cell interaction: a critical component of host defenses to systemic bacterial infections. *J. Leukoc. Biol.* **72**:239-248; 2002.

[9] Buttke, T. M.; Sandstrom, P. A. Oxidative stress as a mediator of apoptosis. *Immunol. Today* **15**:7-10; 1994.

[10] Close, A. H.; Guo, T. L.; Shenker, B. J. Activated human T lymphocytes exhibit reduced susceptibility to methylmercury chloride-induced apoptosis. *Toxicol. Sci.* **49**:68-77; 1999.

[11] Shenker, B. J.; Guo, T. L.; Shapiro, I. M. Low-level methylmercury exposure causes human T-cells to undergo apoptosis: evidence of mitochondrial dysfunction. *Environ. Res.* **77**:149-159; 1998.

[12] Bonni, A.; Brunet, A.; West, A. E.; Datta, S. R.; Takasu, M. A.; Greenberg, M. E. Cell survival promoted by the Ras-MAPK signaling pathway by transcription-dependent and -independent mechanisms. *Science* **286**:1358-1362; 1999.

[13] Seger, R.; Krebs, E. G. The MAPK signaling cascade. *FASEB J.* **9**:726-735; 1995.

[14] Ichijo, H.; Nishida, E.; Irie, K.; ten Dijke, P.; Saitoh, M.; Moriguchi, T.; Takagi, M.; Matsumoto, K.; Miyazono, K.; Gotoh, Y. Induction of apoptosis by ASK1, a mammalian MAPKKK that activates SAPK/JNK and p38 signaling pathways. *Science* **275**:90-94; 1997.

[15] Ichijo, H. From receptors to stress-activated MAP kinases. *Oncogene* **18**:6087-6093; 1999.

[16] Kim, S. H.; Johnson, V. J.; Sharma, R. P. Mercury inhibits nitric oxide production but activates proinflammatory cytokine expression in murine macrophage: differential modulation of NF-kappaB and p38 MAPK signaling pathways. *Nitric. Oxide.* **7**:67-74; 2002.

[17] Lee, J. C.; Young, P. R. Role of CSB/p38/RK stress response kinase in LPS and cytokine signaling mechanisms. *J. Leukoc. Biol.* **59**:152-157; 1996.

[18] Sharif-Askari, E.; Alam, A.; Rheaume, E.; Beresford, P. J.; Scotto, C.; Sharma, K.; Lee, D.; DeWolf, W. E.; Nuttall, M. E.; Lieberman, J.; Sekaly, R. P. Direct cleavage of the human DNA fragmentation factor-45 by granzyme B induces caspase-activated DNase release and DNA fragmentation. *EMBO J.* **20**:3101-3113; 2001.

[19] Araragi, S.; Kondoh, M.; Kawase, M.; Saito, S.; Higashimoto, M.; Sato, M. Mercuric chloride induces apoptosis via a mitochondrial-dependent pathway in human leukemia cells. *Toxicology* **184**:1-9; 2003.

[20] Zhuang, S.; Demirs, J. T.; Kochevar, I. E. p38 mitogen-activated protein kinase mediates bid cleavage, mitochondrial dysfunction, and caspase-3 activation during apoptosis induced by singlet oxygen but not by hydrogen peroxide. *J. Biol. Chem.* **275**:25939-25948; 2000.

[21] Royall, J. A.; Ischiropoulos, H. Evaluation of 2',7'-dichlorofluorescin and dihydrorhodamine 123 as fluorescent probes for intracellular H2O2 in cultured endothelial cells. *Arch. Biochem. Biophys.* **302**:348-355; 1993.

[22] Bernard, G. R. N-acetylcysteine in experimental and clinical acute lung injury. *Am. J. Med.* **91**:54S-59S; 1991.

[23] Wellington, K.; Jarvis, B. Silymarin: a review of its clinical properties in the management of hepatic disorders. *BioDrugs.* **15**:465-489; 2001.

[24] Arbabi, S.; Maier, R. V. Mitogen-activated protein kinases. *Crit Care Med.* **30**:S74-S79; 2002.

[25] Dong, C.; Davis, R. J.; Flavell, R. A. MAP kinases in the immune response. *Annu. Rev. Immunol.* **20**:55-72; 2002.

[26] Baldassare, J. J.; Bi, Y.; Bellone, C. J. The role of p38 mitogen-activated protein kinase in IL-1 beta transcription. *J. Immunol.* **162**:5367-5373; 1999.

[27] Zurgil, N.; Shafran, Y.; Fixler, D.; Deutsch, M. Analysis of early apoptotic events in individual cells by fluorescence intensity and polarization measurements. *Biochem. Biophys. Res. Commun.* **290**:1573-1582; 2002.

[28] Shenker, B. J.; Guo, T. L.; O I; Shapiro, I. M. Induction of apoptosis in human T-cells by methyl mercury: temporal relationship between mitochondrial dysfunction and loss of reductive reserve. *Toxicol. Appl. Pharmacol.* **157**:23-35; 1999.

[29] Kamata, H.; Hirata, H. Redox regulation of cellular signalling. *Cell Signal.* **11**:1-14; 1999.

[30] Shenker, B. J.; Pankoski, L.; Zekavat, A.; Shapiro, I. M. Mercury-induced apoptosis in human lymphocytes: caspase activation is linked to redox status. *Antioxid. Redox. Signal.* **4**:379-389; 2002.

[31] Ueda, S.; Masutani, H.; Nakamura, H.; Tanaka, T.; Ueno, M.; Yodoi, J. Redox control of cell death. *Antioxid. Redox. Signal.* **4**:405-414; 2002.

[32] Lee, M. W.; Park, S. C.; Yang, Y. G.; Yim, S. O.; Chae, H. S.; Bach, J. H.; Lee, H. J.; Kim, K. Y.; Lee, W. B.; Kim, S. S. The involvement of reactive oxygen species (ROS) and p38 mitogen-activated protein (MAP) kinase in TRAIL/Apo2L-induced apoptosis. *FEBS Lett.* **512**:313-318; 2002.

[33] Ajizian, S. J.; English, B. K.; Meals, E. A. Specific inhibitors of p38 and extracellular signal-regulated kinase mitogen-activated protein kinase pathways

block inducible nitric oxide synthase and tumor necrosis factor accumulation in murine macrophages stimulated with lipopolysaccharide and interferon-gamma. *J. Infect. Dis.* **179**:939-944; 1999.

[34] MacKenzie, S.; Fernandez-Troy, N.; Espel, E. Post-transcriptional regulation of TNF-alpha during in vitro differentiation of human monocytes/macrophages in primary culture. *J. Leukoc. Biol.* **71**:1026-1032; 2002.

[35] Lipton, S. A.; Nicotera, P. Calcium, free radicals and excitotoxins in neuronal apoptosis. *Cell Calcium* **23**:165-171; 1998.

[36] Gasso, S.; Cristofol, R. M.; Selema, G.; Rosa, R.; Rodriguez-Farre, E.; Sanfeliu, C. Antioxidant compounds and Ca(2+) pathway blockers differentially protect against methylmercury and mercuric chloride neurotoxicity. *J. Neurosci. Res.* **66**:135-145; 2001.

[37] Marty, M. S.; Atchison, W. D. Elevations of intracellular Ca2+ as a probable contributor to decreased viability in cerebellar granule cells following acute exposure to methylmercury. *Toxicol. Appl. Pharmacol.* **150**:98-105; 1998.

[38] Sakamoto, M.; Ikegami, N.; Nakano, A. Protective effects of Ca2+ channel blockers against methyl mercury toxicity. *Pharmacol. Toxicol.* **78**:193-199; 1996.

[39] Haddad, J. J. Redox regulation of pro-inflammatory cytokines and IkappaB-alpha/NF-kappaB nuclear translocation and activation. *Biochem. Biophys. Res. Commun.* **296**:847-856; 2002.

[40] Woods, J. S.; Dieguez-Acuna, F. J.; Ellis, M. E.; Kushleika, J.; Simmonds, P. L. Attenuation of nuclear factor kappa B (NF-kappaB) promotes apoptosis of kidney

epithelial cells: a potential mechanism of mercury-induced nephrotoxicity. *Environ. Health Perspect.* **110 Suppl 5**:819-822; 2002.

[41] Woods, J. S.; Ellis, M. E.; Dieguez-Acuna, F. J.; Corral, J. Activation of NF-kappaB in normal rat kidney epithelial (NRK52E) cells is mediated via a redox-insensitive, calcium-dependent pathway. *Toxicol. Appl. Pharmacol.* **154**:219-227; 1999.

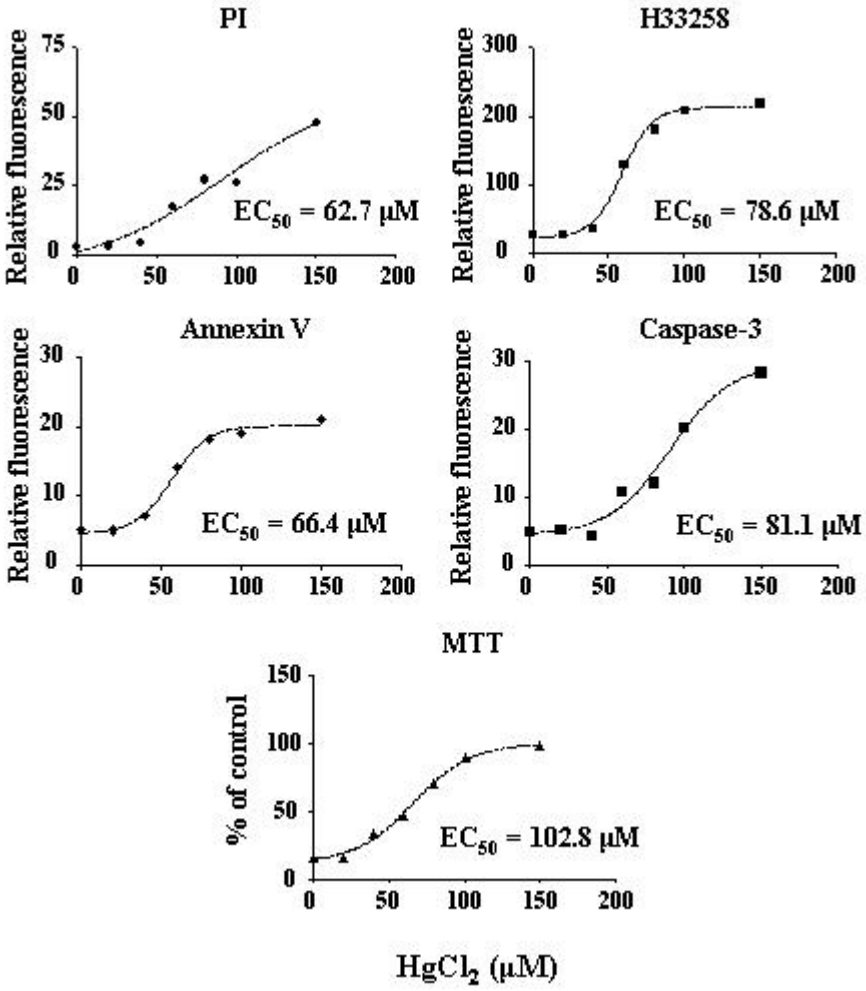


Fig. 4.1. EC_{50} of $HgCl_2$ in various assays. Cells (1×10^5/well in 96-well plates) were incubated with $HgCl_2$ at the indicated concentrations for 24 hr. The value of EC_{50} was calculated by Prism® Version 3.0 (GraphPad Software Inc., San Diego, CA).

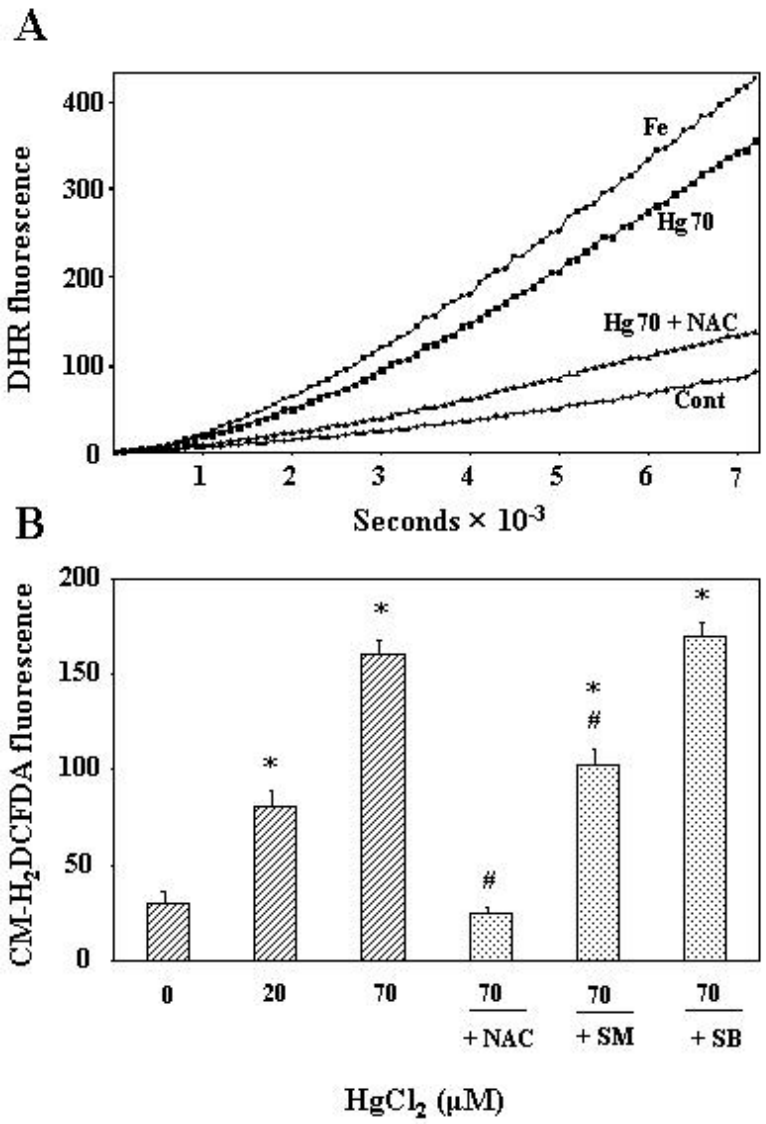


Fig. 4.2. Effects of $HgCl_2$ on ROS production. (A) An illustration of real-time changes up to 2 hr for $HgCl_2$ 70 μM. (B) Fluorescence intensity treated with $HgCl_2$ at the indicated concentrations at 2 hr. All pharmacological agents were added 30 min prior to mercury treatment. Iron ($FeSO_4$, 30 μM) was used as a positive control. Results from a representative experiment of a replicates are expressed as mean ± SE (n=5). *Significantly different than the vehicle-treated cultures at $p < 0.05$. #Significantly different than the mercury-treated cultures at $p < 0.05$. Abbreviations used are SM, silymarin; SB, SB 203580.

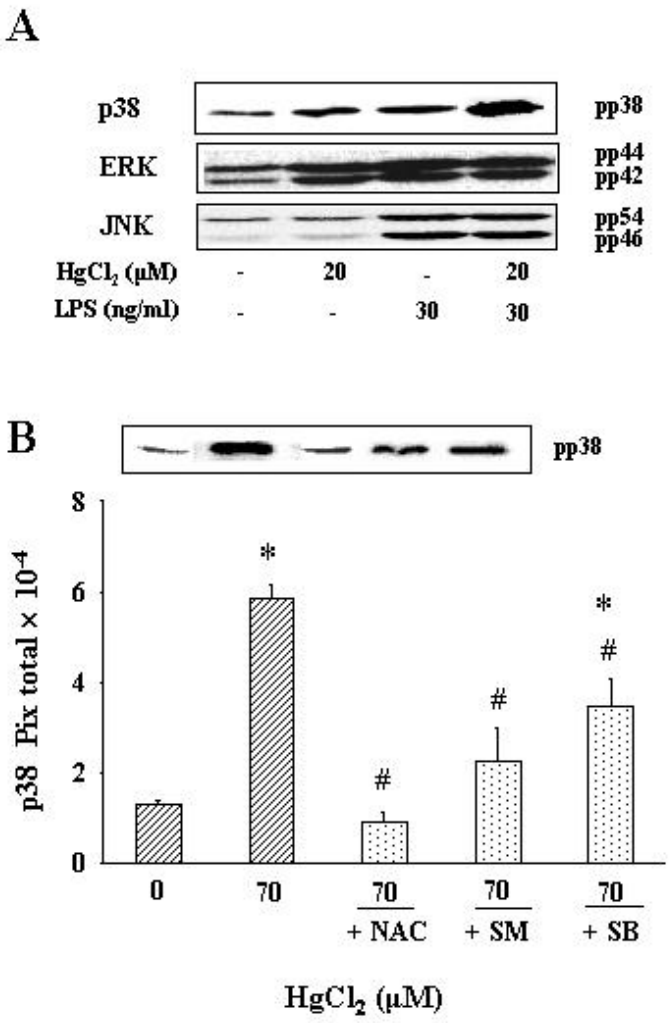


Fig. 4.3. Effect of $HgCl_2$ on MAPKs activation. (A) Cells (1×10^6/well in 6-well plate) were activated 15 min with $HgCl_2$ (20 μM) alone, LPS (30 ng/ml) alone and $HgCl_2$ with LPS. Extract of cell (25 μg) was analyzed by 12% SDS-PAGE, and p38, ERK, and JNK were visualized by western blot analysis. (B) Cells were treated with $HgCl_2$ (70 μM). All pharmacological agents were added 30 min prior to mercury treatment. Results are expressed as mean ± SE. *Significantly different than the control group at $p < 0.05$. #Significantly different than the mercury-treated group at $p < 0.05$. Insert, a representative western blot in the same order as bars. Abbreviations used are same as in Fig. 4.2.

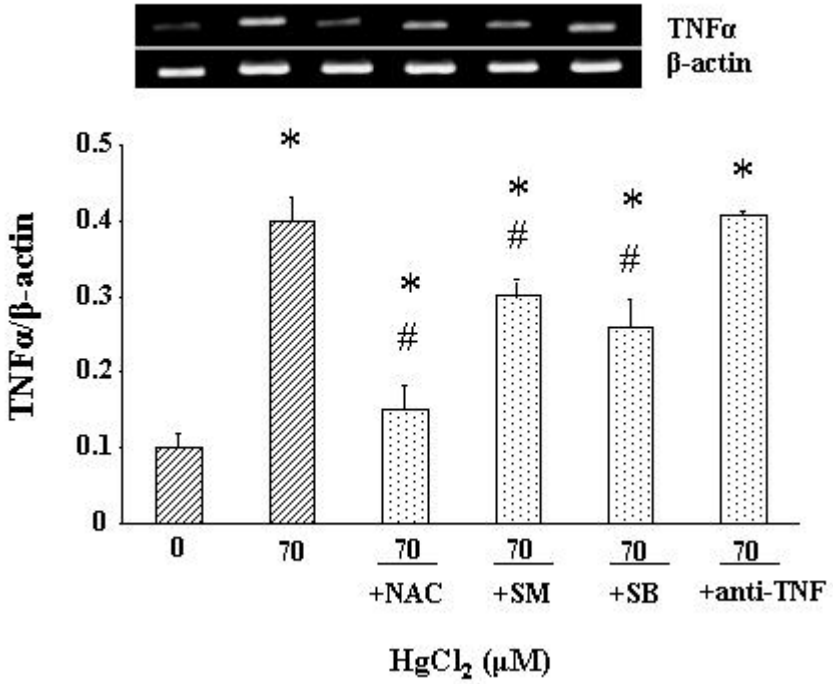


Fig. 4.4. Effect of $HgCl_2$ on TNFα mRNA expression. Cells (5×10^5/well in 24-well plate) were activated 6 hr with $HgCl_2$. Extraction and analysis of mRNA performed as described in Materials and Methods. TNFα mRNA levels were quantified by RT-PCR and normalized against β-actin. All pharmacological agents were added 30 min prior to mercury treatment. Results are expressed as mean ± SE. *Significantly different than the vehicle-treated group at $p < 0.05$. #Significantly different than the mercury-treated group at $p < 0.05$. Abbreviations used are same as in Fig. 4.2.

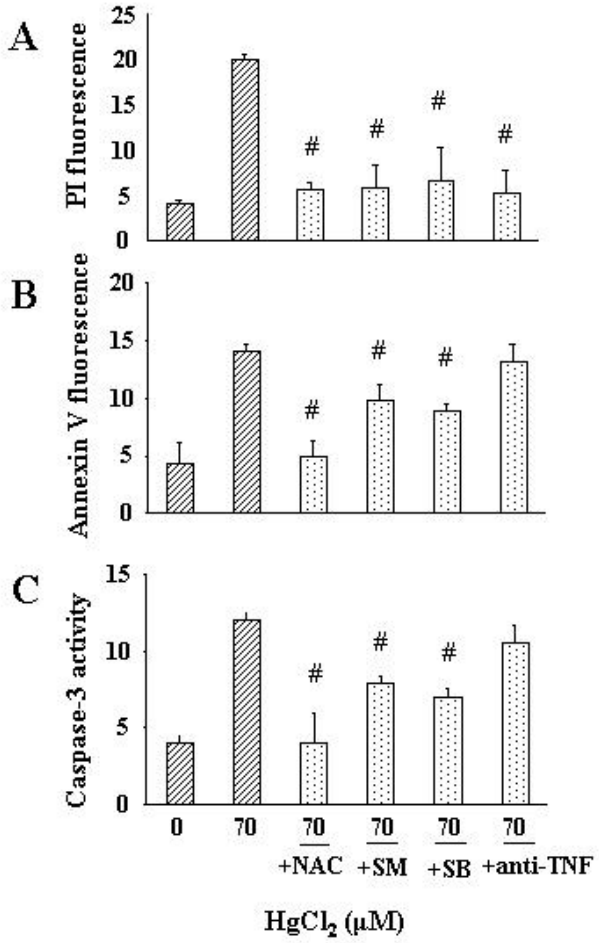


Fig. 4.5. Apoptotic and necrotic effect of $HgCl_2$. Cells (1×10^5/well in 96-well plates) were incubated with mercury for 24 hr then stained with PI (10 μg/ml) (A), or annexin V (5 μl/100 μl) (B) for 15 min. Fluorescent intensity was recorded using fluorescent plate reader. (C) Cells (5×10^5/well in 24-well plates) were treated with mercury for 24 hr. Cell lysation and analysis of caspase-3 activity was performed as described in Materials and Methods. All pharmacological agents (NAC, 1 mM; silymarin, 5 μM; SB 203580, 2 μM; anti-TNFα antibody, 1 μg/ml) were added 30 min prior to mercury treatment. Results are expressed as mean ± SE. #Significantly different than the mercury-treated group at $p < 0.05$. Abbreviations used are same as in Fig. 4.2.

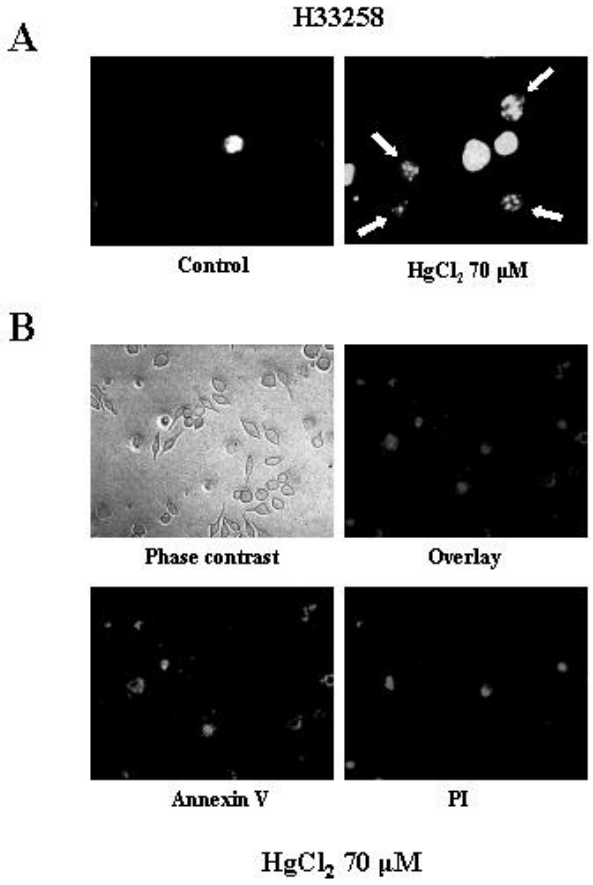


Fig. 4.6. Effect of $HgCl_2$ on nuclear morphology and cell death. Cells (2×10^5/well in 4-well chamber slide) were incubated with 70 μM $HgCl_2$ for 24 hr and stained with fluorescent dye and fluorescence microscopy was performed. (A) For nuclear morphology for detection of apoptotic bodies, cells were stained with H33258 and visualized. Representative photographs display intact nuclei in untreated cell and fragmented nuclei in cells (as indicated by arrows). (B) For comparison of apoptotic and necrotic cell, cells were co-stained with annexin V and PI, and the fluorescence was visualized by fluorescence microscope. Representative photographs show phase contrast (upper left), overlap image of annexin V and PI detection of apoptotic and necrotic cell (upper right), annexin V for apoptosis (lower left), and PI for necrosis (lower right).

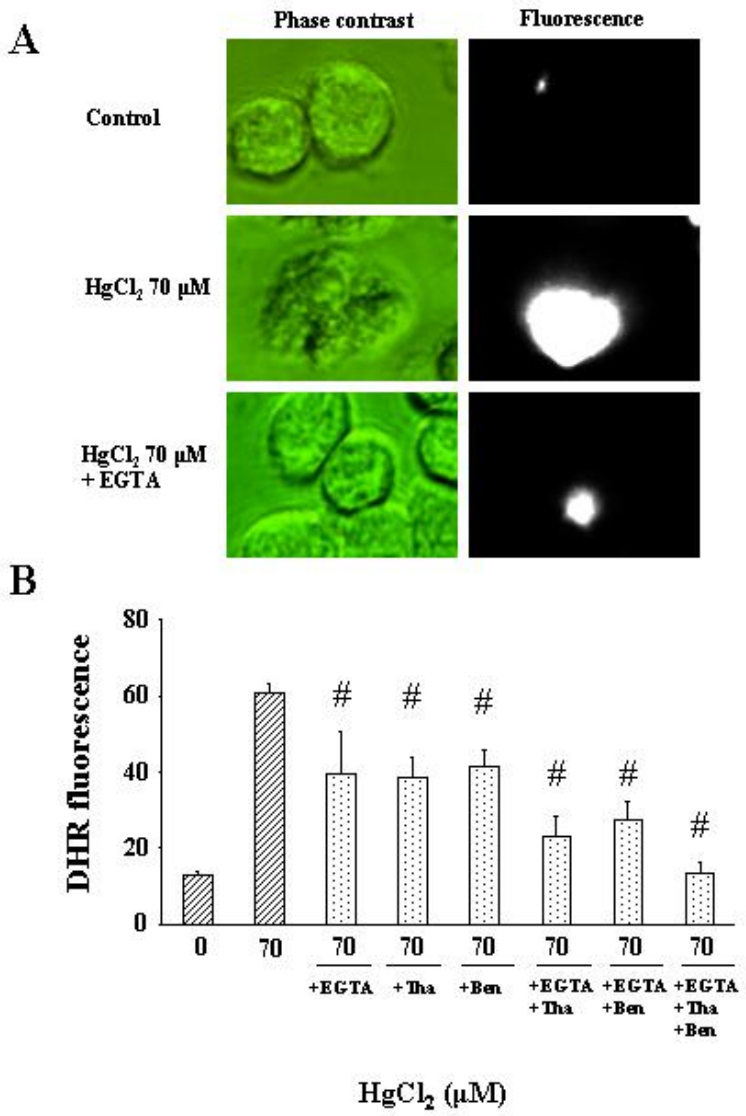


Fig. 4.7. Role of intracellular calcium on ROS production. (A) Cells (1×10^5/well in 96-well plates) were treated with 70 µM $HgCl_2$. Intracellular calcium was stained with Fluo-3/AM after mercury treatment. Morphological and fluorescence change in cells was detected by an inverted microscope. (B) The fluorescent intensity was recorded using fluorescent plate reader at 30 min. All pharmacological agents were added 30 min prior to mercury treatment. Results are expressed as mean ± SE. $^{\#}$Significantly different than the mercury group at $p < 0.05$. Abbreviations used are Tha, thapsigargin; Ben, benzamil.

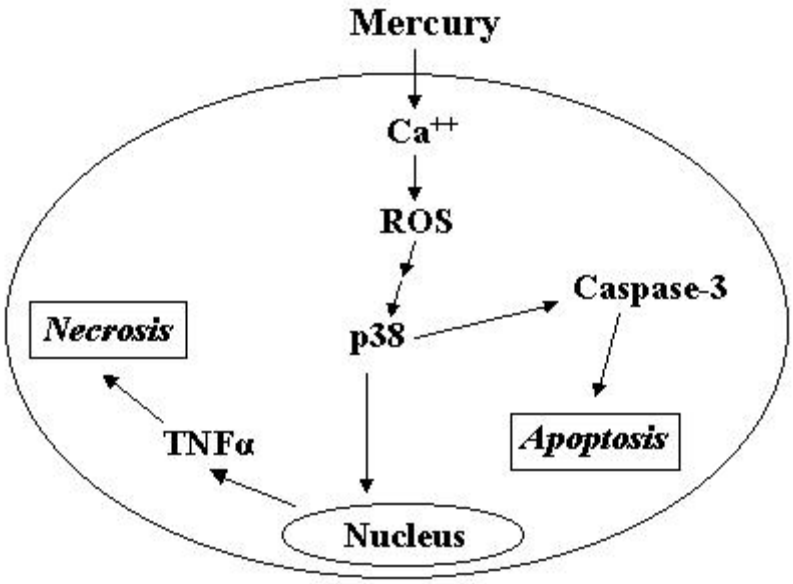


Fig. 4.8. The proposed signaling pathways for $HgCl_2$ toxicity. Mercury-induced cell death is mix of apoptosis and necrosis. Mercury increases ROS production and activates downstream p38 MAPK. Both ROS and p38 MAPK regulates mercury-induced apoptosis and necrosis. p38 mediated caspase-3 activation is the signaling for mercury-induced apoptosis. p38 mediated TNFα is the signaling for mercury-induced necrosis. Intracellular calcium is upstream of mercury-induced ROS production. Because anti- TNFα antibody prevented mercury-mediated necrosis bur not apoptosis, the role of TNFα appears primarily in inducing necrosis in this model.

CHAPTER 5

CYTOTOXICITY OF INORGANIC MERCURY IN MURINE T AND B LYMPHOMA CELL LINES: INVOLVEMENT OF REACTIVE OXYGEN SPECIES, Ca^{2+} HOMEOSTASIS, AND CYTOKINE GENE EXPRESSION[1]

---

[1]Kim S.H., Raghubir P. Sharma. Accepted by *Toxicology in Vitro*.

Abstract

Mercury is a highly toxic heavy metal; exposure to mercury in humans and animals causes damage in several organs or systems including the immune system. To characterize the toxicity of mercury in the immune cells, the cytotoxic effects of inorganic mercury were studied in two distinct lymphoma lines, the murine T lymphoma (EL4) and B lymphoma (A20) cells. Mercury concentration-dependently decreased cell viability, membrane integrity, and proliferation in both EL4 and A20 cells. Mercury increased the reactive oxygen species (ROS) production in both EL4 and A20 cells, and pretreatment with antioxidants reversed mercury-induced ROS generation. Pretreatment of cells with antioxidants *N*-acetylcysteine (NAC) and silymarin decreased mercury-induced lactate dehydrogenase (LDH) release in both types of cells; however, Ca^{2+} channel blocker lanthanum (La^{2+}) decreased it only in A20 cells. The mode of cytotoxicity was a mixture of both apoptosis and necrosis. Mercury-induced apoptosis and necrosis in the two cell lines were indicated by staining with Hoechst 33258, propidium iodide, and co-staining with annexin V and propidium iodide. Both mercury-induced apoptosis and necrosis were attenuated by antioxidants. Mercury increased gene expression of IL-4 and TNFα in EL4 cells; these cytokines were not expressed in A20 cells. Data suggested different pathways of mercury-induced cytotoxicity in T and B lymphoma cells and involvement of ROS, Ca^{2+} homeostasis, and inflammatory cytokine gene expression.

Keywords: mercury, ROS, apoptosis, necrosis, calcium, tumor necrosis factor

1. Introduction

Mercury is a heavy metal widespread in the environment and consequently there are large populations that are exposed to low levels of mercury as a result of ubiquitous environmental factors. Mercury exerts its toxic effect in mammalian organ systems and also elicits autoimmunity (Bagenstose et al., 1999; Hu et al., 1999). There is evidence that mercury interferes with the function of lymphocytes. Mercury induces lymphocyte proliferation, increased level of immunoglobulin, autoantibody production, and immune-complex deposits (Pelletier et al., 1988; Hultman and Enestrom, 1987). Mercury induces protein tyrosine phosphorylation in B lymphoma via interfering lymphocytes signal transduction (Rosenspire et al., 1998).

We recently reported that mercury impaired host defense to bacterial infection via decreasing nitric oxide (NO) production in a murine macrophage cell line (Kim et al., 2002). Low doses of mercury decreased lipopolysaccharide-induced NO production, an important element to host resistance and acquisition of immunity. This effect was related with the inhibition of nuclear factor-κB and alteration of pro-inflammatory cytokines.

Mercury generates reactive oxygen species (ROS) and causes lipid peroxidation, implying that the generated ROS are involved in mercury-induced cytotoxicity in murine hepatocytes and brain cells (Stacey and Kappus, 1982; Hussain et al., 1997). In addition, mercury elevates intracellular Ca^{2+} concentration by promoting extracellular Ca^{2+} entry as well as Ca^{2+} release from intracellular stores in neuronal cells (Denny and Atchison, 1996). Some Ca^{2+} blockers inhibit mercury-mediated cytotoxicity, suggesting the involvement of Ca^{2+} in mercury-mediated cytotoxicity. The role of ROS generation and influx of extracellular Ca^{2+} in mercury-mediated toxicity in lymphocytes remains to be clarified.

Recently, it was reported that mercury treated cells die in a manner consistent with the induction of apoptosis (Shenker et al., 1998, 2002; InSug et al., 1997). Prior to death, mercury treated cells exhibited an elevation of Ca^{2+}, alterations in membrane function, and ROS generation.

Treatment of lymphocytes with mercury modulates the production of many important inflammatory cytokine such as interleukin (IL)-1, IL-2, IL-4, tumor necrosis factor α (TNFα), and interferon γ (IFNγ) (Badou et al., 1997; Hu et al., 1997; Johansson et al., 1997). While several studies have been reported, the findings have been somewhat contradictory and different effects of mercury were shown depending on species, strain and cell type (InSug et al., 1997; Johansson et al., 1997).

The objectives of this study were to define the nature of the mercury-induced cytotoxicity, apoptosis versus necrosis, and involvement of ROS and Ca^{2+} homeostasis in murine lymphocyte cell lines. The results revealed that mercury caused both apoptosis and necrosis. The data suggested that ROS, Ca^{2+}, and related inflammatory cytokine are involved in mercury induced cytotoxicity and the existence of different pathways in various populations of murine lymphocytes.

2. Materials and Methods

2.1. Reagents

$HgCl_2$ (Sigma, St. Louis, MO) was dissolved in distilled water, filtered (0.22 μM filter) and freshly prepared solutions added to cultures at the indicated concentrations. Antioxidant and glutathione (GSH) precursor *N*-acetylcysteine (NAC, Bernard 1991), scavenger and GSH regulator silymarin (Aldrich product number 25492-4, Fraschini et al.,

2002), Ca^{2+} channel blocker lanthanum chloride (source of La^{2+}), Ca^{2+} chelator ethylene glycol-bis(β-aminoethyl ether)-tetraacetic acid (EGTA), and protein synthesis inhibitor cycloheximide (CHX) were purchased from Sigma Chemical Company (St. Louis, MO). All other reagents used were purchased from Sigma and were of tissue culture grade.

2.2. Cell cultures and treatment

C57BL/6 lymphoma cells (EL4, ATCC TIB-181), and BALB/c lymphoma cells (A20, ATCC TIB-208), both from American Type Culture Collection (ATCC, Rockville, MD), were grown in Dulbecco's Modified Eagle's Medium (DMEM) media (Gibco Laboratories, Grand Island, NY) with 10% horse serum or 10% fetal bovine serum (Atlanta Biologicals, Atlanta, GA) in 5% CO_2 at 37^{o}C, respectively. Both cells were passed twice weekly and cells were used between 4-6 passages for treatments. All manipulating agents were added to the cultures 30 min prior to the addition of mercury.

2.3. Assay for viable cells and lactate dehydrogenase release

The cell viability in EL4 and A20 cells was determined by 3(4,5-dimethyl thiazolyl-2)2,5 diphenyl tetrazolium bromide (MTT) assay in 96-well plates as previously described (Kim et al., 2002). Mitochondrial enzyme activity, an indirect measure of the number of viable respiring cells, was determined using MTT assay. Briefly, the cells were incubated with MTT for 4 hr and 100 μl of isopropanol (in 0.04 N hydrochloric acid) was added to dissolve the formazan crystals. The absorbance was read at 570 nm using a Spectra SLT microplate reader (Tecan, Durham, NC). Cell viability was calculated as relative absorbance compared to control.

Both released and total lactate dehydrogenase (LDH) concentrations were determined as described previously (He et al., 2002). For the total LDH determination, the cells were

lysed by adding 1 µl of Triton X-100 (1% final concentration) and incubated for 30 min in the incubator at 37^{o}C. Samples were transferred to plate containing 100 µl of 4.6 mM pyruvic acid in 0.1 M potassium phosphate buffer (pH 7.5). β-NADH (reduced nicotinamide adenine dinucleotide) in 0.1 M potassium phosphate buffer (pH 7.5) was added, mixed, and the absorbance read kinetically using a PowerWave$_x$ Microplate Scanning spectrophotometer (Bio-Tek Instrument, INC, Winooski, VT). The activity of LDH was normalized to the volume, and the released LDH activity was expressed as a percentage of total cellular LDH.

2.4. DNA synthesis

DNA synthesis was used as an index of proliferation of lymphocytes exposed to mercury as described previously (Johnson et al., 2000). EL4 and A20 cells were seeded at 2×10^4 cells/well in 96-well microplates and treated with mercury. After 24 hr of treatment, each well was pulsed with 0.5 µCi of [methyl-^{3}H]thymidine (25 µCi/ml, 6.7 Ci/mmol, DuPont NEN Products, Boston, MA) and incubated for additional 18 hr. Cells were lysed on glass filters and the filters were dehydrated with 95% ethanol. Filter papers were placed in scintillation vials and counted with scintillation counter (Pharmacia, Turku, Finland). Proliferative response (uptake of [^{3}H]thymidine) was expressed as net disintegrations per min (DPM).

2.5. ROS generation

The production of ROS was measured by detecting the fluorescent intensity of oxidant-sensitive probes either after adding dihydroethidium (DHE) or 5-chloromethyl-2`,7`-dichlorodihydrofluorescein diacetate (CM-H_2DCFDA) (Molecular Probes, Eugene, OR) (Rothe and Valet, 1990). Cells were washed with Lock' buffer and aliquoted as 100,000 cells/well in 96-well black microplates. The Lock's buffer was then supplemented with 10

μM DHE or 5 μM CM-H_2DCFDA, and the intracellular and extracellular dye concentrations were allowed to equilibrate at 37°C/5% CO_2 for 30 min. Iron ($FeSO_4$, 30 μM) was used as a positive control. The cells were incubated in the presence of various concentration of mercury and kinetics of fluorescent intensity recorded using Spectramax Gemini® fluorescence plate reader (Molecular Devices, Irvine, CA). The DHE fluorescence was detected by excitation at 505 nm, emission at 610 nm, and CM-H_2DCFDA fluorescence was detected by excitation at 485 nm, and emission at 530 nm. The fluorescence readings were digitized using SoftMax Pro Version 3.1.1. The results were similar in three independent replications and data from a representative experiment (n = 5 wells) have been illustrated.

2.6. Apoptosis and necrosis

The fluorescent probes Hoechst 33258 (H33258) and propidium iodide (PI) were used to measure the apoptotic and necrotic cells, respectively (Arndt-Jovin and Jovin, 1989). The cells were incubated with the various concentration of mercury for 24 hr, then cells were stained with H33258 (10 μg/ml in phosphate buffered saline, PBS) or PI (20 μg/ml in PBS) for 10 min and fluorescent intensity was recorded using Spectramax Gemini® fluorescent plate reader (Molecular Devices). H33258 fluorescence was detected after excitation 350 nm, emission at 450 nm, and that of PI was detected with excitation 535 nm, and emission at 617 nm. Nuclear morphology for detection of apoptotic and necrotic bodies was conducted using Olympus IX71 inverted microscope (Olympus America, Melville, NY). Digital images were acquired using the Magnafire SP digital camera.

Co-staining with annexin V (Molecular Probes) and PI was employed to confirm the nature of mercury-induced cell death. The cells were treated with vehicle or 70 μM mercury. Following 24 hr, cells were co-stained with annexin V (5 μl/100 μl in annexin binding

buffer) and PI (20 μg/ml) for 15 min and fluorescent intensity recorded separately. Annexin V fluorescent was detected by excitation at 495 nm, and emission at 520 nm.

2.7. Semiquantitative analysis of cytokine mRNA expression

Total cellular RNA was isolated from EL4 and A20 cells following 24 hr treatment with mercury using TRI reagent LS (Molecular Research Center, Cincinnati, OH) according to manufacturer's protocol. The first strand complimentary DNA (cDNA) was synthesized using Superscript II reverse transcriptase enzyme (Life Technologies, Grand Island, NY). Reverse-transcriptase polymerase chain reaction (RT-PCR) was used to analyze the expression of mRNA for IL-2, IL-4, TNFα, and β-actin (internal control). The condition for reverse transcription and PCR steps were performed as previously reported (Kim et al., 2002). The respective sense and anti-sense primers chosen by Primer3 program (Whithead Institute, Cambridge, MA) were 5'TCAACCCCCAGCTAGTTGTC3' and 5'GGAGCTCACTCTCTGTGGTG3' for IL-4, 5'CTCTTCAAGGGACAAGGCTG3' and 5'CGGACTCCGCAAAGTCTAAG3' for TNFα, 5'CTCGCATCCTGTGTCACATT3' and 5'ATCCTGGGGAGTTTCAGGTT3' for IL-2, 5'ATGGATGACGATATCGCT3' and 5'ATGAGGTAGTCTGTCAGGT3' for β-actin. The number of cycles was optimized to ensure product accumulation in the exponential range. Amplified products were separated by electrophoresis on 2% agarose gel and documented using a Kodak DC 290 digital camera and digitized using UN-SCAN-IT software (Silk Scientific, Orem, UT). Band intensities for the respective cytokine were normalized to that of β-actin in the same sample.

2.8. Replication, data presentation and statistical analysis

All experiments were repeated 2 to 3 times with similar trends; however, data from a representative trial are depicted in the Results. Statistical analyses were performed using

SAS statistical software (SAS Institute, Cary, NC). Treatment effects were analyzed using one way analysis of variance (ANOVA) followed by Duncan's Multiple Range test. A value of $p \leq 0.05$ was used to indicate significant differences.

3. Results

3.1. Mercury induced cytotoxicity and decreased DNA synthesis in cultures

Mercury-induced cytotoxicity was investigated in both EL4 and A20 cells. The MTT assay was used as an indirect measure of cell viability. Exposure of mercury for 24 hr reduced viability in both cell lines. The viability of EL4 cells exposed to 20 μM of mercury for 24 hr was reduced to 79% of control (Fig. 5.1A), and viability in A20 cells exposed to 10 μM of mercury for 24 hr was reduced to 73% of control (Fig. 5.1D) as determined by MTT assay.

The release of LDH from cells is a pathological manifestation of increased plasma membrane permeability. Up to 50 μM mercury did not increase LDH release in both cells. Only 100 μM of mercury increased LDH release about 4-fold in EL4 cells (Fig. 5.1B) and 3-fold in A20 cells (Fig. 5.1E) compared to control.

DNA synthesis was investigated by [^{3}H]thymidine incorporation in both EL4 and A20 cell lines. Mercury concentration-dependently decreased DNA synthesis in both EL4 and A20 cells. Mercury at 5 μM significantly decreased DNA synthesis in EL4 cell and at 50 μM completely blocked DNA proliferation (Fig. 5.1C). The A20 cells showed a similar trend after mercury exposure. Mercury at 10 μM started to decrease DNA synthesis of A20 cells and at 50 μM completely blocked DNA proliferation (Fig. 5.1F).

3.2. Mercury induced ROS production

Generation of ROS indicated oxidative stress and increased lipid peroxidatioin, and as a consequence, induced cytotoxicity. The generation of ROS increased in a time-dependent manner in both EL4 and A20 cells detected by oxidant-sensitive probes DHE. Mercury at 50 and 100 μM increased ROS generation in both EL4 and A20 cells. The kinetics of ROS production showed that ROS generation started at 80 min after mercury treatment and kept increasing up to 2 hr in EL4 cells (Fig. 5.2A, B), whereas in A20 cells, ROS generation started at 40 min and continued up to 2 hr (Fig. 5.2E, F). To confirm the mercury induced ROS generation and the effect of antioxidants, we used other oxidant-sensitive probes CM-H_2DCFDA. The addition of exogenous iron to cells as a positive control resulted in a significant increase in the generation of ROS in both cells. Pretreatment of cultures (30 min) with NAC and silymarin decreased mercury induced ROS generation in both EL4 (Fig. 5.2C, D) and A20 cells (Fig. 5.2G, H).

3.3. Mercury induced apoptosis and necrosis

The apoptotic and necrotic effects of mercury were examined in both cell lines. We evaluated mercury-induced apoptosis and necrosis using the fluorescent DNA dyes H33258 and PI, respectively. H33258 is excluded from live cells but stains nuclear fragments in apoptotic cells. PI is membrane impermeant and generally excluded from viable cells, and is used for nuclear stain to identify dead cells. Exposure to mercury at 50 and 100 μM increased apoptosis in both cell lines. Mercury at 100 μM showed loss of membrane integrity (Fig. 5.3B, G) with nuclear condensation (Fig. 5.3D, I) in both types of cells employed here. Pretreatment of cells with NAC (Fig. 5.3C, H) and silymarin reversed mercury-induced apoptosis (Fig. 5.3E, J). In addition, incubation with mercury for 24 hr increased necrosis in both cells. Mercury at 100 μM induced necrosis (Fig. 5.4B, G) and

pretreatment of NAC (Fig. 5.4C, H) and silymarin (Fig. 5.4D, I) reversed mercury-induced necrosis in both EL4 and A20 cells.

To confirm the nature of mercury-induced cell death via apoptosis and necrosis, we co-stained cells with annexin V and PI. The externalization of phosphatidyl-serine is an early event in apoptosis and was determined using annexin V staining of cells. A high (cytotoxic) concentration of mercury was desirable to see both types of cellular death. Treatment of cells with 70 μM of mercury for 24 hr increased annexin V binding (Fig. 5.5F, H) and PI binding (Fig. 5.5I, L). Fig. 5N, P represents overlapping image of annexin V and PI fluorescence in both cells. Some cells stained with either annexin V or PI, while other cells were stained both with annexin V and PI.

3.4. Effects of antioxidant, Ca^{2+} channel blocker and protein synthesis inhibitor in mercury induced cytotoxicity

The potent antioxidant NAC and a natural antioxidant silymarin attenuated mercury-induced LDH release. Pretreatment (30 min) of NAC and silymarin significantly reduced mercury-induced (100 μM) LDH release in both EL4 and A 20 cells (Table 5.1).

Mercury is known to alter Ca^{2+} homeostasis; therefore the involvement of the extracellular Ca^{2+} pool on toxicity was examined. Pretreatment with 3 mM EGTA to chelate all extracellular Ca^{2+} did not influence mercury-induced LDH release but significantly increased LDH release in cells not treated with mercury in both EL4 and A20 cells (Table 5.2). We also used the Ca^{2+} channel blocker lanthanum (La^{2+}) to block Ca^{2+} influx from the extracellular milieu. Pretreatment with 100 μM La^{2+} resulted in a significant protection of cells from mercury-induced LDH release in A20 cells but not EL4 cells (Table 5.2). CHX is a potent inhibitor of gene translation and has been used extensively in apoptosis research.

Pretreatment of cells with 0.5 μg/ml of CHX for 30 min prior to the addition of mercury did not alter the mercury-induced LDH release in either EL4 or A20 cells.

3.5. Modulation of cytokine gene expressions by mercury

Mercury is known to modulate production of various cytokines in lymphocytes. In the present study, we treated EL4 and A20 cells with 5 and 10 μM of mercury for 24 hr, the concentrations that were not cytotoxic. Expression of inflammatory cytokines was measured by RT-PCR. Fig. 5.6 shows the effects of mercury on IL-2, IL-4, and TNFα mRNA expressions in EL4 cells. Mercury increased the expression of IL-4 and TNFα mRNA. Gene expression for IL-2 was not altered by mercury treatment. To study the effect of mercury on cytokine gene expressions in B cell, we added mercury (5 and 10 μM) to A20 cells but mercury showed no effect on the expression of cytokines in A20 cells. Indeed, no constitutive expression of these cytokines in A20 cells was observed (data not presented).

4. Discussion

In the present study, we investigated the cytotoxicity of inorganic mercury ($HgCl_2$) using different type of viability assays in both T and B lymphoma cell lines. There is evidence that chronic exposure to low concentrations of mercury results in disruption of the immune functions. Such immunotoxic effects of mercury may lead to immunoregulatory defects, which may result in persistent infection, cancer or autoimmune disease. The previous reports of mercury as immunotoxicant and immunomodulant are contradictory depending on experimental situation, dose and form of mercury, and strain of animal.

Previous studies revealed that ROS generation caused cell death in several cell types (Buttke and Sandstrom, 1995; Sato et al., 1995). Also, low levels of mercury increased ROS

generation in human lymphocytes, hepatocytes, and brain cells (Shenker et al., 1998; Lund et al., 1993; InSug et al., 1997; Stacey and Kappus, 1982; Hussain et al., 1997). Mercury decreased mitochondrial transmembrane potential and increased ROS generation, and consequent depletion of GSH and lipid peroxidation, the latter is the major cause of mercury-induced cytotoxicity. Mercury compromised cytosolic thiol redox system and induced an oxidative burst. On the contrary, Lee et al., (2002) reported that mercury-induced cytotoxicity was not associated with generation of ROS and subsequent lipid peroxidation. To confirm the effect of mercury on the generation of ROS in murine lymphocytes, the production of ROS was monitored using both DHE and CM-H_2DCFDA fluorescent method. We demonstrate here that treatment of mercury induced ROS production in both EL4 and A20 cells, and the antioxidants NAC and silymarin effectively inhibited mercury-induced ROS production. These data suggest that mercury-induced cytotocixity is mediated by modulation of ROS and results in increasing membrane permeability.

Involvement of ROS in mercury-induced cytotoxicity has been reported previously. Mercury-induced apoptosis in human monocytes and T cell, evaluated by the fluorescent probe H33258 (Shenker et al., 1998; InSug et al., 1997). Our data confirm that mercury increased H33258 fluorescence intensity with nuclear condensation and the antioxidants NAC and silymarin decreased such effect in both EL4 and A20 cells. However, treatment of mercury also increased PI fluorescent intensity, which indicated necrosis in both EL4 and A20 cells.

The manner in which a cell dies can have a great impact on the resulting response of the surrounding tissue. Death by necrosis has been shown to act as a natural adjuvant inducing oxidative stress and the production of pro-inflammatory cytokines (Anderson et al.,

2002). This is due to the indiscriminant release of the cytoplasmic contents from the dying cell. The result is an area of inflammation and immune-mediated cell damage in uninvolved cells surrounding the initial insult. In contrast, death by apoptosis is a controlled event usually with minimal loss of membrane integrity until the later stages termed secondary necrosis. Instead, the cytoplasmic contents are systemically degraded from within. This type of cell death often involves phagocytosis by resident tissue macrophages and the release of anti-inflammatory cytokines (Fadok et al., 1998). In our experiment using lymphomas, protein synthesis inhibitor CHX did not reduce mercury-induced LDH release. This suggests that mercury-induced cytotoxicity does not require *de novo* protein synthesis. Taken together, the mode of mercury-induced cytotoxicity appears to be a mix of apoptosis and necrosis; both apoptotic and necrotic effects are mediated by ROS in murine lymphoma cell lines.

It has been established that an increase in Ca^{2+} can lead to cytotoxicity through several downstream reactions, such as ROS generation (Gasso et al., 2001). Mercury was reported to increase intracellular Ca^{2+} concentration (Toimela and Tahti, 2001). Additionally some Ca^{2+} channel blockers protect mercury-induced neurotoxicity (Gasso et al., 2001). It has been known that ROS and lipid peroxidation can increase intracellular Ca^{2+}, although the exact origin of Ca^{2+} is controversial (Suzuki and Forman, 1997). In our experiments, pretreatment of A20 cells with La^{2+} significantly reduced mercury-induced LDH release. Although this effect was not observed when extracellular Ca^{2+} was chelated using EGTA, the finding still indicated that this ion may be involved in the mercury-induced cytotoxicity in A20 cells. Therefore, mercury-mediated oxidative stress leads to cytotoxicity in part through disrupted Ca^{2+} homeostasis.

Treatment of lymphocytes with mercury modulated production of several cytokines such as TNFα, IL-1, IL-2, and IL-4 (Hu et al., 1997; Johansson et al., 1997; Shenker et al., 1992; Badou et al., 1997). In this present study, we used non-cytotoxic levels of mercury to evaluate cytokine production. Our data showing the increases in IL-4 and TNFα gene expression is consistent with other reports (Badou et al., 1997; Johansson et al., 1997). Badou et al, (1997) reported that mercury induced IL-4 gene expression in mouse T cell hybridoma and PKC-dependent Ca^{2+} influx through calcium channels was dominant pathway in the IL-4 gene expression. Also, mercury acts at the transcriptional level without requiring *de novo* protein synthesis. Because of the decreasing cytotoxicity of A20 cells by Ca^{2+} channel blocking, we expected alteration of inflammatory cytokine gene expression in A20 cells. The unresponsiveness of A20 cell to mercury might be due to the insensitivity of A20 cell to low level of mercury or insensitivity of our detection system. The exact mechanisms in relationship between ROS, Ca^{2+}, and cytokine profiles should be further investigated.

Mercury decreases cell GSH content by binding free GSH or by inhibiting its synthesis in human monocytes (InSug et al., 1997). The low GSH level predisposes cells to ROS damage and activates death-signaling pathways. Decrease of mercury-induced ROS production and LDH release by GSH precursor, NAC and GSH regulator, silymarin suggest that GSH is also involved in mercury-induced cytotoxicity in murine lymphocytes.

Oxidative stress evokes apoptosis by mitogen-activated protein kinase (MAPK)-mediated caspase activation (Kamata and Hirata, 1999). MAPKs have important functions as mediators of cellular response to extracellular signals. Mercury inhibits Ras activation during T cell receptor-mediated signal transduction (Mattingly et al., 2001). Previously we reported that mercury increased TNFα gene expression by regulating p38 MAPK in

macrophages (Kim et al., 2002). The mechanistic studies to MAPKs and downstream caspase pathway should be elucidated for further understanding of mercury-induced apoptosis.

In summary, results of the present study show that the involvement of ROS, Ca^{2+} homeostasis in mercury-induced lymphocytes cytotoxicity. Mercury induced cytotoxicity is a mix of apoptosis and necrosis. EL4 and A20 cells showed a similar response to mercury-induced toxicity in involvement of ROS but showed different responses in the involvement of Ca^{2+} and inflammatory cytokines. In EL4 cells inflammatory cytokines may participate in mercury-mediated cytotoxicity.

References

Anderson H.A., Englert R., Gursel I. and Shacter E. (2002) Oxidative stress inhibits the phagocytosis of apoptotic cells that have externalized phosphatidylserine. Cell Death and Differentiation 9, 616-625.

Arndt-Jovin D.J. and Jovin T.M. (1989) Fluorescence labeling and microscopy of DNA. Methods in Cell Biology 30, 417-448.

Badou A., Savignac M., Moreau M., Leclerc C., Pasquier R., Druet P. and Pelletier L. (1997) $HgCl_2$-induced interleukin-4 gene expression in T cells involves a protein kinase C-dependent calcium influx through L-type calcium channels. Journal of Biological Chemistry 272, 32411-32418.

Bernard G.R. (1991) N-acetylcysteine in experimental and clinical acute lung injury. American Journal of Medicine 91, 54-59.

Buttke T.M. and Sandstrom P.A. (1995) Redox regulation of programmed cell death in lymphocytes. Free Radical Research 22, 389-397.

Denny M.F. and Atchison W.D. (1996) Mercurial-induced alterations in neuronal divalent cation homeostasis. Neurotoxicology 17, 47-61.

Fadok V.A., Bratton D.L., Konowal A., Freed P.W., Westcott J.Y. and Henson P.M. (1998) Macrophages that have ingested apoptotic cells in vitro inhibit proinflammatory cytokine production through autocrine/paracrine mechanisms involving TGF-beta, PGE_2, and PAF. Journal of Clinical Investigation 101, 890-898.

Fraschini F., Demartini G. and Esposti D. (2002) Pharmacology of silymarin. Clinical Drug Investigation 22, 51-65.

Gasso S., Cristofol R.M., Selema G., Rosa R., Rodriguez-Farre E. and Sanfeliu C. (2001) Antioxidant compounds and $Ca^{(2+)}$ pathway blockers differentially protect against methylmercury and mercuric chloride neurotoxicity. Journal of Neuroscience Research 66, 135-145.

He Q., Riley R.T. and Sharma R.P. (2002) Pharmacological antagonism of fumonisin B_1 cytotoxicity in porcine renal epithelial cells (LLC-PK_1): a model for reducing fumonisin-induced nephrotoxicity in vivo. Pharmacology and Toxicology 90, 268-277.

Hu H., Moller G. and Abedi-Valugerdi M. (1999) Mechanism of mercury-induced autoimmunity: both T helper 1- and T helper 2-type responses are involved. Immunology 96, 348-357.

Hu H., Abedi-Valugerdi M. and Moller G. (1997) Pretreatment of lymphocytes with mercury in vitro induces a response in T cells from genetically determined low-responders and a shift of the interleukin profile. Immunology 90, 198-204.

Hultman P. and Enestrom S. (1987) The induction of immune complex deposits in mice by peroral and parenteral administration of mercuric chloride: strain dependent susceptibility. Clinical and Experimental Immunology 67, 283-292.

Hussain S., Rodgers D.A., Duhart H.M. and Ali S.F. (1997) Mercuric chloride-induced reactive oxygen species and its effect on antioxidant enzymes in different regions of rat brain. Journal of Environmental Science and Health, Part B 32, 395-409.

InSug O., Datar S., Koch C.J., Shapiro I.M. and Shenker B.J. (1997) Mercuric compounds inhibit human monocyte function by inducing apoptosis: evidence for formation of

reactive oxygen species, development of mitochondrial membrane permeability transition and loss of reductive reserve. Toxicology 124, 211-224.

Johansson U., Sander B. and Hultman P. (1997) Effects of the murine genotype on T cell activation and cytokine production in murine mercury-induced autoimmunity. Journal of Autoimmunity 10, 347-355.

Johnson V.J., Tsunoda M. and Sharma R.P. (2000) Increased production of proinflammatory cytokines by murine macrophages following oral exposure to sodium selenite but not to seleno-L-methionine. Archives of Environmental Contamination and Toxicology 39, 243-250.

Kamata H. and Hirata H. (1999) Redox regulation of cellular signaling. Cell Signaling 11, 1-14.Kim S.H., Johnson V.J. and Sharma R.P. (2002) Mercury inhibits nitric oxide production but activates proinflammatory cytokine expression in murine macrophage: differential modulation of NF-kappaB and p38 MAPK signaling pathways. Nitric Oxide 7, 67-74.

Lee Y.W., Ha M.S. and Kim Y.K. (2001) Role of reactive oxygen species and glutathione in inorganic mercury-induced injury in human glioma cells. Neurochemical Research 26, 1187-1193.

Lund B.O., Miller D.M. and Woods J.S. (1993) Studies on Hg(II)-induced H_2O_2 formation and oxidative stress in vivo and in vitro in rat kidney mitochondria. Biochemical Pharmacology 45, 2017-2024.

Mattingly R.R., Felczak A., Chen C.C., McCabe M.J. Jr and Rosenspire A.J. (2001) Low concentrations of inorganic mercury inhibit Ras activation during T cell receptor-mediated signal transduction. Toxicology and Applied Pharmacology 176, 162-168.

Pelletier L., Pasquier R., Guettier C., Vial M.C., Mandet C., Nochy D., Bazin H. and Druet P. (1988) $HgC1_2$ induces T and B cells to proliferate and differentiate in BN rats. Clinical and Experimental Immunology 71, 336-342.

Rosenspire A.J., Bodepudi S., Mathews M. and McCabe M.J. Jr. (1998) Low levels of ionic mercury modulate protein tyrosine phosphorylation in lymphocytes. International Journal of Immunopharmacology 20, 697-707.

Rothe G. and Valet G. (1990) Flow cytometric analysis of respiratory burst activity in phagocytes with hydroethidine and 2',7'-dichlorofluorescin. Journal of Leukocyte Biology 47, 440-448.

Sato N., Iwata S., Nakamura K., Hori T., Mori K. and Yodoi J. (1995) Thiol-mediated redox regulation of apoptosis. Possible roles of cellular thiols other than glutathione in T cell apoptosis. Journal of Immunology 154, 3194-3203.

Shenker B.J., Guo T.L. and Shapiro I.M. (1998) Low-level methylmercury exposure causes human T-cells to undergo apoptosis: evidence of mitochondrial dysfunction. Environmental Research 77, 149-159.

Shenker B.J., Pankoski L., Zekavat A. and Shapiro I.M. (2002) Mercury-induced apoptosis in human lymphocytes: caspase activation is linked to redox status. Antioxidants and Redox Signaling 4, 379-389.

Shenker B.J., Rooney C., Vitale L. and Shapiro I.M. (1992) Immunotoxic effects of mercuric compounds on human lymphocytes and monocytes. I. Suppression of T-cell activation. Immunopharmacology Immunotoxicology 14, 539-553.

Stacey N.H. and Kappus H. (1982) Cellular toxicity and lipid peroxidation in response to mercury. Toxicology and Applied Pharmacology 63, 29-35.

Suzuki Y.J., Forman H.J. and Sevanian A. (1997) Oxidants as stimulators of signal transduction. Free Radical Biology and Medicine 22, 269-285.

Toimela T.A. and Tahti H. (2001) Effects of mercuric chloride exposure on the glutamate uptake by cultured retinal pigment epithelial cells. Toxicology in Vitro 15, 7-12.

Table 5.1. The effects of antioxidant in mercury-induced LDH release.

		LDH release[a]	
Cell	Pre-treatment	Control	$HgCl_2$ (100 μM)
EL4	None	14.6 ± 3.0	82.7 ± 9.2
	NAC[b]	12.1 ± 2.8	25.4 ± 4.5*
	Silymarin	16.4 ± 4.1	48.6 ± 10.8*
A20	None	16.2 ± 6.1	55.7 ± 13.7
	NAC	15.9 ± 5.2	35.2 ± 4.4*
	Silymarin	17.9 ± 8.8	40.0 ± 1.1*

[a]LDH release was calculated by dividing the LDH units in the supernatant by the LDH units in the lysate and supernatant combined and is expressed as % of total in the respective wells.

[b]NAC (1 mM), silymarin (25 μM) were added to the cultures 30 min prior to the addition of mercury.

*Indicates significant difference from the respective control group at $p < 0.05$.

Table 5.2. The effects of manipulation of extracellular Ca^{2+} on mercury-induced LDH release.

Cell	Pre-treatment	LDH release[a]	
		Control	$HgCl_2$ (100 μM)
EL4	None	20.02 ± 8.57	69.94 ± 22.78
	EGTA[b] (3 mM)	34.93 ± 6.51	74.44 ± 13.92
	La^{2+} (100 μM)	37.94 ± 17.47	70.56 ± 3.99
	CHX (0.5 μg/ml)	30.11 ± 5.12	70.01 ± 9.13
A20	None	5.36 ± 0.91	58.75 ± 5.77
	EGTA (3 mM)	11.77 ± 3.72*	68.57 ± 19.04
	La^{2+} (100 μM)	3.15 ± 1.12	33.60 ± 10.27*
	CHX (0.5 μg/ml)	4.98 ± 2.52	55.55 ± 9.73

[a]LDH release was calculated by dividing the LDH units in the supernatant by the LDH units in the lysate and supernatant combined and is expressed as % of total in the respective well.

[b]All manipulating agents were added to the cultures 30 min prior to the addition of mercury.

*Indicates significant difference from the respective control group at $p < 0.05$.

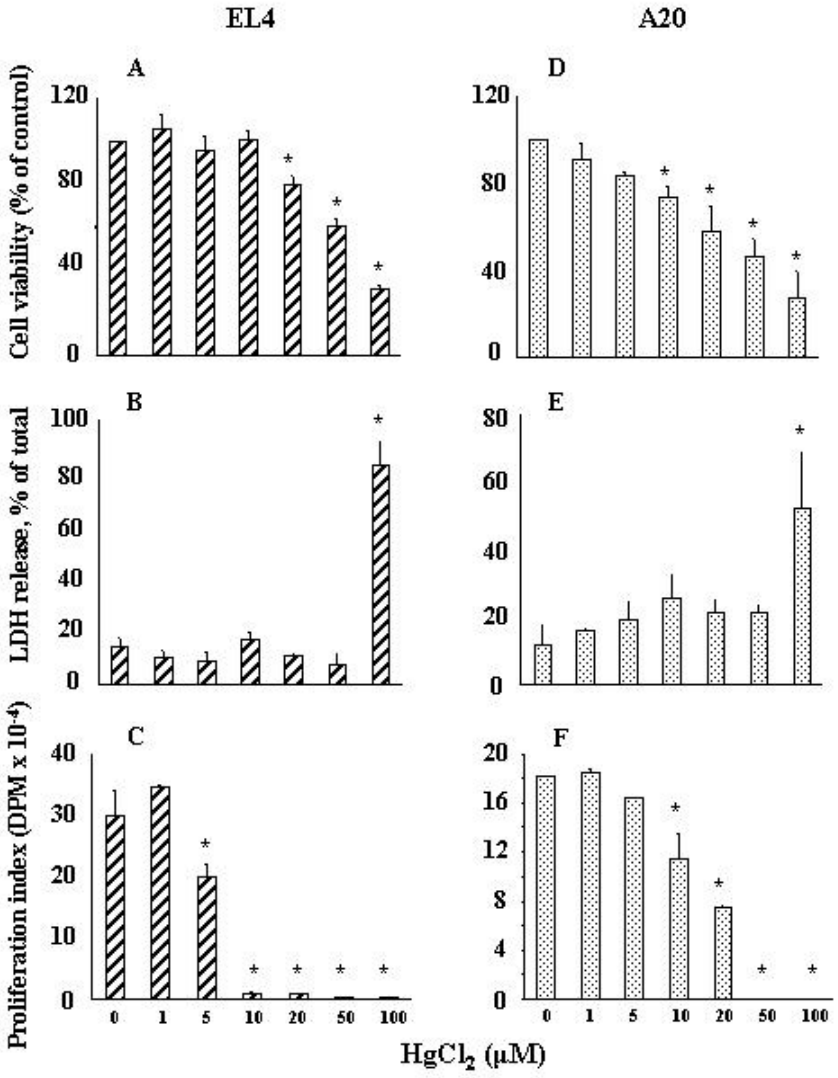
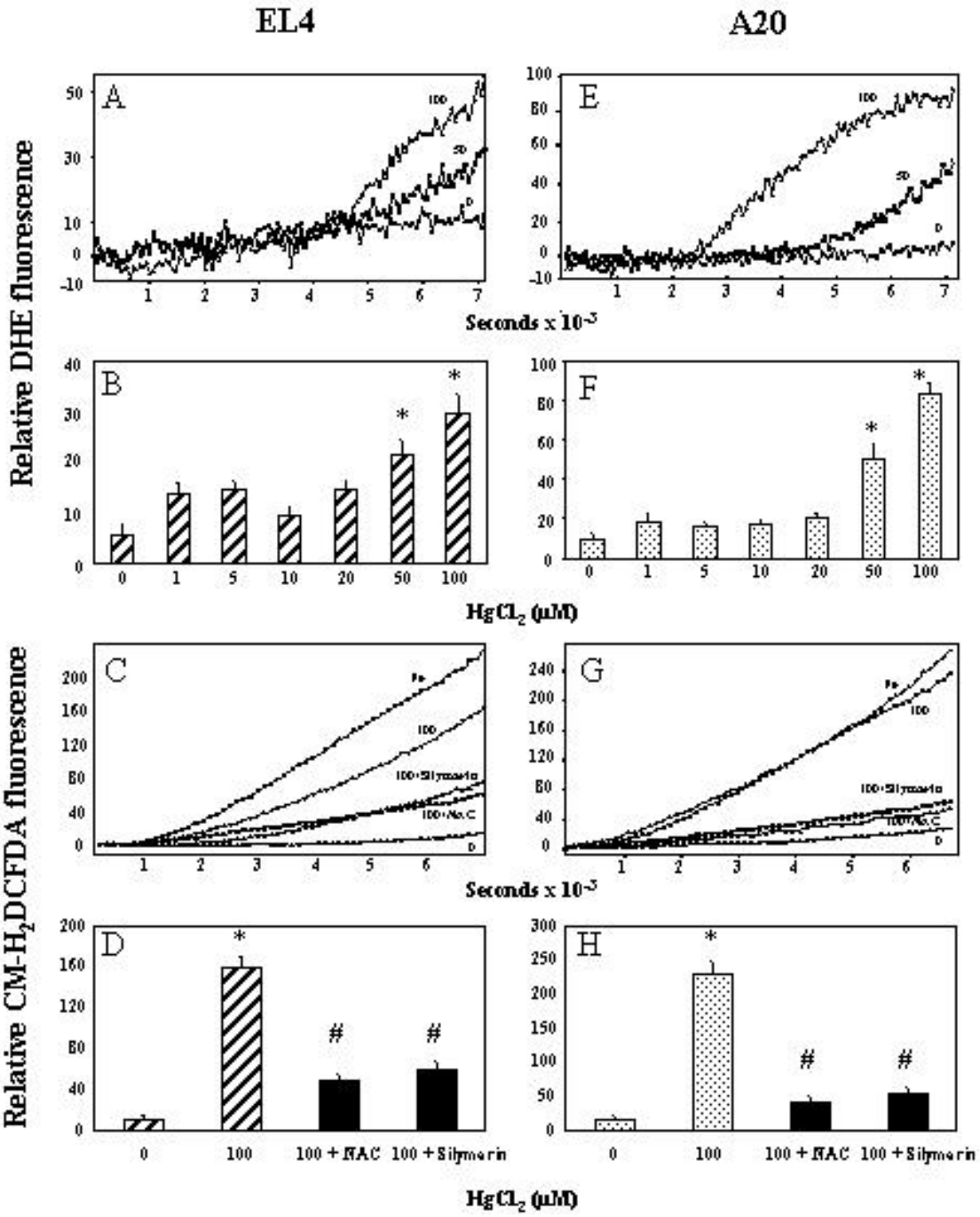


Fig. 5.1. Effects of $HgCl_2$ on cell viability (A, D), LDH release (B, E), and DNA synthesis (C, F) in EL4 cells and A20 cells. EL4 cell (4×10^4/well in 96-well plate) and A20 cell (8×10^4) were treated with various concentration of $HgCl_2$ for 24 hr. Cell viability was represented by relative absorbance compared to control. The LDH release was expressed as a percentage of total cellular LDH. DNA synthesis of lymphocytes was measured by [^{3}H]thymidine incorporation. Results from a representative experiment are expressed as mean ± SE (n=3). *Significantly different than the control cultures at $p < 0.05$.

Fig. 5.2. Effects of $HgCl_2$ on ROS production. DHE fluorescence: An illustration of real-time change is provided in the top panel (A, E) for selected $HgCl_2$ concentrations. The lower graph (B, F) represents the concentration-related fluorescence change at 2 hr. CM-H_2DCFDA fluorescence: An illustration of real-time change is provided in the top panel (C, G) for iron (positive control), mercury, and mercury with antioxidant. The lower graph (D, H) represents the fluorescence intensity at 2 hr. Results from a representative experiment are expressed as mean ± SE (n=5). *Significantly different than the vehicle-treated cultures at $p < 0.05$, $^{\#}$significantly different than the mercury-treated cultures at $p < 0.05$.


EL4
A20
A
E
100
50
0
Relative DHE fluorescence
Seconds x 10⁻³
B
F
HgCl₂ (μM)
C
G
Pe
100
100+Silymarin
100+N.A.C
Relative CM-H₂DCFDA fluorescence
D
H
#
100 + NAC
100 + Silymarin
HgCl₂ (μM)

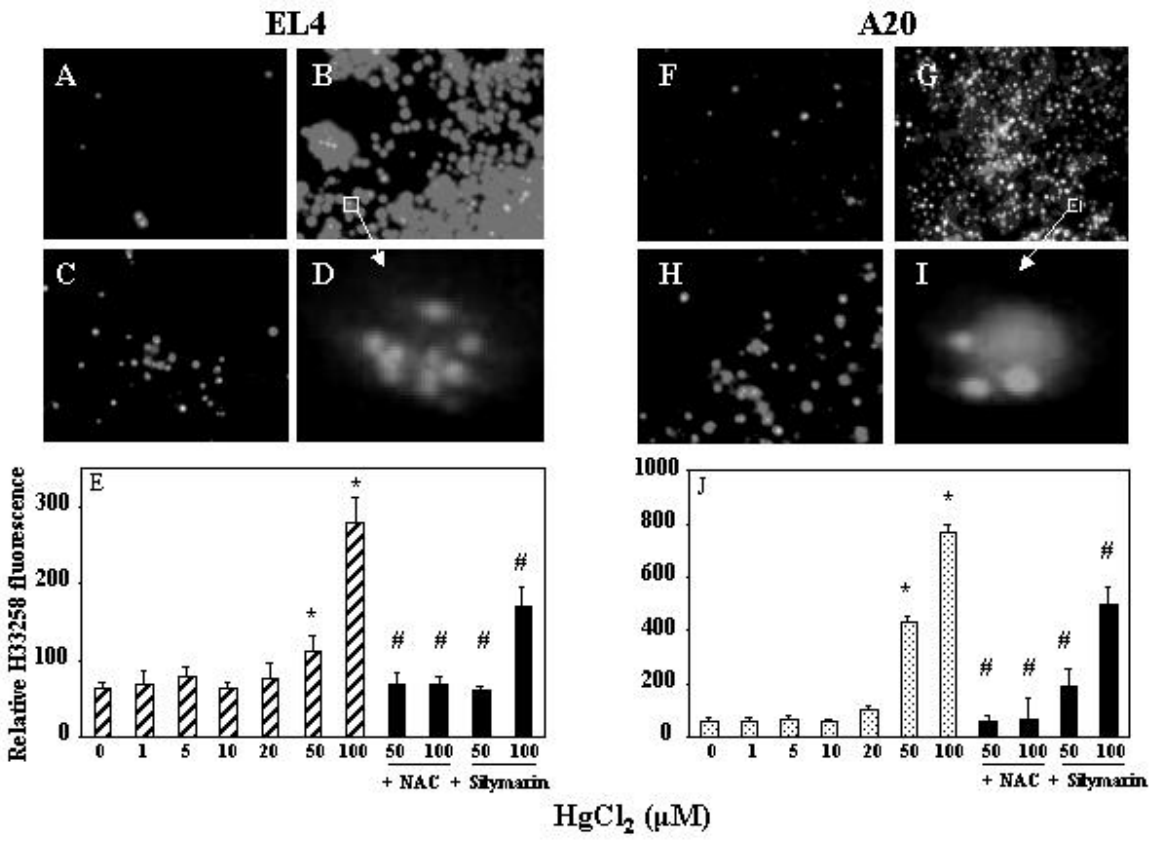


Fig. 5.3. Apoptotic effect of $HgCl_2$. EL4 cell (4×10^4/well in 96-well plate) and A20 cell (8×10^4) were treated with various concentration of $HgCl_2$ for 24 hr. Antioxidants were added 30 min prior to mercury treatment. Cells were stained with H33258 and the fluorescence intensity was measured by fluorescence reader, and visualized by fluorescence microscope. Representative photographs show increased nuclear staining with H33258 in cells exposed to 0 μM mercury (A, F), 100 μM mercury (B, G), 100 μM mercury and NAC (C, H), and fragmented nuclei in cells exposed to 100 μM mercury (D, I). The lower graphs (E, J) represent the fluorescence change at 10 min after H33258 staining. Results from a representative experiment are expressed as mean ± SE (n=5). *Significantly different than the control cultures at $p < 0.05$, #significantly different than the mercury-treated cultures at $p < 0.05$.

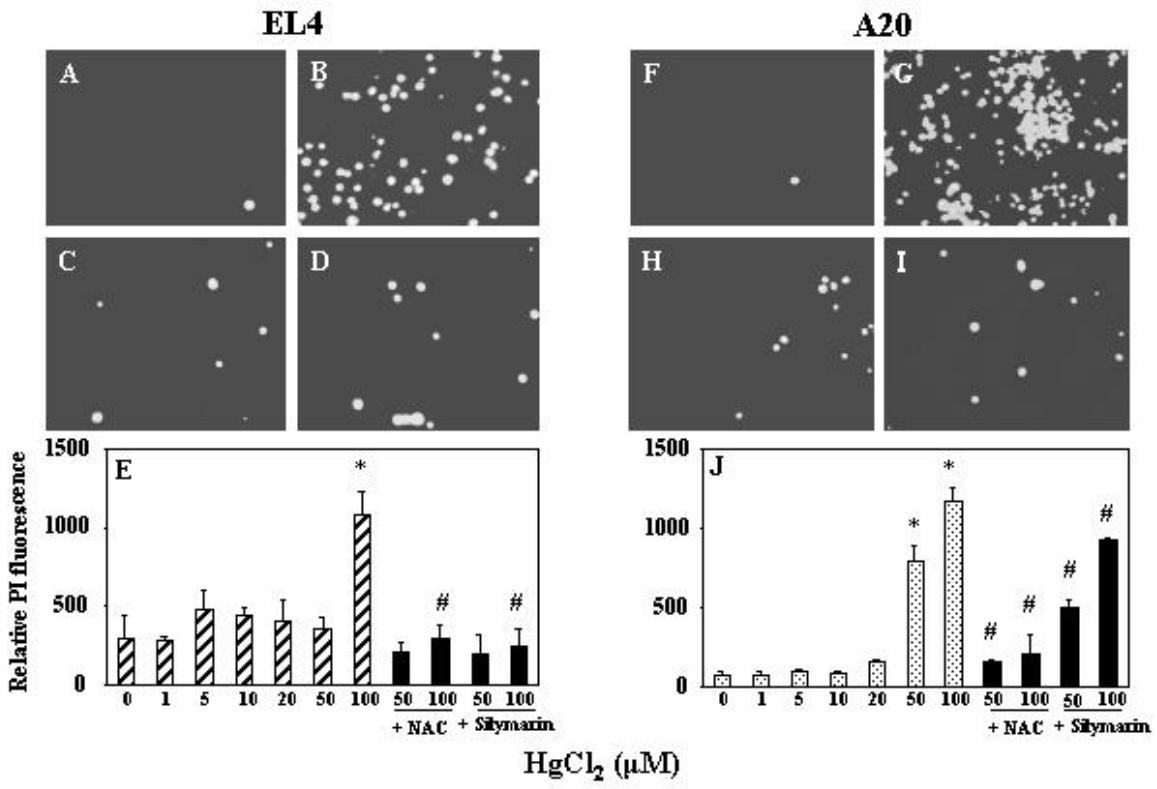
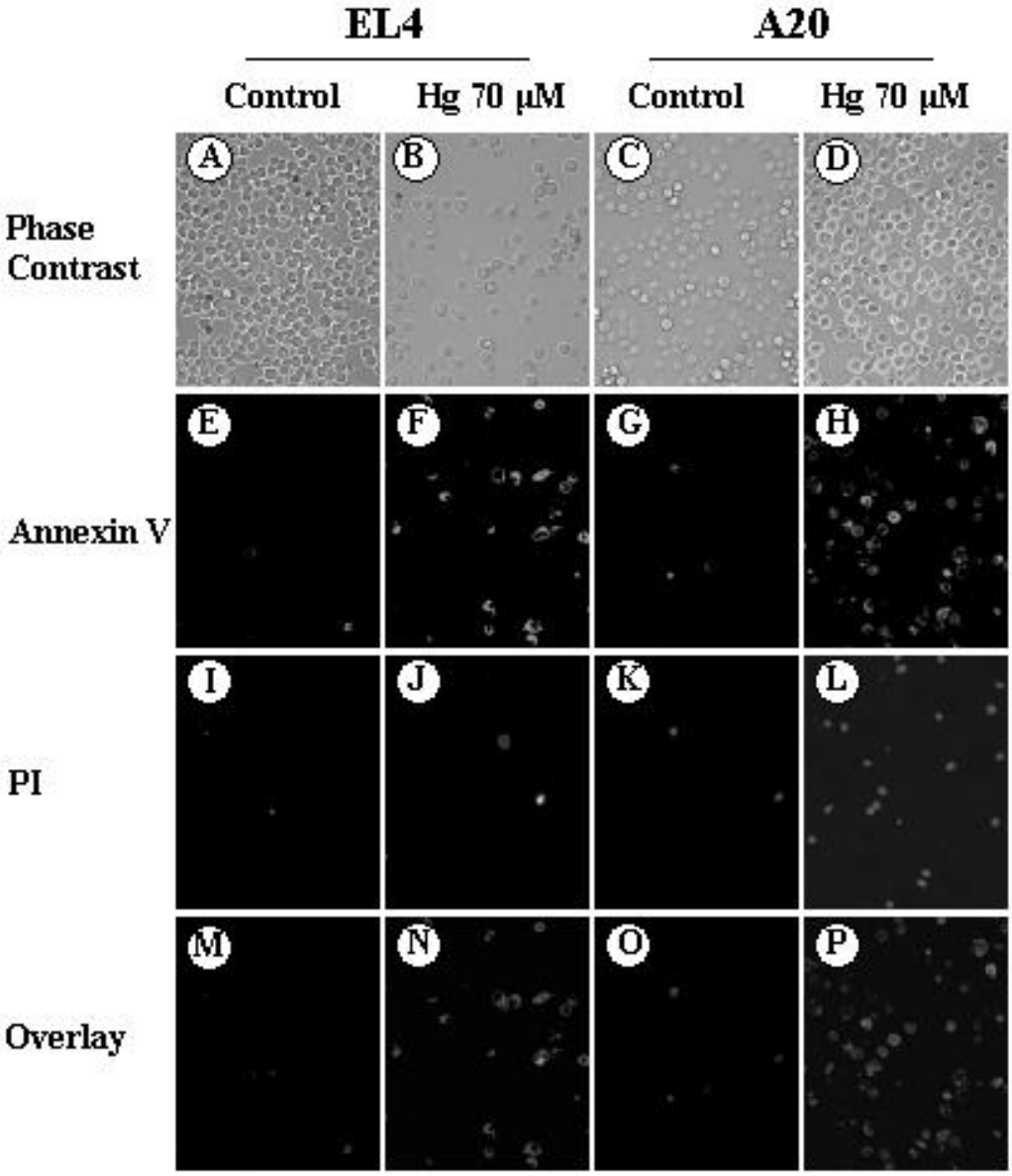


Fig. 5.4. Necrotic effect of $HgCl_2$. EL4 cell (4×10^4/well in 96-well plate) and A20 cell (8×10^4) were treated with various concentration of $HgCl_2$ for 24 hr. Antioxidants were added 30 min prior to mercury treatment. Cells were stained with PI and the fluorescence intensity was measured by fluorescence reader, and visualized by fluorescence microscope. Representative photographs show increased nuclear staining with PI in cells exposed to 0 μM mercury (A, F), 100 μM mercury (B, G), 100 μM mercury and NAC (C, H), and 100 μM mercury and silymarin (D, I). The lower graphs (E, J) represent the fluorescence change at 10 min after PI staining. Results from a representative experiment are expressed as mean ± SE (n=5). *Significantly different than the control cultures at $p < 0.05$, #significantly different than the mercury-treated cultures at $p < 0.05$.

Fig. 5.5. Comparison of $HgCl_2$-induced cell death. EL4 cell (1.5×10^5/well in chamber slide) and A20 cell (3×10^5) were treated with 70 μM of $HgCl_2$ for 24 hr. Cells were co-stained with annexin V and PI, and the fluorescence was visualized by fluorescence microscope. Representative photographs show phase contrast in cells exposed to 0 μM mercury (A, C), 70 μM mercury (B, D), annexin V exposed to 0 μM mercury (E, G), 70 μM mercury (G, H), PI exposed to 0 μM mercury (I, K), 70 μM mercury (J, L), and overlap image of annexin V and PI exposed to 0 μM mercury (M, O), 70 μM mercury (N, P). Micrographs are representative of two independent experiments with similar results.


EL4
A20
Control
Hg 70 µM
Control
Hg 70 µM
Phase Contrast
Annexin V
PI
Overlay
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P

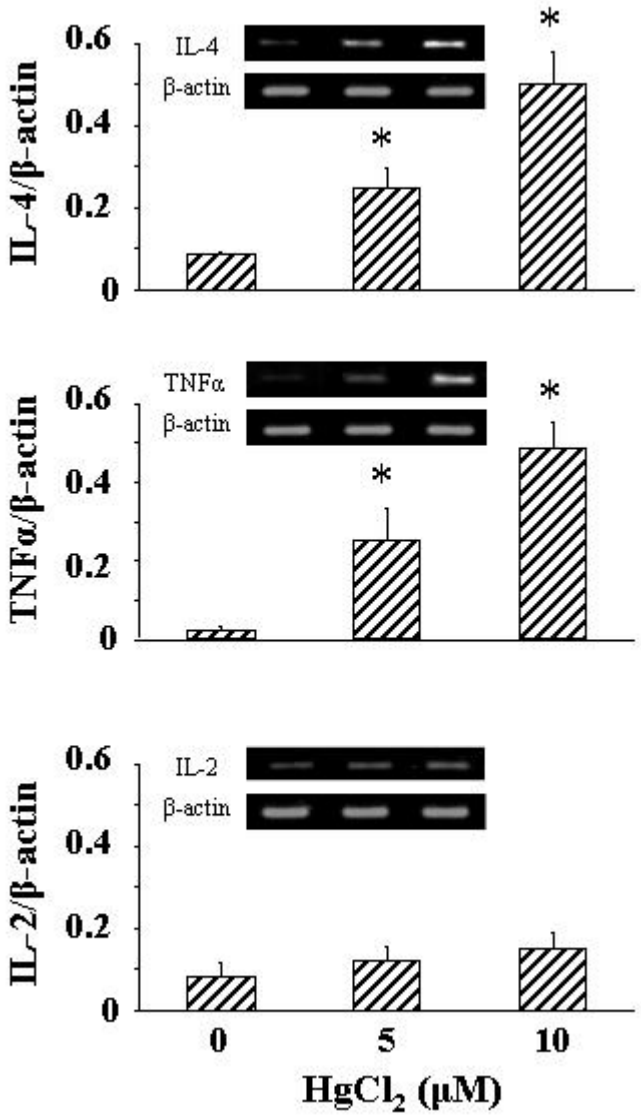


Fig. 5.6. Effects of $HgCl_2$ on inflammatory cytokine gene expressions in EL4 cells. EL4 cells (5×10^5/well in 24-well plate) were treated with 5 and 10 μM of $HgCl_2$ for 24 hr. Extraction and analysis of mRNA performed as described in Materials and Methods, and IL-4, IL-2, and TNFα mRNA levels were determined by RT-PCR. Band density for each gene expressions was normalized against β-actin. Results are expressed as mean ± SE (n=3). Representative gel photographs are shown as inset in the same order. *Significantly different than the control group at $p < 0.05$.

CHAPTER 6

ORAL EXPOSURE TO INORGANIC MERCURY ALTERS T-LYMPHOCYTE PHENOTYPES AND CYTOKINE GENE EXPRESSION IN BALB/c MICE[1]

---

[1]Kim, S.H., Victor J. Johnson, Raghubir P. Sharma. Submitted to *Archives Toxicology*

Abstract

Mercury is a well-recognized health hazard and an environmental contaminant. Mercury suppresses immune responses, but the mechanisms responsible for these effects are still unclear. Male BALB/c mice were exposed continuously to 0, 0.3, 1.5, 7.5, or 37.5 ppm of mercury in drinking water for 14 days. Body weight was reduced at the highest dose of mercury whereas the relative kidney and spleen weights were significantly increased. The dose-range of mercury used did not cause hepatotoxicity as indicated by circulating alanine aminotransferase and aspartate aminotransferase. Circulating blood leukocytes were elevated in mice treated with the highest dose of mercury. Single-cell splenocyte cultures were used to determine the effects of mercury treatment on mitogen-induced lymphocyte blastogenesis. Mercury at 1.5 ppm decreased basal splenocyte proliferation in treated mice causing increases in PHA and LPS stimulation indices for T and B lymphocytes, respectively, Mercury ranging from 1.5 to 37.5 ppm dose-dependently decreased $CD3^+$ T lymphocytes in spleen; both $CD4^+$ and $CD8^+$ single positive lymphocyte populations were decreased. Exposure to 7.5 and 37.5 ppm of mercury decreased the $CD8^+$ T lymphocyte population in the thymus, whereas double positive $CD4^+/CD8^+$ and $CD4^+$ thymocytes were not altered. Mercury altered the expression of inflammatory cytokines (tumor necrosis factor α, interferon γ, and interleukin-12), c-myc, and major histocompatibility complex II, in various organs. Results indicated that decreases in T lymphocyte populations in immune organs and altered cytokine gene expression may contribute to the immunosuppressive effects of inorganic mercury.

Keyword: Mercury, T lymphocyte, Inflammatory cytokine, Gene expression, Immunosuppression, Major histocampatibility.

1. Introduction

Mercury is widespread in the environment and chronic exposure to low levels of mercury is common due to contamination of food and drinking water supplies. Mercury is highly toxic and moderate levels of exposure to mercury can cause neurotoxic manifestations, nephrotoxicity, immune system alterations, and decreased host resistance to viral infections in mice [1-3]. Oral exposure of rabbits to mercury resulted in significantly lower antibody titers after inoculation with viral agents [4]. Prolonged exposure of mice to relatively low concentrations of mercury also increased susceptibility to a variety of viruses [5]. Mercury exposure was shown to increase serum IgG_1, and IgE levels in certain strains of mice after subcutaneous injection [6,7], and caused auto-antibody formation to glomerular basement membrane in rats [8].

Recently, there has been growing concern regarding the human health effects of low level of environmental mercury contamination [9]. Although mercury is broadly sulfhydryl reactive, surprisingly little is understood as to the specific cellular targets and/or biochemical pathways targeted by the metal. Clinical studies on industrial workers exposed to mercury levels within World Health Organization guidelines have shown immune system abnormalities in the absence of gross neurological dysfunction [10]. The immune system may be an important target for mercury intoxication as well as a particularly revealing sentinel organ system in which to investigate the biochemical effects of low level mercury exposure. We recently reported that non-cytotoxic concentrations of inorganic mercury inhibited nitric oxide (NO) production and altered proinflammatory cytokine gene expressions in murine macrophages in vitro [11]. Mercury impaired host defense to bacterial infection via decreasing NO production. Mercury altered cytokine production and activated

DNA synthesis in both human and murine lymphocytes treated in vitro [12,13]. Treatment of lymphocytes with mercury modulated cytokine production such as those of interleukin-1 (IL-1), IL-2, IL-4, and interferon γ (IFNγ), and proto-oncoprotein c-myc [13-17].

Mercury exists in different forms, e.g., elemental, inorganic and organic mercury compounds. They have some properties in common but differ in their metabolism and toxicity. The distribution of mercury within the body and specific organs varies with the chemical form, dose and time after exposure [18]. Additionally, the route of administration affects the organ distribution of absorbed mercury [19,20]. Although the use of inorganic mercury compounds has decreased in recent years and precautions against industrial emissions have increased, future human exposure to inorganic mercury will probably result in few individuals, including those occupationally exposed, and populations exposed to low levels from dental amalgam or from food or drinking water containing mercury [21]. Parenteral administration of soluble mercury salts has been the commonly used exposure route in past animal studies despite natural human exposure via the oral route, i.e., drinking water and food [19].

The current study was conducted to investigate the effects of mercury in the murine immune system. Previous studies with mercury immunotoxicity have primarily investigated alterations in immunoglobulins and development of autoimmunity. Little is known about specific lymphocyte populations and immune modulation in peripheral organs after mercury treatment. We hypothesized that the immunotoxic effects of low levels of mercury are induced by alterations in cellular subpopulations and interactions reflected by the modulation of major cytokines. Male BALB/c mice were treated via drinking water to determine the immunotoxicity of mercury to closely reflect a normal exposure route. Several functional

parameters were examined, including T-lymphocyte phenotypes, cytokine gene expressions in various organs, and lymphocyte blastogenesis. In addition to the peripheral immune organs, i.e., spleen and thymus, we investigated the cytokine production in liver and kidney, the organs that have their local network of immunologic responses and are often the target of mercury intoxication.

2. Materials and methods

2.1. Animal care and handling

Inbred male BALB/c mice (specific pathogen free, Harlan Inc., Indianapolis, IN), 6 weeks of age and an average body weight of 20 g were procured. Mice were randomly assigned to treatment groups (four per cage) and acclimated for 1 week in the housing facility maintained at 21^{o}C with a 12 h light/dark cycle. The mice were housed in polycarbonate shoe box-style cages lined with wood chip bedding (Betachip, Northeastern Products Corporation, Warrensberg, NY) which was changed every third day. Rodent chow, Harlan Teklad 22/5 rodent chow (Harlan Teklad, Madison, WI) and water were supplied *ad libitum*. Food and water consumption as well as body weight gain were recorded daily. The care and treatment of the mice were in accordance with the guidelines established by the Public Health Service Policy on the Humane Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee.

2.2. Treatment

Mercuric chloride (Sigma, St. Louis, MO) was administered in the deionized drinking water as 0, 0.3, 1.5, 7.5, and 37.5 ppm as mercury. Mice were continuously provided the mercury-containing water for 14 days; the water was replaced every other day with freshly

prepared solution. At the end of the treatment period, mice were fasted overnight and euthanized by decapitation. Trunk blood was collected, and spleen, thymus, liver, and kidneys were aseptically excised and weighed.

2.3. Hematology and estimation of liver enzymes in plasma

Total blood erythrocyte and leukocyte cell counts were determined using an electronic counter (Coulter Electronics, Hialeah, FL). Levels of plasma alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were determined using a Hitachi 912 automatic autoanalyzer (Roche Diagnostics Corp, Indianapolis, IN).

2.4. Preparation of single-cell lymphocyte populations

Single-cell lymphocyte populations were prepared from spleen and thymus as described previously [22] with modifications as noted below. Organs were maintained in 10 ml of cold complete RPMI [RPMI 1640 (Gibco-Life Technologies, Grand Island, NY) with 10% heat-inactivated fetal bovine serum (FBS, Gibco) and 1% Pen-Strep (penicillin-streptomycin, Gibco)]. Monocellular suspensions were prepared using a Stomacher laboratory blender (STOM 80, Seward, London), and connective tissue removed by passing through 120 μm screen. The cell suspension was washed twice with RPMI for 10 min at room temperature. The cell pellet was resuspended in complete RPMI and lymphocyte cell counts performed using a hemocytometer. Cell viability was determined using the trypan blue (Gibco) exclusion and was greater than 95%.

2.5. Mitogen-induced lymphocyte proliferation

The response of lymphocytes to mitogens following treatment of animals to mercury was examined using the lymphocyte blastogenesis assay described earlier [23]. The

mitogens were reconstituted in complete RPMI at the following optimized concentrations: concanavalin A (Con A), 5 μg/ml; phytohemagglutinin (PHA-P), 10 μg/ml; and lipopolysaccharide (LPS), 50 μg/ml. Cultures were plated in triplicate in 96-well flat-bottomed microtiter plates by adding 100 μl spleen cell suspension and 100 μl of the desired mitogen or complete RPMI for controls. An additional control consisting of 200 μl of complete RPMI was also used. The microtiter plates were incubated in a humidified incubator at 37°C with 5% CO_2 for 48 h. After 48 h, each well was pulsed with 20 μl of [methyl-^{3}H]thymidine (25 μCi/ml, 6.7 Ci/mmol, DuPont NEN Products, Boston, MA) and incubated for an additional 18 h. Following the incubation, cells were harvested onto glass fiber filter paper (Cambridge Technology, Watertown, MA) using a cell harvester (PHD, Cambridge). The harvested cells were lysed with deionized water and dried with 95% ethanol. The filter papers were placed in scintillation vials containing 3 ml of liquid scintillation cocktail (Ready-Solv, Beckman, Fullerton, CA) and counted in a liquid scintillation counter (Pharmacia, Turku, Finland). Proliferative responses (uptake of [^{3}H]thymidine) were expressed as net disintegrations per min (DPM) or as stimulation indices (DPM with mitogen/DPM without mitogen).

2.6. Flow cytometric phenotyping of splenic and thymic lymphocyte populations

Three-color flow cytometry was used to determine the prevalence of specific lymphocyte populations in the thymus and spleen. Monoclonal antibodies were conjugated to fluorescein isothiocyanate (FITC, emission at 525 nm), R-phycoerythrin (R-PE, emission at 575 nm) or TRI-COLOR™ (PE-Cy5 tandem transfer dye, emission at 650 nm). Antibodies to cell-specific receptors (purchased from Caltag Laboratories, Burlingame, CA) included hamster anti-mouse CD3-FITC (pan T-lymphocyte), rat anti-mouse CD45/B220-R-

PE (pan B-lymphocyte), rat anti-mouse CD4-R-PE (T-helper lymphocyte) and rat anti-mouse CD8-TRI-COLOR (T-cytotoxic/suppressor lymphocyte). Mouse anti-mouse CD32/16 antibodies were used to block non-specific binding of Fcγ II/III receptors. Cells were washed in phosphate buffered saline (PBS) and antibodies were added to samples and vortexed gently followed by incubation at 4^{o}C for 30 min. Cells were washed three times in PAB (PBS with 1% bovine serum albumin and 0.1% NaN_3) and then resuspended in 0.5% formalin in PBS while vortexing. Fixed cells were maintained at 4^{o}C in the dark until acquisition (< 24 h). Cells were acquired (20,000 events) using an EPICS XL-MCL flow cytometer (Coulter Cytometry, Hialeah, FL) equipped with a 488 nm argon ion laser and Lysis II acquisition software. Analysis was performed using WinMDI^{TM} flow analysis software.

2.7. RNA isolation and semi-quantitative estimation of mRNA expression

RNA was isolated from the tissue using the protocol described earlier [25]. First strand complimentary DNA (cDNA) was synthesized using Superscript II reverse transcriptase enzyme (Life Technologies, Grand Island, NY). Reverse-transcriptase polymerase chain reaction (RT-PCR) was used to analyze the expression of mRNA for TNFα, IFNγ, IL-1β, IL-4, IL-12, major histocompatibility class II (MHC II), c-myc, TGFβ, and β-actin (internal control). The condition for RT-PCR were as previously reported [11,24]. Cycle number was optimized to ensure the product accumulation in an exponential range. The number of cycles was 26-40 cycles based on preliminary trials. Amplified products were separated by electrophoresis on 2% agarose gel containing ethidium bromide. The gels were documented using a Kodak DC 290 digital camera and digitized using UN-

SCAN-IT software (Silk Scientific, Orem, UT). Band intensities for the genes of interest were normalized to that of β-actin in the same sample.

2.8. Statistical analysis

All statistical analyses were performed using SAS statistical software (SAS Institute, Cary, NC). Treatment effects were analyzed using one-way analysis of variance (ANOVA) followed by Duncan's Multiple Range test. A value of $P < 0.05$ was used to indicate significant differences.

3. Results

3.1. Mercury decreased food and water consumption and changed body and organ weights

Food and water consumption decreased in a dose-dependent manner in mice exposed to mercury in the drinking water. Exposure to 0.3 and 1.5 ppm of mercury as mercuric chloride did not alter the water consumption, but mercury at 7.5 and 37.5 ppm decreased daily water consumption by 13.9 and 40.5%, respectively, compared to the control. Food consumption was decreased by 14.4% in mice treated with 37.5 ppm of mercury (Table 1).

Mercury affected body weight gain and relative organ weights (Table 2). Mice treated with 37.5 ppm of mercury for 14 days exhibited 2.1% reduction in body weight compared to an 8.0% gain in the control mice. However, the relative kidney and spleen (organ/body weight ratios) weights were significantly increased in mice exposed to mercury at 37.5 ppm. The relative liver and thymus weights were not altered.

Hematological parameters were altered by mercury exposure. Total erythrocyte counts were significantly decreased at all concentration of mercury treatment (Table 3).

Leukocyte counts were increased at the highest dose of mercury. Oral treatment with mercury did not change plasma ALT and AST levels, indicating a lack of overt hepatotoxicity.

3.2. Mercury treatment altered mitogen-induced lymphoproliferation

Continuous oral exposure to mercury in the drinking water slightly decreased the basal rate of proliferation in splenic lymphocytes (Table 4). In the group treated with 1.5 ppm of mercury, the basal rate of proliferation was reduced to an extent that it resulted in an increase in calculated stimulation indices for PHA-P and LPS.

3.3. Mercury decreased lymphocyte populations in spleen and thymus

Exposure to mercury by drinking water dose-dependently decreased specific lymphocyte populations in spleen and thymus of mice. Oral exposure to mercury at 1.5, 7.5, and 37.5 ppm decreased $CD3^+$ T lymphocytes in the spleen by 14.1, 20.5, and 25.4 %, respectively, whereas population of $CD45^+$ B lymphocytes remained unchanged (Table 5). Dose-dependent decreases in both $CD4^+$ and $CD8^+$ T lymphocyte population in the spleen paralleled the decreases in the total $CD3^+$ T lymphocyte population. Oral exposure to 7.5 and 37.5 ppm of mercury decreased the population of $CD8^+$ cytolytic T lymphocytes in thymus by 46.9 and 50.0%, respectively (Table 6). The double positive $CD4^+/CD8^+$ and the single positive $CD4^+$ thymocyte populations did not decrease significantly.

3.4. Mercury altered the expression of cytokines, c-myc, and MHC II in various organs

In the spleen, a peripheral immune organ, oral exposure to mercury decreased gene expression for IFNγ, and MHC II in a dose-dependent manner (Fig. 1). At the highest dose of mercury, the expression of c-myc was increased. Expression of other cytokines in the

spleen such as TNFα, IL-1β, IL-4, IL-12, and TGFβ were not be altered by mercury. In thymus, the results were different. Expression of TNFα, IL-12, and c-myc were significantly increased by mercury treatment at higher doses of mercury (Fig. 2). Mercury decreased the gene expression for MHC II at the highest dose used here. Expression of other cytokines in thymus, including IFNγ, IL-1β, IL-4, and TGFβ, were not be altered by mercury treatment.

Liver and kidney, other target organs for mercury toxicity, showed different results compared to spleen and thymus, for cytokine expression. In liver, oral exposure to mercury increased expression of TNFα, IFNγ, IL-12, and MHC II (Fig. 3). Other pro-inflammatory cytokines IL-1β and IL-4 (the latter produced by Th2 lymphocytes) were not changed following mercury exposure. Changes in expression of various cytokines in the kidney were similar to that observed in the liver. Exposure to 37.5 ppm of mercury significantly increased expression of TNFα, IFNγ, IL-12, and MHC II (Fig. 4). Expression of IL-1β, and IL-4 in kidney were not be altered by mercury exposure.

4. Discussion

Results of this study indicated that inorganic mercury given to mice via drinking water caused immunologic alterations, i.e., changes in T-lymphocyte phenotypes and cytokine expression, and in hematological parameters in BALB/c mice. The immune responses to mercury exposure were complex, depending in part on the dose of mercury and the genetic characteristics of exposed strains. Administration of 14.8 mg mercury/kg/day as mercuric chloride to B6C3F mice for 2 weeks showed immune suppression, including a decrease in thymus weight [25]. To simulate closely to human exposure, we treated mice with relatively low level of mercury, ranging from 0.06 to 4.81 mg/kg/day, via drinking

water. The dose of mercury used in this study did not induce hepatotoxicity as suggested by a lack of changes in circulating liver enzymes. This suggests that the doses of mercury that were not cytotoxic in liver; nevertheless, altered immunologic signaling. The doses used in the current study were spaced logarithmically and provided a sudden drop in body weights between the two highest doses. In future studies it may be desirable to investigate the doses at closely spaced intervals to define the dose-response in observed changes.

Exposure to mercury in the drinking water affected lymphocyte blastogenesis of splenocytes only marginally. Contradictory effects have been reported earlier on splenic lymphocyte proliferation. After 2 weeks of treatment with 2 ppm mercury via drinking water, decreased spleen lymphocyte proliferation and response to mitogens were observed in Swiss Webster mice [26]. Increased lymphoproliferative responses of splenocytes after stimulation with mitogens was seen in SJL mice at 5 ppm mercury (0.7 mg/kg/day) [27]. In the present study, mercury treatment resulted in a decrease in the spontaneous rate of splenocyte proliferation (without mitogens) at only 1.5 ppm of mercury (0.31 mg/kg/day). Such a decrease was reflected by increased PHA-P and LPS-stimulated lymphocyte proliferation indices, even though the net incorporation of thymidine in cells was not altered in the presence of mitogens. The decreased spontaneous proliferation of splenocytes at 1.5 ppm of mercury needs further confirmation in separate studies. It has been reported that mitogen-activated lymphocytes become refractory to the immunotoxic effects of mercury [28]. Mitogen-stimulated proliferation of lymphocytes occurs through multiple activation pathways that require distinct lymphokines [29].

Mercury is known to elicit a spectrum of immunological responses ranging from immunosuppression to autoimmunity [4,30]. It has been reported that 14 days of treatment

with inorganic mercury altered cervical lymph node lymphocyte phenotypes [31]. In mercury-susceptible BN rats, mercury decreased $CD4^+$ and $CD8^+$ populations without changing the population of B cells and $CD4^+/CD8^+$ double positive T lymphocytes. Our current data showing a decrease in $CD3^+$ T lymphocyte population, and among the T lymphocytes, both $CD4^+$ and $CD8^+$ T lymphocytes without changing number of B lymphocytes in spleen are consistent to this early report in rats. No changes were seen in the ratio of $CD4^+$ and $CD8^+$ T lymphocytes, which indicated that general impact of mercury on T lymphocyte is not a skewing of the subtype. Furthermore, the effect is T lymphocyte dependent, because mercury significantly decreased T lymphocytes without changes in B lymphocytes in spleen. In the current study a lack of effect of mercury on B cell population and a decrease in T cells (both helper and suppressor) in the spleen, and a selective depletion of T helper cells in thymus, indicate the possible pathways involved in mercury-induced divergent immunotoxic responses.

Mercury decreased proliferation of $CD4^+$ lymph node T lymphocytes, IL-3 and IFNγ secretion, leading to apoptotic cell death [32]. In other studies, mercury inhibited antigen-induced T cell responses but enhanced mitogen-induced responses and directly stimulated T cells, leading to apoptosis or activation [12,33]. In the present study, decrease in specific types of lymphocyte is not likely because of mercury-induced apoptosis in peripheral lymphoid organs, as mercury did not decrease the weight of thymus and increased the weight of spleen. The number of apoptosis-sensitive double-positive T lymphocyte in thymus remained unchanged. It has been reported that mercury caused thymus atrophy and changes in thymocyte subpopulations, and the atrophy of thymus was not due to apoptotic effect of mercury [34].

Decreased expression of MHC II and IFNγ in spleen may be responsible for the decrease of the lymphocyte subpopulation. In previous reports cytokines such as TNFα, IL-1, IL-4, IL-12, and IFN-γ were induced after in vivo or in vitro treatments and may have contributed to the immunologic and cell proliferative responses [16,35,36]. MHC II is also involved in both cytokine production and proliferation [15]. The IFNγ is an important cytokine that stimulates macrophages, and MHC II promotes the ability of stimulated macrophages to serve as antigen presenting cells to T lymphocytes [37]. The involvement of MHC II suggests that the two cell types, T cells and macrophages, are required for polyclonal proliferation, and may contribute to mercury-induced immunological responses, including autoimmunity [16,35]. In our present results, relatively high concentration of mercury suppressed gene expression of MHC II, which is important for T lymphocyte-mediated immune response. This result is consistent with the decrease of $CD4^+$, and $CD8^+$ lymphocyte populations, and decrease in IFNγ, which may be related to T lymphocyte mediated activation of macrophages. In thymus the highest dose of mercury decreased expression of MHC II and increased the expression of TNFα, IL-12, and c-myc. Treatment of mouse fetal thymic organ culture with IL-12 caused a significant increase in both the percentage and cell number of $CD8^+$ thymocytes [38]. These results may explain the increase of IL-12 mRNA in thymus by mercury. Because $CD8^+$ thymocytes were significantly decreased by mercury, IL-12 was produced in response for stimulating thymus to differentiate $CD8^+$ lymphocytes. The highest dose of mercury increased expression of c-myc in both spleen and thymus. These data are in accordance with previous reports showing increased expression of protooncogene c-myc after mercury treatment, which is known to be responsible for carcinogenesis [17].

Liver and kidney are major targets of mercury toxicity [20,39]. In both liver and kidney, relatively high concentrations of mercury increased expressions of TNFα, IL-12, IFN-γ, and MHC II. Increases of IFNγ and MHC II expression in liver and kidney were opposite to decreases in spleen and thymus. This disparity may be due to the structural and functional differences of different organs. Instead of immunosuppressive effects, intoxication of mercury may stimulate localized immune response in liver and kidney suggesting mild inflammatory changes and resulting increased cytokine expression. Madrenas et al [40] reported that mercury increased renal MHC II expression and treatment of anti-IFNγ antibody blocked this MHC II induction. This report suggested that the mercury-induced MHC II induction was mediated by IFNγ in renal system and supports our data showing increases of both IFNγ and MHC II in kidney.

It should be emphasized that the changes reported here were measured after a 2-week treatment with mercury. Cytokine changes may occur early during the treatment period and it is desirable to investigate the time-related alterations in cytokine expression. Such kinetics of cytokine expression will be evaluated in future studies.

Because of the broad range of immunomodulant properties, mercury is considered as paradigm of xenobiotic immunotoxicity, and may be a useful tool for investigation of the cytokine network and as a model for autoimmune disease [41]. Our present data suggest that mercury contamination in the drinking water caused immunotoxicity. The subtype populations of peripheral lymphoid T lymphocyte and cytokine expression in various organs were altered by mercury exposure. The molecular mechanism of mercury-induced immunomodulation remains to be elucidated. To the best of our knowledge this is a first

report of cytokine network and pathological investigations in liver and kidney with low doses of mercury exposure.

References

1. Christensen MM, Ellermann-Eriksen S, Rungby J, and Mogensen SC. Influence of mercuric chloride on resistance to generalized infection with herpes simplex virus type 2 in mice. Toxicology 1996; 114:57-66.
2. Gerstner HB and Huff JE. Clinical toxicology of mercury. J Toxicol Environ Health 1977; 2:491-526.
3. Wild LG, Ortega HG, Lopez M, and Salvaggio JE. Immune system alteration in the rat after indirect exposure to methyl mercury chloride or methyl mercury sulfide. Environ Res 1997; 74:34-42.
4. Koller LD. Immunosuppression produced by lead, cadmium, and mercury. Am J Vet Res 1973; 34:1457-8.
5. Koller LD. Methylmercury: effect on oncogenic and nononcogenic viruses in mice. Am J Vet Res 1975; 36:1501-4.
6. Pietsch P, Vohr HW, Degitz K, and Gleichmann E. Immunological alterations inducible by mercury compounds. II. $HgCl_2$ and gold sodium thiomalate enhance serum IgE and IgG concentrations in susceptible mouse strains. Int Arch Allergy Appl Immunol 1989; 90:47-53.
7. Prouvost-Danon A, Abadie A, Sapin C, Bazin H, and Druet P. Induction of IgE synthesis and potentiation of anti-ovalbumin IgE antibody response by $HgCl_2$ in the rat. J Immunol 1981; 126:699-792.
8. Sapin C, Druet E, and Druet P. Induction of anti-glomerular basement membrane antibodies in the Brown-Norway rat by mercuric chloride. Clin Exp Immunol 1977; 28:173-9.

9. Kaiser, J. Mercury report backs strict rules. Science 2000; 289:371-2.
10. Perlingeiro RC and Queiroz ML. Polymorphonuclear phagocytosis and killing in workers exposed to inorganic mercury. Int J Immunopharmacol 1994; 16:1011-1017.
11. Kim SH, Johnson VJ, and Sharma RP. Mercury inhibits nitric oxide production but activates proinflammatory cytokine expression in murine macrophage: differential modulation of NF-kB and p38 MAPK signaling pathways. Nitric Oxide 2002; 7:67-74.
12. Jiang Y and Moller G. In vitro effects of HgCl2 on murine lymphocytes. I. Preferable activation of CD4+ T cells in a responder strain. J Immunol 1995; 154:3138-46.
13. Shenker BJ, Rooney C, Vitale L, and Shapiro IM. Immunotoxic effects of mercuric compounds on human lymphocytes and monocytes. I. Suppression of T-cell activation. Immunopharmacol Immunotoxicol 1992; 14:539-53.
14. Badou A, Savignac M, Moreau M et al. $HgCl_2$-induced interleukin-4 gene expression in T cells involves a protein kinase C-dependent calcium influx through L-type calcium channels. J Biol Chem 1997; 272:32411-8.
15. Hu H, Moller G, and Abedi-Valugerdi M. Major histocompatibility complex class II antigens are required for both cytokine production and proliferation induced by mercuric chloride in vitro. J Autoimmun 1997; 10:441-6.
16. Johansson U, Sander B, and Hultman P. Effects of the murine genotype on T cell activation and cytokine production in murine mercury-induced autoimmunity. J Autoimmun 1997; 10:347-55.

17. Vamvakas S, Bittner D, and Koster U. Enhanced expression of the protooncogenes c-myc and c-fos in normal and malignant renal growth. Toxicol Lett 1993; 67:161-72.

18. Cember H, Gallagher P, and Faulkner A. Distribution of mercury among blood fractions and serum proteins. Am Ind Hyg Assoc J 1968; 29:233-7.

19. Nielsen JB and Andersen O. Oral mercuric chloride exposure in mice: effects of dose on intestinal absorption and relative organ distribution. Toxicology 1989; 59:1-10.

20. Nielsen JB and Andersen O. Disposition and retention of mercuric chloride in mice after oral and parenteral administration. J Toxicol Environ Health 1990; 30:167-80.

21. Nielsen JB. Toxicokinetics of mercuric chloride and methylmercuric chloride in mice. J Toxicol Environ Health 1992; 37:85-122.

22. Johnson VJ and Sharma RP. Gender-dependent immunosuppression following subacute exposure to fumonisin B_1. Int Immunopharmacol 2001; 1:2023-34.

23. Johnson VJ, Rosenberg AM, Lee K, and Blakley BR. Increased T-lymphocyte dependent antibody production in female SJL/J mice following exposure to commercial grade malathion. Toxicology 2002; 170:119-29.

24. Sharma RP, Bhandari N, Riley RT, Voss KA, and Meredith FI. Tolerance to fumonisin toxicity in a mouse strain lacking the P75 tumor necrosis factor receptor. Toxicology 2000; 143:183-94.

25. National Toxicology Program. Toxicology and carcinogenesis studies of mercuric chloride in F344/N rats and B6CF1 mice. 1993; NIH publication, 91-3139.

26. Gaworski CL and Sharma RP. The effects of heavy metals on [^{3}H]thymidine uptake in lymphocytes. Toxicol Appl Pharmacol 1978; 46:305-13.

27. Hultman P and Johansson U. Strain differences in the effect of mercury on murine cell-mediated immune reactions. Food Chem Toxicol 1991; 29:633-8.

28. Shenker BJ, Berthold P, Rooney C, Vitale L, DeBolt K, and Shapiro IM. Immunotoxic effects of mercuric compounds on human lymphocytes and monocytes. III. Alterations in B-cell function and viability. Immunopharmacol Immunotoxicol 1993; 15:87-112.

29. Pecanha LM and Dos Reis GA. Murine polyclonal T-lymphocyte activation induced by phytohemmagglutinin; differential lymphokine requirements of two unusual activation pathways defined by resistance to blockade by barium and by cyclosporin A. Int J Immunopharmacol 1989; 11:149-56.

30. Koller LD. Immunotoxicology of heavy metals. Int J Immunopharmacol 1980; 2:269-79.

31. Kosuda LL, Whalen B, Greiner DL, and Bigazzi PE. Mercury-induced autoimmunity in Brown Norway rats: kinetics of changes in RT6+ T lymphocytes correlated with IgG isotypes of circulating autoantibodies to laminin 1. Toxicology 1998; 125:215-31.

32. Shen X, Lee K, and Konig R. Effects of heavy metal ions on resting and antigen-activated CD4(+) T cells. Toxicology 2001; 169:67-80.

33. Guo TL, Miller MA, Shapiro IM, and Shenker BJ. Mercuric chloride induces apoptosis in human T lymphocytes: evidence of mitochondrial dysfunction. Toxicol Appl Pharmacol 1998; 153:250-7.

34. Kosuda LL, Hannigan MO, Bigazzi PE, Leif JH, and Greiner DL. Thymus atrophy and changes in thymocyte subpopulations of BN rats with mercury-induced renal autoimmune disease. Autoimmunity 1996; 23:77-89.

35. Pollard KM and Hultman P. Effects of mercury on the immune system. Met Ions Biol Syst 1997; 34:421-40.

36. Reardon CL and Lucas DO. Heavy-metal mitogenesis: Zn^{++} and Hg^{++} induce cellular cytotoxicity and interferon production in murine T lymphocytes. Immunobiology 1987; 175:455-69.

37. Locksley RM, Wakil AE, Corry DB, Pingel S, Bix M, and Fowell DJ. The development of effector T cell subsets in murine *Leishmania major* infection. Ciba Found Symp 1995; 195:110-7.

38. Godfrey DI, Kennedy J, Gately MK, Hakimi J, Hubbard BR, and Zlotnik A. IL-12 influences intrathymic T cell development. J Immunol 1994; 152:2729-35.

39. Zalups RK. Molecular interactions with mercury in the kidney. Pharmacol Rev 2000; 52:113-43.

40. Madrenas J, Parfrey NA, and Halloran PF. Interferon γ-mediated renal MHC expression in mercuric chloride-induced glomerulonephritis. Kidney Int 1991; 39:273-81.

41. Bigazzi PE. Mercury. In: Zelikoff J, Thomas P (Eds.), Metal Immunotoxicology, Taylor and Francis, London, UK. 1998; p. 131-161.

Table 6.1. Exposure to mercury and water and food consumption

Mercury in Water (ppm)	Hg dose (mg/kg/day)[a]	Water Consumption (ml/day/group)	Food Consumption (g/day/group)
0	0	19.57 ± 0.86	13.84 ± 0.77
0.3	0.06	18.50 ± 0.81	14.21 ± 0.46
1.5	0.31	19.14 ± 1.14	14.45 ± 0.59
7.5	1.39	16.85 ± 0.90*	13.45 ± 1.58
37.5	4.81	11.64 ± 1.26*	11.85 ± 0.45*

Mean ± SE (n = 4).

[a] Calculated dose based on water consumption.

*Significantly different from the control group at $P < 0.05$.

Table 6.2. Effects of oral mercury exposure on body weight gain and relative organ weights in male BALB/c mice

Mercury in Water (ppm)	Body Weight Gain, g (% change from initial Body Weight)	Organ/Body Weight Ratio (g/100 g) for			
		Liver	Kidney	Spleen	Thymus
0	1.72 ± 0.51 (8.0)	4.70 ± 0.08	1.65 ± 0.02	0.31 ± 0.01	0.21 ± 0.04
0.3	1.72 ± 0.24 (8.6)	4.59 ± 0.11	1.62 ± 0.01	0.35 ± 0.02	0.27 ± 0.02
1.5	1.65 ± 0.27 (7.9)	4.72 ± 0.04	1.64 ± 0.04	0.34 ± 0.01	0.27 ± 0.01
7.5	1.45 ± 0.19 (7.1)	4.70 ± 0.04	1.83 ± 0.01*	0.38 ± 0.02	0.25 ± 0.01
37.5	-0.45 ± 0.43 (-2.1)*	4.78 ± 0.17	2.05 ± 0.06*	0.40 ± 0.02*	0.26 ± 0.01

Mean ± SE (n = 4).

*Significantly different from the control group at $P < 0.05$.

Table 6.3. Effects of oral mercury exposure on circulating erythrocyte and leukocyte, and liver enzymes in male BALB/c mice

Mercury in Water (ppm)	RBC ($\times 10^{-6}$/mm^3)	WBC ($\times 10^{-3}$/mm^3)	ALT (Units/Liter)	AST (Units/Liter)
0	7.61 ± 0.20	2.20 ± 0.19	38.25 ± 3.09	215.25 ± 31.76
0.3	6.64 ± 0.32*	2.70 ± 0.57	38.75 ± 0.75	228.75 ± 7.90
1.5	6.61 ± 0.13*	3.30 ± 0.65	40.25 ± 4.11	201.75 ± 26.85
7.5	6.75 ± 0.08*	2.53 ± 0.50	40.00 ± 6.46	217.75 ± 52.89
37.5	6.15 ± 0.23*	4.21 ± 0.76*	29.50 ± 2.53	143.25 ± 22.96

Mean ± SE (n = 4).

Abbreviations: RBC, red blood cell; WBC, white blood cell; ALT, alanine aminotransferase; AST, aspartate aminotransferase.

*Significantly different from the control at $P < 0.05$.

Table 6.4. Effects of oral mercury exposure on mitogen-stimulated lymphocyte proliferation of splenic lymphocytes, as measured by [^{3}H]thymidine incorporation

Mercury concentration (ppm) in Water	No mitogen DPM[a] × 10^{-3}	Mitogens					
		PHA-P		LPS		Con A	
		Net DPM[b] × 10^{-3}	SI[c]	Net DPM × 10^{-3}	SI	Net DPM × 10^{-3}	SI
0	0.93 ± 0.13	2.69 ± 0.85	4.14 ± 1.18	18.55 ± 4.54	22.01 ± 6.22	59.17 ± 6.12	67.91 ± 10.81
0.3	0.99 ± 0.14	3.48 ± 0.39	4.70 ± 0.58	22.54 ± 1.34	25.10 ± 3.69	57.95 ± 4.41	63.77 ± 11.93
1.5	0.58 ± 0.07*	3.97 ± 0.37	8.15 ± 0.73*	24.70 ± 3.58	44.27 ± 2.25*	51.94 ± 2.97	97.48 ± 15.44
7.5	0.83 ± 0.07	1.94 ± 0.89	3.44 ± 1.18	20.47 ± 5.58	26.13 ± 7.51	47.87 ± 2.06	59.90 ± 6.29
37.5	0.74 ± 0.05	3.75 ± 0.90	6.04 ± 1.19	27.69 ± 4.37	39.76 ± 7.61	54.13 ± 2.62	75.33 ± 7.12

Mean ± SE (n = 4).

Significantly different from the control at $P < 0.05$.

[a]DPM = disintegrations/minute.

[b]Net DPM = DPM with mitogen - DPM without mitogen.

[c]SI = stimulation index = DPM with mitogen / DPM without mitogen.

Table 6.5. Effects of oral mercury exposure on lymphocyte populations in the spleen of male BALB/c mice. Populations are expressed as absolute cell numbers expressing a given receptor

Mercury in Water (ppm)	Splenocytes ($\times 10^{-5}$) per spleen expressing				
	$CD3^+$ (T-lymphocytes)	$CD45/B220^+$ (B-lymphocytes)	$CD4^+$ (T-helper)	$CD8^+$ (T-supperssor/cytotocxic)	$CD4^+/CD8^+$ (Ratio)
0	47.69 ± 0.93	52.80 ± 1.04	31.29 ± 0.64	14.08 ± 0.27	2.22 ± 0.04
0.3	43.59 ± 0.64	53.87 ± 2.30	28.75 ± 0.55	12.93 ± 0.53	2.22 ± 0.07
1.5	41.01 ± 1.65*	53.44 ± 1.25	26.92 ± 1.20*	12.02 ± 0.53*	2.24 ± 0.03
7.5	37.91 ± 1.81*	55.52 ± 1.81	25.04 ± 1.30*	10.89 ± 0.58*	2.30 ± 0.06
37.5	35.56 ± 0.94*	58.81 ± 2.63	23.14 ± 0.61*	10.69 ± 0.35*	2.16 ± 0.04

Mean ± SE (n = 4).

*Significantly different from the control group at $P < 0.05$.

Table 6.6. Effects of oral mercury exposure on lymphocyte populations in the thymus of male BALB/c mice. Populations are expressed as absolute cell numbers expressing a given receptor

Mercury in Water (ppm)	Thymocytes ($\times 10^{-5}$) per thymus expressing		
	$CD4^+/CD8^+$ (double positive)	$CD4^+/CD8^-$ (helper)	$CD4^-/CD8^+$ (suppressor/cytotoxic)
0	63.35 ± 4.94	8.58 ± 0.82	2.81 ± 0.29
0.3	56.54 ± 6.71	7.06 ± 1.06	2.08 ± 0.29
1.5	54.08 ± 7.21	6.63 ± 0.82	1.98 ± 0.23
7.5	63.02 ± 2.92	6.56 ± 0.43	1.49 ± 0.15*
37.5	59.23 ± 6.21	6.05 ± 0.69	1.38 ± 0.16*

Mean ± SE (n = 4).

*Significantly different from the control group at $P < 0.05$.

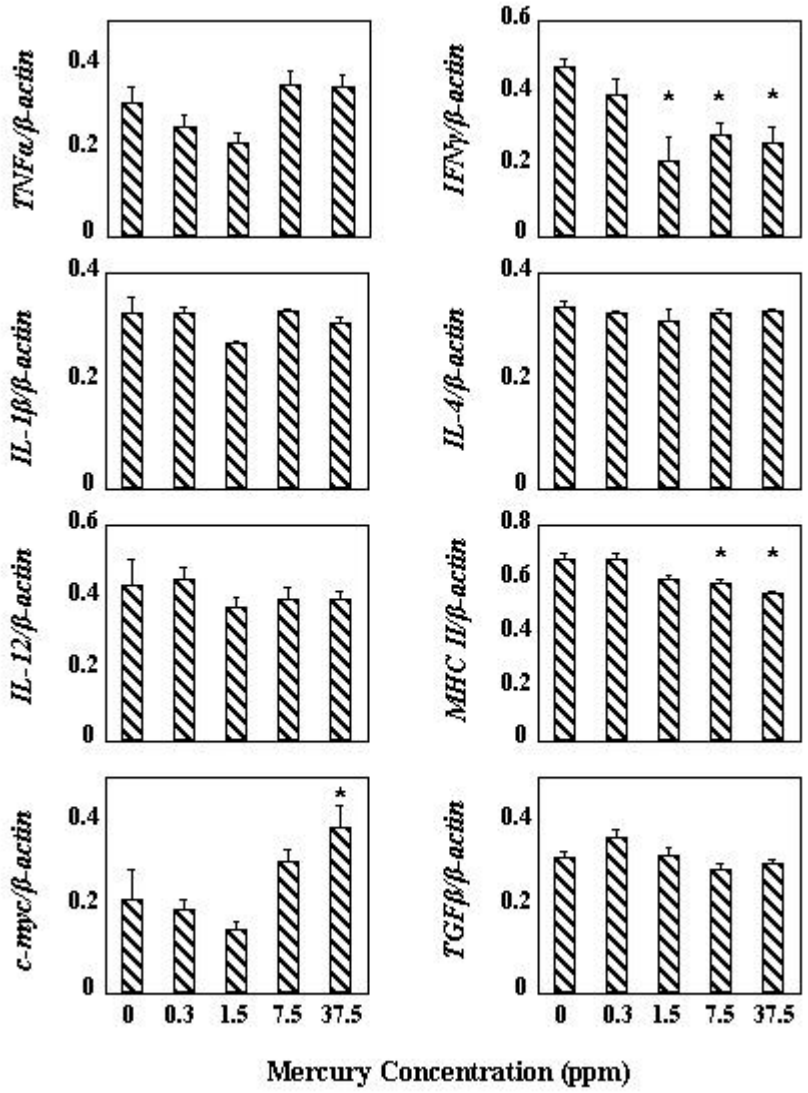


Fig. 6.1. The effect of inorganic mercury on the expression of various cytokines and other factors in spleen. Male BALB/c mice were treated with 0.3, 1.5, 7.5, 37.5 ppm of mercury in the drinking water for 14 days. Extraction and analysis of mRNA performed as described under Materials and methods. Each gene expression was analyzed by RT-PCR. Results are expressed as mean ± SE (n=4). *Significantly different than the control group at $P < 0.05$.

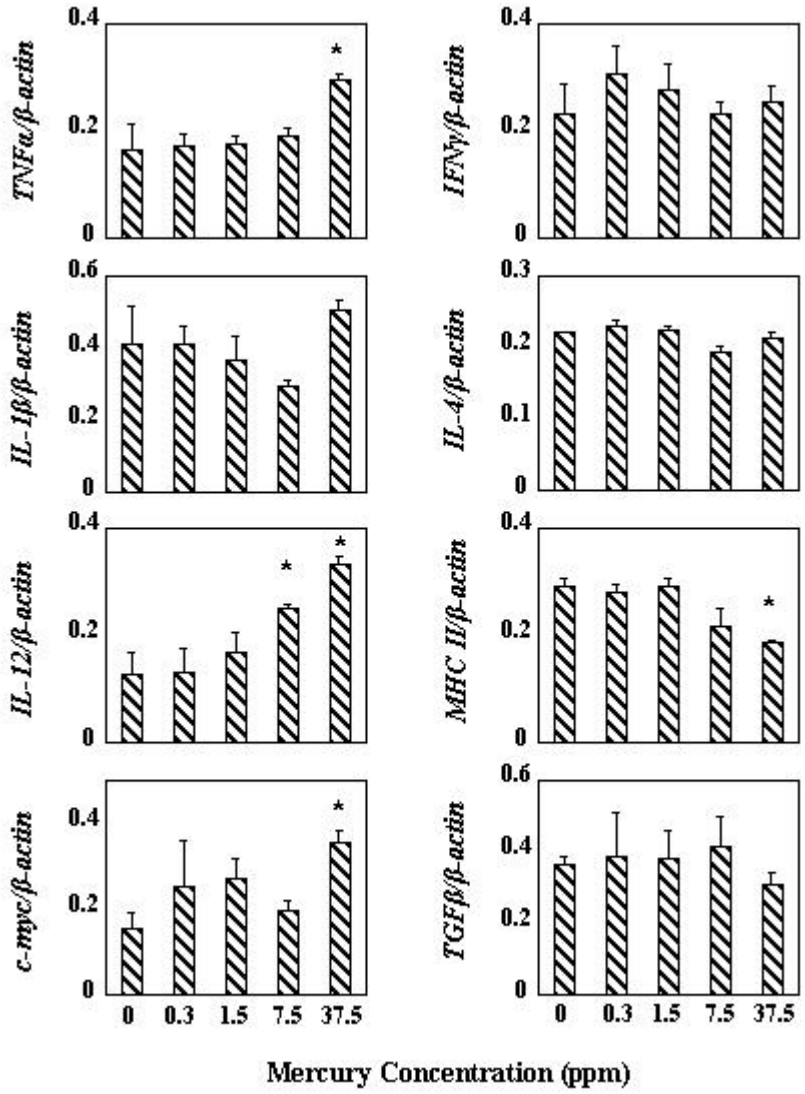


Fig. 6.2. The effect of inorganic mercury on the expression of cytokines and related factors in thymus. Male BALB/c mice were treated with 0.3, 1.5, 7.5, 37.5 ppm of mercury in the drinking water for 14 days. Extraction and analysis of mRNA performed as described under Materials and methods. Each gene expression was analyzed by RT-PCR. Results are expressed as mean ± SE (n=4). *Significantly different than the control group at $P < 0.05$.

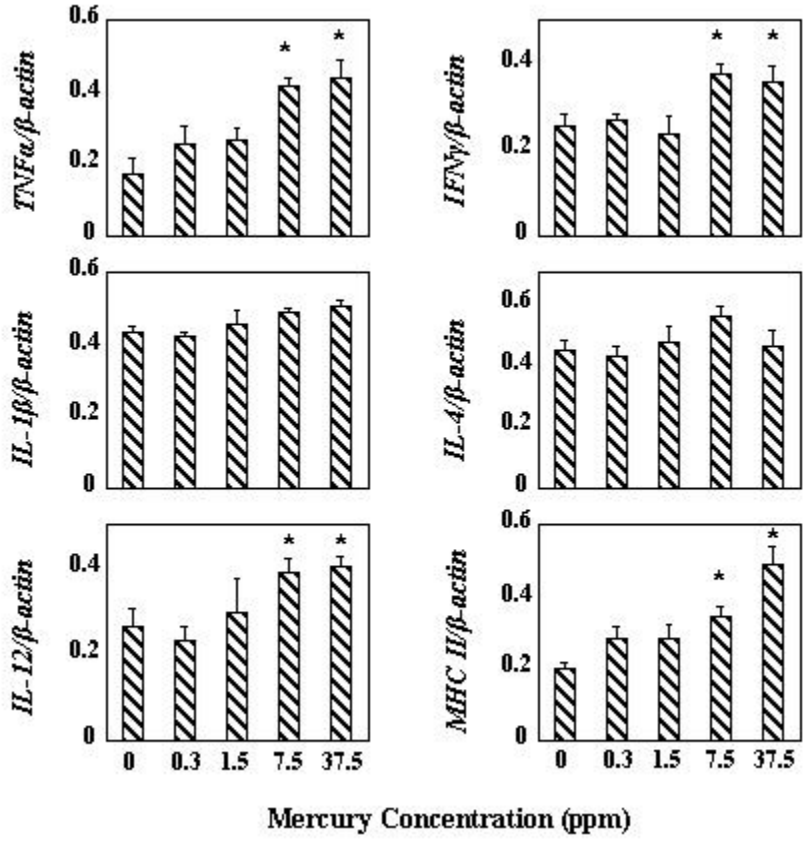


Fig. 6.3. The effect of inorganic mercury on the expression of cytokines and related factors in liver. Male BALB/c mice were treated with 0.3, 1.5, 7.5, 37.5 ppm of mercury in the drinking water for 14 days. Extraction and analysis of mRNA performed as described under Materials and methods. Each gene expression was analyzed by RT-PCR. Results are expressed as mean ± SE (n=4). *Significantly different than the control group at $P < 0.05$.

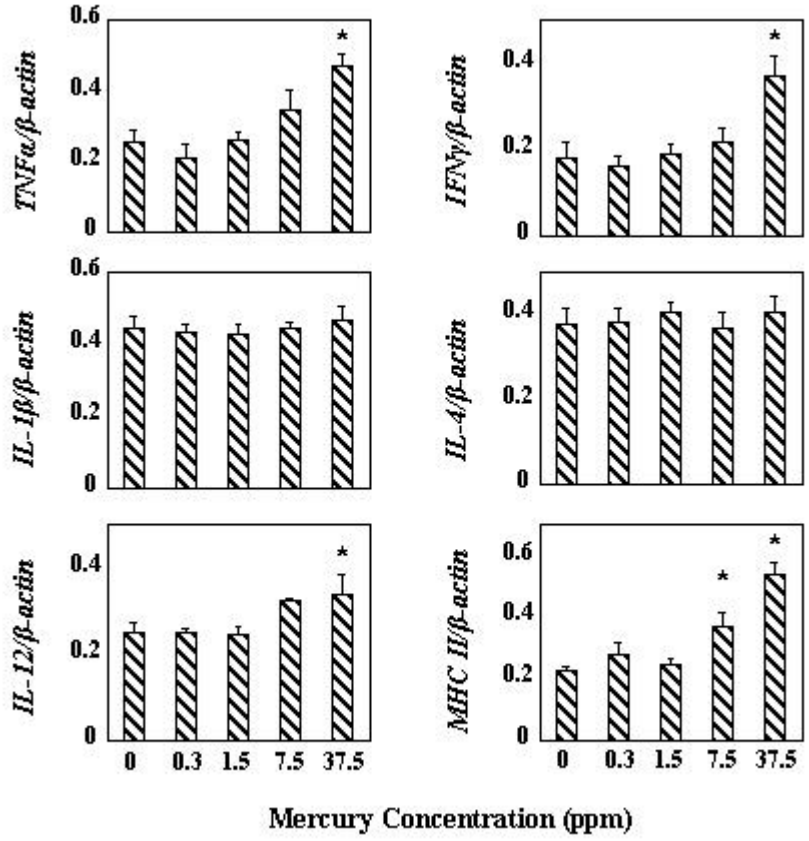


Fig. 6.4. The effect of inorganic mercury on the expression of cytokines and other factors in kidney. Male BALB/c mice were treated with 0.3, 1.5, 7.5, 37.5 ppm of mercury in the drinking water for 14 days. Extraction and analysis of mRNA performed as described under Materials and methods. Each gene expression was analyzed by RT-PCR. Results are expressed as mean ± SE (n=4). *Significantly different than the control group at $P < 0.05$.

CHAPTER 7

MERCURY ALTERS ENDOTOXIN INDUCED INFLAMMATORY CYTOKINE EXPRESSION IN LIVER: DIFFERENTIAL ROLE OF p38 AND EXTRACELLULAR SIGNAL-REGULATED KINASE MITOGEN-ACTIVATED PROTEIN KINASE[1]

---

[1]Kim S.H., Raghubir P. Sharma. Submitted to *Life Sciences*.

Abstract

Mercury is a widespread metal in the environmental and consequently there are large populations that currently exposed to low levels of mercury. Endotoxin is a component of the gram-negative bacteria and promotes inflammatory responses. We previously reported that mercury modulates the production of nitric oxide and various inflammatory cytokines induced by endotoxin in macrophage cell lines (Nitric Oxide 7:67, 2002). The present study was designed to determine the impact of mercury on endotoxin-induced inflammatory cytokine expression and corresponding signal transduction in liver. Male BALB/c mice were exposed continuously to 0, 0.3, 1.5, 7.5, or 37.5 ppm of mercury in drinking water for 14 days and at the end of the treatment period, lipopolysaccharde (LPS, 0.5 mg/kg) was injected intraperitoneally 2 hr prior to euthanasia. The doses of mercury and LPS did not cause hepatotoxicity as indicated by circulating alanine aminotransferase and aspartate aminotransferase. Mercury decreased liver glutathione (GSH) and potentiated LPS-induced GSH depletion. Mercury activated p38 mitogen-activated protein kinase (MAPK) and additively increased LPS-induced p38 MAPK phosphorylation. In contrast, mercury inhibited LPS-induced activation of extracellular signal-regulated kinase (ERK) but had no effect alone. Mercury increased the gene expression of tumor necrosis factor α (TNFα) and potentiated LPS-induced TNFα expression. Mercury did not alter LPS-induced interleukin-1β (IL-1β) expression. Mercury decreased LPS-induced IL-6 expression. These results indicate that low levels of mercury augment LPS-induced TNFα expression by altering GSH and p38 MAPK. Mercury modulates LPS-induced p38 and ERK activation, and downstream TNFα and IL-6 expression in liver, respectively.

Keyword: Mercury, Lipopolysaccharide, Glutathione, Mitogen-activated protein kinase, Tumor necrosis factor α, Inflammation.

Introduction

The component of cell walls of gram-negative bacteria, lipopolysaccharide (LPS), mediates many of the pathophysiological alterations in tissues, including liver. Systemic exposure to LPS results in a cascade of events involving cellular and soluble mediators of inflammation that leads to injury to organs. Endotoxin promotes the activation of immune cells which are important sensors of infections [13,32]. Non-toxic and non-injurious doses of LPS augment the hepatotoxicity of certain xenobiotic agents such as alcohol and metals [4,17]. Exposure to small amounts of LPS that do not normally evoke overt liver damage are nevertheless capable of initiating aspects of the inflammatory response [38].

Mercury is widespread in the environment and chronic exposure to low levels of mercury is common due to contamination of food and drinking water supplies. The known critical organ of toxicity for the mercuric chloride is kidney, however, the hepatotoxic effects of mercury have been reported. Oral administration of mercuric chloride increases hepatic lipid peroxidation and decrease glutathione peroxidase [31]. Acute exposure to mercuric chloride causes liver damage by binding sulfhydryl groups [8]. The property of bonding to sulfydryl group causes alteration in cell signaling. Mercury depletes cellular glutathoine (GSH) content and causes cellular and tissue damage [33,39]. The GSH has an important role in the reduction of reactive oxygen species (ROS) and the maintaining intracellular redox equilibrium in the liver [12,39]. Modified redox signaling regulates LPS-induced mitogen-activated protein kinases (MAPKs) activation and MAPK-mediated proinflammatory cytokines such as tumor necrosis factor α (TNFα), interleukin (IL)-1β, and IL-6 [14,15,22]. p38 and extracellular

signal-regulated kinase (ERK) mediate responses to cellular stress such as endotoxin and inflammatory signals [23,24].

We recently reported that non-cytotoxic dose of mercury altered macrophages response to bacterial infection [21]. Low dose of mercury decreased LPS-induced nitric oxide (NO) production, an important element to host resistance and acquisition of immunity. Mercury potentiated LPS-induced TNFα expression by regulating p38 MAPK. Excess induction of TNFα causes liver injury [16,25]. However, the mechanism of mercury-induced proinflammatory cytokine production, especially TNFα, has not been well characterized in liver.

Clinical studies on industrial workers exposed to mercury levels within World Health Organization guidelines have shown immune system abnormalities such as deficiency of myeloperoxidase in neutrophils and stimulation of immunogloblin in the absence of liver injury and neurological dysfunction [29,30]. It is known that immune response initiates liver damage by hepatic neutrophil accumulation, and liver injury is caused partly by activation of the local cytokine network, because the liver has a complete innate immune system consisting of Kupffer cells and hepatic NK cells [20]. Hence we hypothesized that the low doses of mercury, known immunotoxic agent, may alter LPS-mediated immune signaling in liver. The results of this study revealed that mercury potentiated LPS-mediated depletion of GSH, followed by alteration of MAPKs-mediated inflammatory cytokine expression. Mercury differentially modulated LPS-mediated MAPKs and inflammatory cytokine expression.

Materials and methods

Animal care and handling

Inbred male BALB/c mice (specific pathogen free, Harlan Inc., Indianapolis, IN), 6 weeks of age and an average body weight of 20 g were procured. Mice were randomly assigned to treatment groups (four per cage) and acclimated for 1 week in the housing facility maintained at 21^{o}C with a 12 h light/dark cycle. The mice were housed in polycarbonate shoe box-style cages lined with wood chip bedding (Betachip, Northeastern Products Corporation, Warrensberg, NY) which was changed every third day. Rodent chow, Harlan Teklad 22/5 rodent chow (Harlan Teklad, Madison, WI) and water were supplied *ad libitum*. Food and water consumption as well as body weight gain were recorded daily. The care and treatment of the mice were in accordance with the guidelines established by the Public Health Service Policy on the Humane Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee.

Treatment

Mercuric chloride (Sigma, St. Louis, MO) was administered in the drinking water ranging 0, 0.3, 1.5, 7.5, and 37.5 ppm as mercury. Mice were continuously fed water supplemented with freshly prepared mercury solution and this solution was changed every other day for 14 days. At the end of the treatment period, mice were fasted overnight and LPS (0.5 mg/kg) was injected intraperitoneally 2 hr prior to euthanasia the mice. Mice were decapitated, trunk blood was collected, and liver was aseptically excised and weighed.

Assessment of hepatotoxicity

Hepatic toxicity was assessed by measuring plasma alanine aminotransferase (ALT) and aspartate aminotransferase (AST) using a Hitachi 912 Automatic Analyzer (Roche Diagnostics, Indianapolis, IN).

GSH assay

Levels of GSH in liver were determined by measuring the total reduced GSH content in the liver as described by Baker [2]. The tissue was homogenized in 5 vol of 5% of sulfosalicylic acid (SSA) to precipitate macromolecules and extract GSH from tissues and centrifuged for 15 min at 12,000 × g. The GSH was detected by the color change associated with 5,5-dithiobis-(2-nitrobenzoic acid) reduction using PowerWave$_x$ Microplate Scanning spectrophotometer (Bio-Tek Instruments, Winooski, VT) at 405 nm. The GSH concentration was calculated by GSH standard calibration and expressed as GSH equivalents per gram of liver tissue.

Western blot analysis

Liver samples were homogenized in lysis buffer (20 mM Tris, 137 mM NaCl, 2 mM EDTA, 10% glycerol, 1% Triton X-100, 1mM Na_3VO_3, 100 μM DTT, 100 mM PMSF, 100 μg/ml leupeptin, 10 μg/ml aprotinin) and centrifuged for 10 min at 12,000 × g. After homogenizing, 30 μg of protein was precipitated with cold acetone at –80^{o}C for 1 hr. The precipitated proteins were electrophoresed using 12% SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) as previously described [21] and then transferred to nitrocellulose membrane. The p38 and ERK activation were assayed using (1:1000) anti-phospho-p38 and anti-phospho-ERK antibody (Cell Signaling, Beverly,

MA). Immunodetection was performed using enhanced chemiluminescence (ECL) detection kit (Amersham Pharmacia, Piscataway, NJ).

Semiquantitative analysis of gene expression

Total RNA was isolated from the tissue using the protocol described earlier [18]. First strand complimentary DNA (cDNA) was synthesized using Superscript II reverse transcriptase enzyme (Life Technologies, Grand Island, NY). Reverse-transcriptase polymerase chain reaction (RT-PCR) was used to analyze the expression of mRNA for TNFα, IL-1β, IL-6, and β-actin (internal control). The condition for reverse transcription and PCR steps were performed as previously reported [21]. The respective primer sets were chosen by Primer3 program (Whithead Institute, Cambridge, MA) and are shown in Table 1. Cycle number was optimized to ensure product accumulation in an exponential increase. Amplified products were separated by electrophoresis on 2% agarose gel and documented using a Kodak DC 290 digital camera and digitized using UN-SCAN-IT software (Silk Scientific, Orem, UT). Band intensities for the genes of interest were normalized to that of β-actin in the same sample.

Statistical analysis

All statistical analyses were performed using SAS statistical software (SAS Institute, Cary, NC). Treatment effects were analyzed using one way analysis of variance (ANOVA) followed by Duncan's Multiple Range test. A value of $p < 0.05$ was used to indicate significant differences.

Results

For finding the subtoxic dose of mercury and LPS, we first examined the dose-dependent effects. Administration of 0.3, 1.5, 7.5, 37.5 ppm of mercury (0.06, 0.31, 1.39, 4.81 mg/kg/day, respectively) for 2 weeks and 0.5 mg/kg of LPS for 2 hr did not cause hepatic necrosis as indicated by plasma ALT and AST increase (Table 2). Doses of mercury and LPS used did not cause hepatic apoptosis as indicated by terminal UTP nucleotide transferase end-labeling (TUNEL) assay (data not shown). Mercury affected body weight gain and liver weights. Mice treated with 37.5 ppm of mercury for 2 weeks exhibited significantly smaller body weight gain compared to the control mice. However, the relative liver weight (organ/body weight ratio) was significantly increased in mice exposed to mercury at 7.5 and 37.5 ppm.

Administration of 37.5 ppm of mercury for 2 weeks to mice decreased the hepatic GSH level to same extent as 0.5 mg/kg of LPS in 2 hr. Mercury potentiated LPS-induced hepatic GSH depletion (Fig. 1).

Treatment of mice with LPS activated p38 and ERK in liver (Fig. 2). Treatment of 37.5 ppm of mercury for 2 weeks increased the phosphorylation of p38 in mice liver. Mercury additively increased LPS-induced p38 activation. Treatment of 37.5 ppm of mercury had no effect on the phosphorylation of ERK, in contrast, mercury from 1.5 to 37.5 ppm decreased LPS-induced ERK activation.

LPS increased the expression of all three types of proinflammatory cytokines, i.e., TNFα, IL-1β, and IL-6 (Fig. 3). Mercury at 37.5 ppm increased TNFα expression and with LPS additively increased TNFα expression. Mercury had no effect on IL-1β

expression. Mercury at 37.5 ppm did not alter IL-6 expression but mercury from 1.5 to 37.5 ppm decreased LPS-induced IL-6 expression.

Discussion

Our hypothesis in this study was that the low levels of mercury exposure exacerbate the cellular signaling in liver elicited by subtoxic dose of LPS. Results from our study clearly demonstrated that oral exposure to mercury altered inflammatory cytokine expression during endotoxin treatment. The outcome of exposure to large doses of LPS is a host inflammatory response that results in self-destruction, which is a chain of inflammatory events initiating the elaboration of a cascade of secondary mediators that amplify the response to the initial insult of the liver [11]. Exposure to small doses of LPS is an initiation of inflammatory response that do not evoke tissue damage in liver such as hepatic neutrophil accumulation and TNFα induction [34].

Mercury is well known to alter cellular GSH content and causes cellular and tissue damage [33,39]. The antioxidant and buffering agent of oxidative stress, GSH, is responsible for the diverse properties including regulation of the activation of redox-sensitive transcription factors such as MAPKs [15,36]. p38 and ERK are thought to be necessary for optimal cytokine gene expression in LPS-stimulated cell and tissue, and that the MAPK pathways play a critical role in the inflammatory response. p38 is stress-induced MAPK and one of the most important members of the family in control of inflammatory responses. ERK is thought to have an important role in proliferation, transformation, and differentiation [1]. Numerous of reports show that MAPKs regulate transcription of inflammatory cytokines, although the network regulation of MAPKs on cytokines gene expression is controversial [1,6]. There is complex cross-talk and signal

convergence between p38 and ERK, and they have feedback loops affecting their activation status [40]. Endotoxin activates both p38 and ERK but the end points of signaling cascade is different. It has been reported that while p38 plays an essential role in the NO synthesis, ERK play a minor role. While p38 promotes induction of IL-12, ERK suppresses LPS-mediated IL-12 transcription in mouse macrophages [9]. Activation of ERK is not essential for LPS-induced NO and IL-1β production [5,37]. It may imply that p38 rather than ERK have been postulated to be important in the control of inflammatory responses. Indeed, our results showed that mercury activated p38 MAPK and with LPS additively activated LPS-induced p38 MAPK (Fig. 2). Mercury alone did not alter activation of ERK, instead, mercury decreased LPS-induced ERK phosphorylation. These results strongly suggest that p38 but not ERK is responsible for the augmentation of mercury in LPS-mediated proinflammatory cytokine expression.

Endotoxin-induced liver injury is associated with activation of the cytokine cascade in the mouse liver [35]. Haddad [14] reported that LPS-induced p38 regulates TNFα and IL-6 production and GSH regulates this p38-mediated cytokine production. The role of TNFα in liver injury has been extensively studied. Stimulation of TNFR1 (TNFα receptor 1) or CD95 independently trigger liver failure by causing apoptosis of murine hepatocytes [25]. Kupffer cells mediate liver injury through TNFα and TNFR1. LPS stimulation of Kupffer cells leads to increase in Fas ligand mRNA [27]. These reports suggest that liver macrophage-induced TNFα is the key molecule in liver injury. Our data showed that mercury increased TNFα expression and with LPS additively increased TNFα mRNA (Fig. 3A). These data consistence with our previous report [21] showed that the effect of mercury on LPS-induced p38 activation and downstream TNFα

transcription in macrophage cells. Our data suggest that p38 and TNFα are the major pathway to mercury-induced inflammatory response.

Another important outcome of this study is the reduction of LPS-induced ERK and IL-6 expression by mercury. It has been reported that IL-6 is critical for liver regeneration [26], plays a protective role in regulating TNFα cytotoxicity in alcohol hepatitis [3]. Treatment of non-cytotoxic levels of mercury to microglia cells leads to IL-6 release from adjacent astrocytes to protect microglia cells [7]. The relation between ERK and IL-6 is still not clear. Nguyen and Gao [28] reported that ERK regulates IL-6 signaling in hepatocytes. The dose-responses of mercury between ERK activation and IL-6 expression from our data imply that ERK regulates IL-6 transcription in mouse liver. The effect of mercury on both TNFα and IL-6 suggests that in addition to increase cytotoxic cytokine TNFα, mercury decreased LPS-induced ERK and downstream IL-6, which have a protective property to LPS-induced liver damage.

The exact reason of the unresponsiveness of mercury to IL-1β is unknown, although IL-1β is also important proinflammatory cytokine. No change of IL-1β expression by mercury in liver is same with our previous report showing no effect of mercury on LPS-induced IL-1β in macrophage cells [21].

Lipid peroxidation and oxidative products participate in the regulation of proinflammatory cytokine levels. Endotoxin and lipid peroxidation activate the transcription factor NF-κB which then influences gene expression for proinflammatory cytokine such as TNFα and IL-6, and result in liver injury [19]. Depletion of GSH in hepatocytes causes enhanced activation of NF-κB [10]. Since NF-κB is important mediator in mercury-induced cytotoxicity in many cell types, more studies are warranted

to define the role of NF-κB signaling in mercury-induced and oxidative stress involved liver damage.

In this present report, we show that mercury differentially altered inflammatory cytokine expression by different modulation of MAPKs in liver. Our data suggest that increase of oxidative stress by depletion of GSH, concomitant activation of p38 MAPK, and downstream TNFα expression is the major pathway of mercury-induced exacerbation of LPS-mediated inflammatory signaling. We, for the first time, showed the involvement of p38 MAPK on mercury-induced TNFα expression in liver. More studies are required to further our understanding of regulation of MAPKs on inflammatory cytokine production while mercury contamination.

Reference

1. Arbabi S and Maier RV. Mitogen-activated protein kinases. Crit Care Med 2002; 30:S74-S79.
2. Baker MA, Cerniglia GJ, and Zaman A. Microtiter plate assay for the measurement of glutathione and glutathione disulfide in large numbers of biological samples. Anal Biochem 1990; 190:360-5.
3. Batey RG and Wang J. Molecular pathogenesis of T lymphocyte-induced liver injury in alcoholic hepatitis. Front Biosci 2002; 7:d1662-d1675.
4. Bhagwandeen BS, Apte M, Manwarring L, and Dickeson J. Endotoxin induced hepatic necrosis in rats on an alcohol diet. J Pathol 1987; 152:47-53.
5. Chen CC and Wang JK. p38 but not p44/42 mitogen-activated protein kinase is required for nitric oxide synthase induction mediated by lipopolysaccharide in RAW 264.7 macrophages. Mol Pharmacol 1999; 55:481-8.
6. Dong C, Davis RJ, and Flavell RA. MAP kinases in the immune response. Annu Rev Immunol 2002; 20:55-72.
7. Eskes C, Honegger P, Juillerat-Jeanneret L, and Monnet-Tschudi F. Microglial reaction induced by noncytotoxic methylmercury treatment leads to neuroprotection via interactions with astrocytes and IL-6 release. Glia 2002; 37:43-52.
8. Farina M, Brandao R, de Lara FS et al. Profile of nonprotein thiols, lipid peroxidation and delta-aminolevulinate dehydratase activity in mouse kidney and liver in response to acute exposure to mercuric chloride and sodium selenite. Toxicology 2003; 184:179-87.

9. Feng GJ, Goodridge HS, Harnett MM et al. Extracellular signal-related kinase (ERK) and p38 mitogen-activated protein (MAP) kinases differentially regulate the lipopolysaccharide-mediated induction of inducible nitric oxide synthase and IL-12 in macrophages: Leishmania phosphoglycans subvert macrophage IL-12 production by targeting ERK MAP kinase. J Immunol 1999; 163:6403-12.
10. Fernandez-Checa JC, Kaplowitz N, Garcia-Ruiz C, and Colell A. Mitochondrial glutathione: importance and transport. Semin Liver Dis 1998; 18:389-401.
11. Ghosh S, Latimer RD, Gray BM, Harwood RJ, and Oduro A. Endotoxin-induced organ injury. Crit Care Med 1993; 21:S19-S24.
12. Gilot D, Loyer P, Corlu A et al. Liver protection from apoptosis requires both blockage of initiator caspase activities and inhibition of ASK1/JNK pathway via glutathione S-transferase regulation. J Biol Chem 2002; 277:49220-9.
13. Gordon S. The role of the macrophage in immune regulation. Res Immunol 1998; 149:685-8.
14. Haddad JJ. The involvement of L-gamma-glutamyl-L-cysteinyl-glycine (glutathione/GSH) in the mechanism of redox signaling mediating MAPK(p38)-dependent regulation of pro-inflammatory cytokine production. Biochem Pharmacol 2002; 63:305-20.
15. Haddad JJ and Land SC. Redox/ROS regulation of lipopolysaccharide-induced mitogen-activated protein kinase (MAPK) activation and MAPK-mediated TNF-alpha biosynthesis. Br J Pharmacol 2002; 135:520-36.

16. Hewett JA, Jean PA, Kunkel SL, and Roth RA. Relationship between tumor necrosis factor-alpha and neutrophils in endotoxin-induced liver injury. Am J Physiol 1993; 265:G1011-G1015.
17. Hewett JA and Roth RA. Hepatic and extrahepatic pathobiology of bacterial lipopolysaccharides. Pharmacol Rev 1993; 45:382-411.
18. Johnson VJ and Sharma RP. Gender-dependent immunosuppression following subacute exposure to fumonisin B_1. Int Immunopharmacol 2001; 1:2023-34.
19. Jokelainen K, Reinke LA, and Nanji AA. NF-kappaB activation is associated with free radical generation and endotoxemia and precedes pathological liver injury in experimental alcoholic liver disease. Cytokine 2001; 16:36-9.
20. Kaneda K and Wake K. Distribution and morphological characteristics of the pit cells in the liver of the rat. Cell Tissue Res 1983; 233:485-505.
21. Kim SH, Johnson VJ, and Sharma RP. Mercury inhibits nitric oxide production but activates proinflammatory cytokine expression in murine macrophage: differential modulation of NF-kappaB and p38 MAPK signaling pathways. Nitric Oxide 2002; 7:67-74.
22. Koropatnick J and Zalups RK. Effect of non-toxic mercury, zinc or cadmium pretreatment on the capacity of human monocytes to undergo lipopolysaccharide-induced activation. Br J Pharmacol 1997; 120:797-806.
23. Lee JC, Laydon JT, McDonnell PC et al. A protein kinase involved in the regulation of inflammatory cytokine biosynthesis. Nature 1994; 372:739-46.
24. Lee JC and Young PR. Role of CSB/p38/RK stress response kinase in LPS and cytokine signaling mechanisms. J Leukoc Biol 1996; 59:152-7.

25. Leist M, Gantner F, Bohlinger I, Tiegs G, Germann PG, and Wendel A. Tumor necrosis factor-induced hepatocyte apoptosis precedes liver failure in experimental murine shock models. Am J Pathol 1995; 146:1220-34.

26. Li W, Liang X, Leu JI, Kovalovich K, Ciliberto G, and Taub R. Global changes in interleukin-6-dependent gene expression patterns in mouse livers after partial hepatectomy. Hepatology 2001; 33:1377-86.

27. Muschen M, Warskulat U, Douillard P, Gilbert E, and Haussinger D. Regulation of CD95 (APO-1/Fas) receptor and ligand expression by lipopolysaccharide and dexamethasone in parenchymal and nonparenchymal rat liver cells. Hepatology 1998; 27:200-8.

28. Nguyen VA and Gao B. Cross-talk between alpha(1B)-adrenergic receptor (alpha(1B)AR) and interleukin-6 (IL-6) signaling pathways. Activation of alpha(1b)AR inhibits IL-6-activated STAT3 in hepatic cells by a p42/44 mitogen-activated protein kinase-dependent mechanism. J Biol Chem 1999; 274:35492-8.

29. Perlingeiro RC and Queiroz ML. Polymorphonuclear phagocytosis and killing in workers exposed to inorganic mercury. Int J Immunopharmacol 1994; 16:1011-7.

30. Queiroz ML, Perlingeiro RC, Dantas DC, Bizzacchi JM, and De Capitani EM. Immunoglobulin levels in workers exposed to inorganic mercury. Pharmacol Toxicol 1994; 74:72-5.

31. Rana SV and Boora PR. Antiperoxidative mechanisms offered by selenium against liver injury caused by cadmium and mercury in rat. Bull Environ Contam Toxicol 1992; 48:120-4.
32. Roth RA, Harkema JR, Pestka JP, and Ganey PE. Is exposure to bacterial endotoxin a determinant of susceptibility to intoxication from xenobiotic agents? Toxicol Appl Pharmacol 1997; 147:300-11.
33. Shenker BJ, Mayro JS, Rooney C, Vitale L, and Shapiro IM. Immunotoxic effects of mercuric compounds on human lymphocytes and monocytes. IV. Alterations in cellular glutathione content. Immunopharmacol Immunotoxicol 1993; 15:273-90.
34. Spitzer JA, Zhang P, and Mayer AM. Functional characterization of peripheral circulating and liver recruited neutrophils in endotoxic rats. J Leukoc Biol 1994; 56:166-73.
35. Tsutsui H, Matsui K, Kawada N et al. IL-18 accounts for both TNF-alpha- and Fas ligand-mediated hepatotoxic pathways in endotoxin-induced liver injury in mice. J Immunol 1997; 159:3961-7.
36. Ueda S, Masutani H, Nakamura H, Tanaka T, Ueno M, and Yodoi J. Redox control of cell death. Antioxid Redox Signal 2002; 4:405-14.
37. Watters JJ, Sommer JA, Pfeiffer ZA, Prabhu U, Guerra AN, and Bertics PJ. A differential role for the mitogen-activated protein kinases in lipopolysaccharide signaling: the MEK/ERK pathway is not essential for nitric oxide and interleukin 1beta production. J Biol Chem 2002; 277:9077-87.

38. Yee SB, Kinser S, Hill DA et al. Synergistic hepatotoxicity from coexposure to bacterial endotoxin and the pyrrolizidine alkaloid monocrotaline. Toxicol Appl Pharmacol 2000; 166:173-85.

39. Zalups RK, Barfuss DW, and Lash LH. Disposition of inorganic mercury following biliary obstruction and chemically induced glutathione depletion: dispositional changes one hour after the intravenous administration of mercuric chloride. Toxicol Appl Pharmacol 1999; 154:135-44.

40. Zhang H, Shi X, Hampong M, Blanis L, and Pelech S. Stress-induced inhibition of ERK1 and ERK2 by direct interaction with p38 MAP kinase. J Biol Chem 2001; 276:6905-8.

Table 7.1. Primers and PCR conditions for gene expression analyzed in the liver following exposure to mercury and LPS

	Primers		Annealing temperature (^{o}C)	# of cycles
[a]TNFα	Sense	5'CTCTTCAAGGGACAAGGCTG3'	55	27
	Anti-sense	5'CGGACTCCGCAAAGTCTAAG3'		
IL-1β	Sense	5'GCAACTGTTCCTGAACTCA3'	55	28
	Anti-sense	5'CTCGGAGCCTGTAGTGCAG3'		
IL-6	Sense	5'TTCCATCCAGTTGCCTTCTT3'	53	32
	Anti-sense	5'CAGAATTGCCATTGCACAAC3'		
β-actin	Sense	5'ATGGATGACGATATCGCT3'	48	29
	Anti-sense	5'ATGAGGTAGTCTGTCAGGT3'		

[a]Selected by Primer3 program (Whithead Institute, Cambridge, MA).

Table 7.2. The effect of oral mercury supplementation and LPS injection on body weight gain, liver weights, and liver enzymes in male BALB/c mice

Mercury in Water (ppm)	LPS	Body Weight Gain, g (% change from initial Body Weight)	Liver/Body Weight Ratio (g/100g)	ALT (Units/Liter)	AST (Units/Liter)
0	-	2.35 ± 0.25 (11.3)	5.08 ± 0.12	27.75 ± 1.84	168.50 ± 22.24
37.5	-	0.58 ± 0.13 (2.1)*	5.44 ± 0.05*	29.50 ± 2.53	143.25 ± 22.96
0	+	2.52 ± 0.19 (12.2)	5.26 ± 0.03	32.75 ± 3.25	203.75 ± 36.10
0.3	+	1.50 ± 0.38 (7.2)	5.20 ± 0.06	31.25 ± 0.85	171.50 ± 11.06
1.5	+	2.32 ± 0.31 (10.6)	5.07 ± 0.07	37.75 ± 5.76	199.00 ± 35.49
7.5	+	2.01 ± 0.30 (9.3)	5.43 ± 0.03*	32.00 ± 2.94	153.50 ± 21.68
37.5	+	0.70 ± 0.29 (3.2)*	5.50 ± 0.03*	29.00 ± 1.41	151.25 ± 10.20

Mercury treated in drinking water for 14 days and LPS (0.5 mg/kg) was injected i.p. 2 hr prior to euthanasia the mice.

ALT: alanine aminotransferase, AST: aspartate aminotransferase.

Mean ± SE (n=4).

* Significantly different from the control (no treatment) at $p < 0.05$.

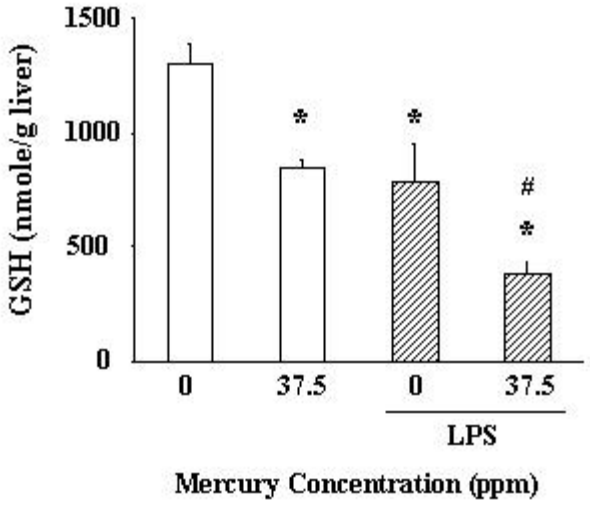


Fig. 7.1. Hepatic GSH levels in mice exposed to mercuric chloride and LPS (0.5 mg/kg, i.p.). Male BALB/c mice were treated with 37.5 ppm of mercury in the drinking water for 14 days. Animals were sacrificed 2 hr after LPS injection to measure hepatic GSH levels. Results are expressed as mean ± SE (n=4). *Significantly different than the control group at $p < 0.05$. #Significantly different than the LPS alone group at $p < 0.05$.

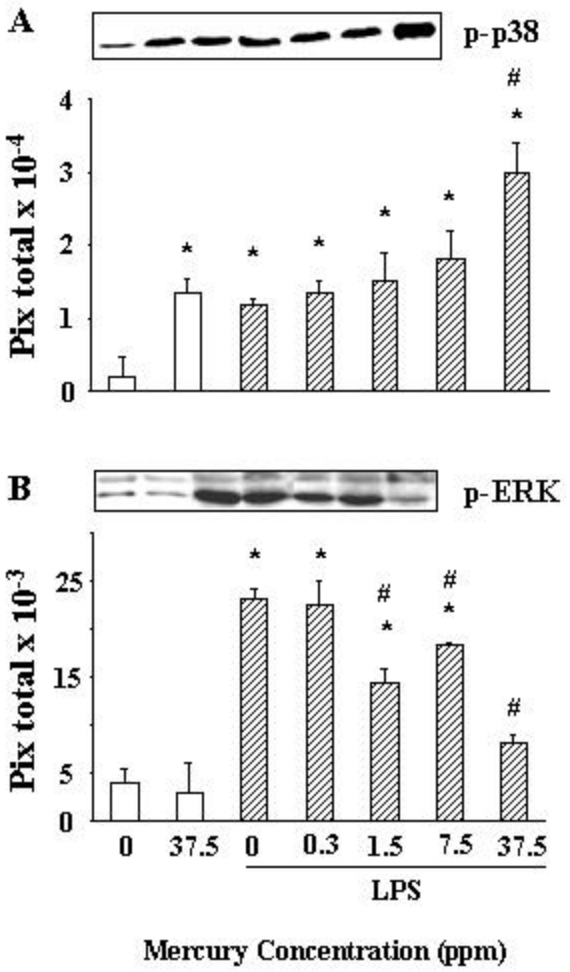


Fig. 7.2. Hepatic p38 and ERK MAPK activation in mice exposed to mercuric chloride and LPS (0.5 mg/kg, i.p.). Male BALB/c mice were treated with 0.3, 1.5, 7.5, 37.5 ppm of mercury in the drinking water for 14 days. Animals were sacrificed 2 hr after LPS injection. Protein (30 μg) was analyzed by 12% SDS-PAGE, and p38 (A) and ERK (B) MAPK was visualized by western blot analysis. Results are expressed as mean ± SE (n=4). *Significantly different than the control group at $p < 0.05$. #Significantly different than the LPS alone group at $p < 0.05$. Insert, a representative western blot in the same order.

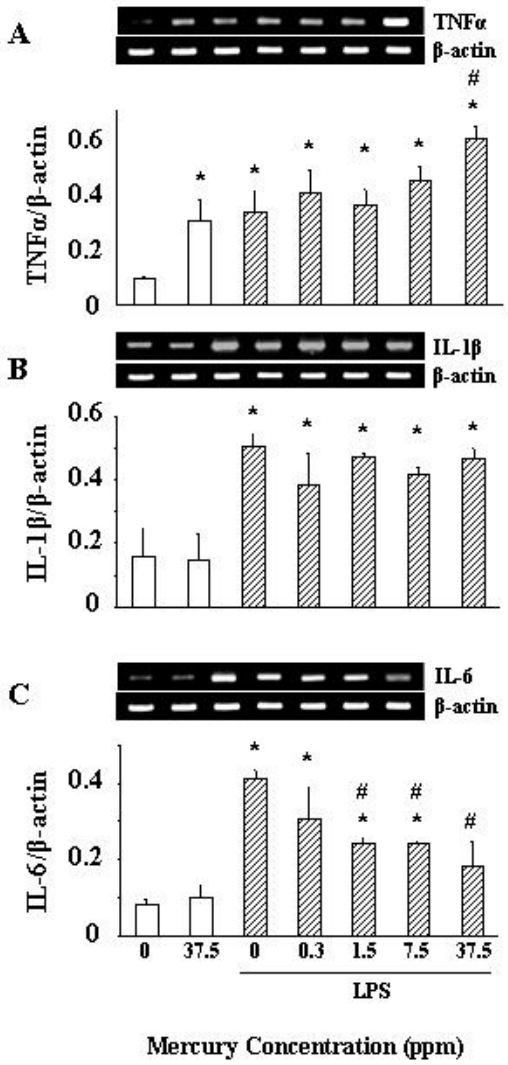


Fig. 7.3. Hepatic TNFα, IL-1β, and IL-6 mRNA in mice exposed to mercuric chloride and LPS (0.5 mg/kg, i.p.). Male BALB/c mice were treated with 0.3, 1.5, 7.5, 37.5 ppm of mercury in the drinking water for 14 days. Animals were sacrificed 2 hr after LPS injection. Extraction and analysis of mRNA performed as described in Materials and Methods. TNFα (A), IL-1β (B), and IL-6 (C) mRNA levels were quantified by RT-PCR and normalized against β-actin. Results are expressed as mean ± SE (n=4). *Significantly different than the control group at $p < 0.05$. #Significantly different than the LPS alone group at $p < 0.05$. Insert, a representative RT-PCR blot in the same order.

CHAPTER 8

SUMMARY AND CONCLUSIONS

The objectives of the present study were to 1) investigate the effect of mercury on endotoxin induced nitric oxide (NO) production and proinflammatory cytokine expression such as tumor necrosis factor α (TNFα), interleukin-1β (IL-1β), and IL-6 in macrophages, 2) determine the effect of mercury on reactive oxygen species (ROS), and mitogen-activated protein kinases (MAPKs), and define the mode of mercury induced cell death, 3) define the impact of mercury induced cytotoxicity in T and B lymphocytes, 4) examine the effect of mercury on T lymphocyte population change and cytokine gene expression in mice, 5) determine the impact of mercury on endotoxin-induced inflammatory response and signaling pathways in mice.

In the first study we demonstrated that treatment of murine macrophages with non-cytotoxic concentration of mercury as $HgCl_2$ resulted in a marked decrease in endotoxin induced NO production. A decrease was also observed in inducible nitric oxide synthase (iNOS) mRNA and protein production. The decrease in NO and iNOS production correlated with a decrease in activation of nuclear factor κB (NF-κB) which indicates that the impact of mercury on this transcription factor is likely responsible for the observed effect on NO production. Interestingly, mercury stimulated TNFα expression and increased endotoxin-induced TNFα and IL-6 mRNA. Further mechanistic studies revealed that mercury activated p38 MAPK and additively increased endotoxin-induced p38 MAPK phosphorylation. These data indicate that mercury suppresses NO synthesis by inhibition of NF-κB pathway and modulates cytokine expression by p38 MAPK activation in macrophages.

The second study delineated the mode of mercury induced cell death, apoptosis versus necrosis, and the related signaling such as calcium, ROS, MAPK, TNFα, and caspase cascade in macrophages. Mercury did not cause cytotoxicity up to 20 μM of concentration

and 96 hr of incubation. The EC_{50} of mercury was in the range of 62.7 to 102.8 μM by various cytotoxicity assays such as MTT assay, annexin V binding, propidium iodide (PI) binding, Hoechst 33258 (H33258) binding, and caspase-3 activation. It was clearly evident that cells were dying by a mix of apoptosis and necrosis in response to mercury treatment. Mercury induced the translocation of phosphatidyl serine from the inner leaflet to the outer leaflet of the plasma membrane and also increased caspase-3 like enzyme activity thus supporting apoptosis as a mechanism of cell death. Nuclear condensation and fragmentation was obtained upon microscopic examination of cells treated with mercury. Additionally, mercury increased PI binding which indicates necrotic cell death. Mercury increased intracellular Ca^{2+} and ROS production, and pretreatment of Ca^{2+} antagonist decreased mercury-induced ROS. Mercury activated p38 MAPK and this effect was attenuated by pretreatment of antioxidant *N*-acetylcysteine (NAC) and silymarin. Mercury increased gene expression of TNFα, and antioxidant and specific p38 inhibitor decreased this effect. Pretreatment of antioxidant, p38 inhibitor, and anti-TNFα antibody decreased mercury-induced necrosis, however only antioxidant and p38 inhibitor decreased mercury-induced apoptosis. These data indicate that mercury-induced cell death is mix of apoptosis and necrosis with different pathway. Mercury-induced ROS and downstream p38 regulate both apoptosis and necrosis. p38 mediated caspase activation regulates mercury-induced apoptosis and p38 mediated TNFα regulates necrosis.

The third study investigated the cytotoxic effect of mercury on T and B lymphocytes. Mercury concentration-dependently decreased cell viability, membrane integrity, and proliferation of both lymphocytes. Mercury increased the ROS production in both cells, and pretreatment with antioxidant reversed mercury induced ROS production. Pretreatment of

antioxidant decreased mercury-induced lactate dehydrogenase (LDH) release in both cells, and Ca^{2+} channel blocker decreased only in B cells. The mode of cytotoxicity was a mix of apoptosis and necrosis. Mercury induced both apoptosis and necrosis in both cells as indicated by H33258 and PI staining, and this cytotoxicity was reduced by antioxidant. Additionally, mercury increased gene expression of IL-4 and TNFα in T cells but no changes in B cells. These data indicate that mercury-induced cytotoxicity has different pathways on T and B cells, and the ROS, Ca^{2+} homeostasis, and inflammatory cytokine gene expression are involved in mercury-induced cytotoxicity.

The fourth study determined the effect of mercury on T lymphocyte phenotype population and cytokine gene expression in BALB/c mice. Oral exposure of relatively low level of mercury in drinking water for 2 weeks did not cause hepatotoxicity as indicated by circulating alanine aminotransferase and aspartate aminotransferase. However mercury decreased the $CD8^+$ T lymphocyte population in the thymus. Mercury decreased $CD3^+$ T lymphocytes in spleen; both $CD4^+$ and $CD8^+$ single positive lymphocyte numbers were decreased. Mercury altered the expression of inflammatory cytokines (TNFα, IFNγ, IL-12), c-myc, and major histocompatibility complex II (MHC II) in liver, kidney, spleen, and thymus. Results indicated that decreases in T lymphocyte populations in immune organs and altered cytokine gene expression contribute to the immunosuppressive effects of mercury.

The final study determined the effect of mercury on endotoxin-induced inflammatory response and signaling in liver tissue. Oral exposure of mercury in drinking water for 2 weeks decreased total glutathione (GSH) content in liver, and with endotoxin, it additively decreased GSH content. Similar trend of results were obtained in p38 MAPK activation, downstream TNFα expression, and apoptosis. However mercury decreased endotoxin-

induced ERK phosphorylation and IL-6 expression. These results implicate that the balance of p38 and ERK mediates mercury-induced inflammatory signaling.

Overall, the data presented in this dissertation indicate the significance of immunotoxicity of low levels of mercury. The oxidative stress plays an important role in mercury toxicity. Because of the strong binding to sulfhydryl group, mercury causes oxidative stress and results in cytotoxicity. A new mechanism potentially regulating the immunotoxicity of mercury has been identified. Oxidative stress-mediated MAPKs regulate mercury-induced cell damage. Ultimately MAPK-mediated inflammatory cytokines and caspase cascade regulate mercury-induced immunotoxicity.