2003

Mechanisms of action of arsenic-induced cardiovascular disease

Melisa Bunderson

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MECHANISMS OF ACTION OF ARSENIC-INDUCED CARDIOVASCULAR DISEASE

By

Melisa Bunderson

B.S. Animal Science 1997
Utah State University, Logan, Utah

M.S. Toxicology 2000
Utah State University, Logan, Utah

Presented in partial fulfillment of the requirements for the Doctor of Philosophy in Pharmaceutical Sciences degree

The University of Montana
Missoula, Montana

August 2003

Approved by:

Howard D. Beall
Chairperson, Board of Examiners

David A. Strobel
Dean of the Graduate School

8-25-03
Date
ABSTRACT

Epidemiological evidence suggests that exposure to the metalloid arsenic constitutes a risk factor for cardiovascular disease. Some of the cardiovascular effects attributed to arsenic are decreased cardiac function, peripheral vascular diseases and atherosclerosis. While the epidemiological evidence linking arsenic to cardiovascular (CVD) is abundant, the mechanisms of action are primarily unknown. The purpose of this study is to begin elucidating those mechanisms by (1) defining the changes in key atherogenic regulatory molecules upon exposure to arsenic in bovine aortic endothelial cells (BAE) (2) investigating the effects of arsenite on the formation of reactive oxygen (ROS) and reactive nitrogen species (RNS) in endothelial cells and (3) determining the effects of arsenite on atherogenesis and expression of key CVD regulatory molecules in the ApoE^+ /LDLr^+ atherosclerotic mouse model. This model develops severe atherosclerotic plaques early in life. We were able to use them in our studies to show the first laboratory-controlled evidence that arsenic increases the severity of arterial occlusion indicative of atherosclerosis. In order to understand the mechanisms behind this occurrence, we looked at various components of atherogenesis that might be influenced by the presence of arsenic. Sodium arsenite, the reduced form of inorganic arsenic was used in these studies. We found that production of peroxynitrite, a strong oxidant formed from the coupling of nitric oxide and superoxide anion, is significantly increased in BAE cells exposed to sodium arsenite. In addition, the biological marker for peroxynitrite, 3-nitrotyrosine, is increased in the atherosclerotic plaque of arsenite treated ApoE^+/LDLr^- mice. Expression of the inflammatory mediator, cyclooxygenase-2 (COX-2) is also upregulated in response to arsenite exposure, indicating an increase in the inflammatory response in BAE cells. This result was supported by an increase in the generation of prostaglandin E2 (PGE2) following exposure to arsenite in BAE cells. There was also an increase in the synthesis of the COX-2 product, prostaglandin I2 (PGI2), in the serum of arsenite exposed mice, although we were unable to detect an increase in PGE2 in this model. Since peroxynitrite is capable of nitrating protein tyrosine residues, peroxynitrite formation can be confirmed by determining the extent of nitrotyrosine formation in a protein. Nitration of COX-2 was detected in arsenite-treated cells, but not in untreated control cells, supporting the evidence that an increase in peroxynitrite results from arsenite exposure in BAE cells. Furthermore, an increase in the proinflammatory protein 5-lipoxygenase (5-Lo) results from arsenite exposure in BAE cells. This is mirrored by an increase in LTE4, a product of the 5-Lo pathway, in the serum of mice chronically exposed to arsenite as well as in BAE cells. The findings in this report show that arsenic definitively increases the severity of occluded arteries in atherogenic mice and suggests an increase in reactive species, notably peroxynitrite, in BAE cells exposed to arsenic. Furthermore, induction of important inflammatory mediators such as COX-2 and 5-Lo may exacerbate the inflammatory state typical of atherosclerosis.
ACKNOWLEDGEMENTS

I would first like to thank the Department of Pharmaceutical Sciences and Center for Environmental Health Sciences for providing me with the opportunity for an outstanding higher education and the chance to discover Montana.

I offer my heartfelt gratitude to the members of my dissertation committee: Drs. Beall, Coffin, Lodmell, Parker, and Sugden.

I would especially like to thank my mentor, Dr. Howard D. Beall for his patience, perseverance and guidance in helping me to complete this project.

I would also like to acknowledge the American Foundation for Pharmaceutical Education (AFPE) for providing financial assistance with an APFE Pre-doctoral Fellowship from 2001-2003.

Finally, I want to thank my family and all my friends who feel like family. Thank you for your faith and inspiration. I couldn’t have done this without you.
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LITERATURE REVIEW

Rationale

The focus of our lab is to develop a better understanding of the mechanisms behind arsenic-induced cardiovascular disease. This knowledge will aid in future prevention endeavors and treatment of people who are exposed to environmental contaminants that result in heart disease. The objective of these studies is to define the changes in the atherosclerotic lesions of ApoE^{-}/LDLr^{-} mice and endothelial cell homeostasis in response to arsenic exposure that may result in initiation, onset or progression of atherosclerosis. We focus on the ability of arsenic to cause an oxidative and inflammatory environment within the endothelium that will result in an increase in CVD. To address these issues we use cultured endothelial cells as well as ApoE^{-}/LDLr^{-} mice susceptible to atherogenesis. This study is unique in that it progresses from the chemical changes resulting from arsenic exposure to a molecular and finally pathological outcome by using both in vitro and in vivo techniques.

Specific Aim 1: Define the changes in key atherogenic regulatory proteins upon exposure to arsenic in bovine aortic endothelial cells.

Specific Aim 2: Investigate the effects of arsenic on the formation of peroxynitrite in endothelial cells.

Specific Aim 3: Determine the effects of arsenic on atherogenesis and expression of key CVD regulatory molecules in the ApoE^{-}/LDLr^{-} atherosclerotic mouse model.
Arsenic

*Environmental Importance.* Arsenic constitutes approximately 5 ppm of the earth’s crust with inorganic arsenic being the major type found in drinking water. Arsenic is released into the environment through natural and manmade endeavors such as mining, smelting, and pesticide applications. Contamination “pockets” resulting from improper hazardous waste management, primarily from mining, has created an immense challenge for the United States and the Environmental Protection Agency (EPA). The western part of Montana, in particular, is home to the Clark Fork Complex, the largest group of superfund sites in the country (Moore and Luoma, 1990). The hazardous waste problem in this area was produced by more than 127 years of copper and silver mining and subsequent smelting activities. Typically, ore containing valuable mineral is separated by milling and flotation with as much as 90% being discarded as “tailings”. Subsequent refining processes result in flue dust and slag which can contain “thousands of times the natural levels of arsenic, cadmium, copper, lead and zinc”. In fact, the Clark Fork Complex could contain as much as 9,000 metric ton (MT) of arsenic, 200 MT of cadmium, 90,000 MT copper, 20,000 MT lead, 200 MT silver, and 50,000 MT zinc as deposits in tailings ponds.

Smelting is one of the most destructive stages in the mining process, resulting in $3.7 \times 10^6$ MT of air pollutants per year in conjunction with the ground and water contamination (Moore and Luoma, 1990). Anaconda, Montana is an example of the damage caused by smelting techniques, where in 1902, cattle, sheep and horses started displaying signs of arsenic poisoning only months after a new copper smelter began
production. In fact, it was due to the incidence of arsenic in the groundwater that the Milltown Reservoir Superfund Site (MRSS), near Milltown, Montana was established.

Located just outside of Missoula, Montana MRSS is an artifact of the Milltown Dam, built at the confluence of the Clark Fork and Blackfoot Rivers in 1907. Acting as a repository for sediment and mining wastes, the reservoir is highly contaminated and caused the formation of a groundwater arsenic plume that impacted Milltown's drinking water supply (USEPA). EPA added the site to its National Priorities List (NPL) in September 1983 and is addressing the problem through the combined actions of federal and state agencies and the Potentially Responsible Parties (PRPs), primarily the Atlantic Richfield Company (ARCO) and the Northwestern Energy Corporation.

In 1983, concern for the health of the Milltown residents resulted in an organized effort of volunteers to supply residences with water service on a biweekly basis for three months. In 1984, EPA provided replacement water for Milltown and in 1985, EPA replaced household water supply equipment that remained a source of contamination, as well as continued sampling of individual residences. However, the problem of the huge sediment deposit sitting behind the Milltown Dam remains an issue. Recently, the EPA has released a proposed plan for public review and comment which would result in removal of the dam as well as limited sediment removal. The potential benefits of this plan would be “permanent, long-term protection of public health and the environment, recovery of the Milltown drinking water aquifer, substantial elimination of contaminant release from ice-scouring and catastrophic events”, as well other environmental benefits such as the return of the Clark Fork and Blackfoot rivers to a free-flowing state and unrestricted fish passage (USEPA, 2003). The process of evaluating risk and developing
recovery and management plans for the MRSS has lasted ten years and is still an ongoing process. Once a remediation plan has been established and a record of decision finalized, site preparation will begin. In the year 2006, removal of 2.6 million cubic yards of sediment will begin, a process that is expected to take three years. Under the proposed plan, the sediments would be relocated less than a mile downstream from the Dam in a lined repository. Finally, the dam would be removed in 2009 and stabilization and revegetation of the Clark Fork and Blackfoot channels would begin in 2010. The cleanup of the MRSS is estimated to cost $95 million!

Pesticide application is another source of man-made contamination of the environment by arsenic. Arsenic compounds have traditionally been used to control plant pests in cotton and grape crops (Gao and Burau, 1997). Arsenic can accumulate to phytotoxic levels in the soil after continuous use of arsénical pesticides and herbicides and then be released into the environment after transformation from microbial activity (Gao and Burau, 1997). This long-time practice has contributed to the overall environmental load of arsenic and its prevalence as a human health problem.

The oxidized form of arsenic, arsenate (As[V]), is the predominant species in water followed by arsenite (As[III]). Arsenate is reduced in vivo to arsenite (As[III]), which can be further metabolized to mono- and dimethylated derivatives (Abernathy et al., 1999). Arsenite is considered to be the most toxic form of inorganic arsenic (Buchet and Lison, 2000) and has been linked to a number of diseases, including bladder, lung and skin cancers, various cardiovascular diseases, hepatic and renal pathologies, diabetes and neuropathy (Szymanska-Chabowska et al., 2002).
Toxicology. Arsenic is a known human carcinogen and has also been linked to other health effects such as hypertension, various vascular diseases, diabetes, and polyneuropathy (Chabowska et al., 2002). In general, mechanisms of arsenic toxicity are attributed to its ability to uncouple oxidative phosphorylation by inhibition of succinic dehydrogenase and α-ketoglutarate dehydrogenase activity, interference with the regulation of stress proteins, and inhibition of enzymes critical for the cellular response to oxidative stress, such as thioredoxin reductase (Chabowska et al., 2002). However, the toxicity of arsenicals has been shown to be specific to the valency state of arsenic, tissue type, and species of animal affected (Fierro et al., 1999; Vahter, 2002).

It has been suggested that arsenic partially induces its toxic effect by the formation of reactive oxygen and nitrogen species (Liu and Jan, 2000). This is supported by studies demonstrating the cytoprotective effects of the antioxidant glutathione against trivalent arsenical-induced (arsenic trioxide and sodium arsenite) endothelial cell injury (Chang et al., 1991) and by the wide array of stress-induced heat shock proteins that are upregulated in response to arsenic exposure (Razo et al., 2001). For example, in bovine aortic endothelial (BAE) cells treated with sodium arsenite concentrations of 5 µM and above for 4 h, DNA strand breaks (DSB) (including double-strand breaks, single-strand breaks, and alkali-labile sites) were significantly increased as detected by single-cell alkaline electrophoresis (Liu and Jan, 2000). This effect of DNA strand breakage was concentration dependent up to 20 µM arsenic. Reduction of DSB as a result of arsenic exposure was accomplished by the addition of superoxide dismutase as well as the NO synthase inhibitor, Nω-nitro-L-arginine methyl ester. Furthermore, addition of uric acid, a peroxynitrite scavenger resulted in fewer DSB (Liu and Jan, 2000). Similarly, porcine
aortic endothelial cells (PAEC) treated with 5 μM arsenic (arsenic trioxide) had an increase in NF-κB dependent transcription as measured by luciferase expression as well as an increase in H₂O₂-dependent tyrosine phosphorylation observed by immunoblots (Barchowsky et al., 1999). These effects were seen with a concurrent increase in reactive oxygen species generation as observed by EPR spectroscopy. These data suggest that low levels of oxidants generated in response to low concentrations of arsenic exposure may stimulate cell signaling and transcriptional activation (Barchowsky et al., 1999).

Generation of reactive species in response to arsenic also results in an increase in lipid peroxidation that is inversely related to a depletion of cellular GSH (Ramos et al., 1995). This important observation was seen in liver, kidney tissue, and heart tissue (Ramos et al., 1995).

The carcinogenic effects of arsenic could be attributed to a wide range of possible mechanisms. Arsenic has been reported to cause chromosomal aberrations that may lead to cancer, including increased presence of micronuclei and sister chromatid exchanges (reviewed in Kitchin, 2001). Increased oxidative stress, altered growth factor synthesis, increased cell proliferation, and altered DNA repair are additional possible mechanisms involved in arsenic-induced carcinogenesis (Kitchin, 2001). One hypothesis regarding the mechanisms of arsenic carcinogenesis involves its ability to cause hypomethylation of DNA resulting in aberrant replication. This is supported by a study using the human colon cancer cell line, Caco-2. Caco-2 cells were treated with 0, 1 or 2 μM arsenic followed by measurement of DNA methylation. Cells treated with 0 or 2 μM arsenic were significantly less methylated than cells treated with 1 μM arsenic. However, when methylation of the p53 tumor suppressor gene was accounted for singularly, cells treated
with 2 μM arsenic were significantly more hypomethylated than cells treated with 1 μM arsenic (Davis et al., 2000). These results suggest a wide discrepancy in the effects of arsenic toxicity depending on the concentration of exposure (Davis et al., 2000).

Arsenic may induce its cellular effects by modifying a variety of important factors necessary for normal function. In a study where mice received sodium arsenite in their drinking water for a period of 8 weeks there was a marked increase in AP-1 DNA binding activity at concentrations ranging from 20-100 μg/ml (Simeonova et al., 2001). In mice that received 0.5-10 μg/ml there was a slight increase in AP-1 DNA binding activity, although it was not as striking as the higher dosage effects. In this same study, histopathological changes within the epithelial lining of the bladder were noted at arsenic concentrations between 50 and 100 μg/ml and included simple hyperplasia, and eosinophilic, cytoplasmic inclusions. The authors suggested a non-linear dose-response curve for predicting arsenic-induced bladder cancer that included the idea that lower concentrations of arsenic may cause cellular abnormalities that are not readily visible with histology (Simeonova et al., 2001). In a separate study, the human phospholipid hydroperoxide glutathione peroxidase (PHGPx) promoter was transfected into the human epidermoid carcinoma A431 cell line. There was a significant downregulation in the mRNA, protein expression, and enzyme activity of PHGPx after exposure to 30 μM arsenic for 9 h (Huang et al., 2002). A concomitant upregulation of the mRNA and protein expression of p21, a CDK inhibitor involved in the arrest of cells in the G1 phase, was also seen. These effects were significantly prevented by addition of the antioxidant N-acetyl-L-cysteine, suggesting that reactive oxygen species were involved in these processes (Huang et al., 2002).
Although arsenic is well accepted as a carcinogen in humans, studies using rodent models have typically had limited success. However, a recent transplacental study done in mice demonstrated an increased incidence in the occurrence of hepatocellular carcinoma and adrenal tumors in male offspring with increases in ovarian tumors and cancers of the lung, uterus and oviduct in the female offspring as well (Waalkes et al., 2003). In these animals exposure occurred through the dam during gestation only, suggesting that arsenic may have embryonic effects in rodents that had not previously been recognized. Furthermore, cellular events that have been linked to cancer in humans are being discovered in animal models. For example, low concentrations of arsenic (0.5-10 μM) were recently shown to cause an accumulation of low-molecular-weight protein conjugates that had been ubiquitinated in both rabbit renal-cortical slices and human embryonic kidney 293 cells (Kirkpatrick et al., 2003). This phenomenon could have an array of consequences on cellular signaling events that could lead to arsenic’s ability to cause cancer and cardiovascular disease.

Metabolism. Metabolism of arsenic in living systems usually involves a series of oxidation-reduction and methylation reactions (Scott et al., 1993). The oxidized form of arsenic, arsenate (AsV) is reduced to the more toxic inorganic form, arsenite (AsIII) via an As(III)-(GS)₃ complex. Furthermore, glutathione complexes form subsequent to the reduction of arsenate and may be substrates for the methylating enzymes (Scott et al., 1993). Inorganic arsenite and arsenate are metabolized by rat liver cytosol fractions through methylation using S-adenosylmethionine as the methyl group donor and reduced glutathione (Buchet and Lauwerys, 1988). The first methylation step, forming monomethylarsenite (MMA), is the rate-limiting step and may be catalyzed by GSH.
There is a separate methylating enzyme responsible for further metabolism to the dimethylarsenite (DMA) form (Buchet and Lauwerys, 1988).

Human and rat hepatocytes exposed to trivalent inorganic arsenic at a concentration of 0.1 μM for 24-48 h had a peak intracellular arsenite concentration after exposure for 1 h followed by a steady decline (Styblo et al., 1999). However, the cellular concentrations of methyl- and dimethylarsenite peaked after 9 and 24 h respectively. There was a steady decline in the inorganic arsenite concentration within the cell medium throughout the exposure period with MMA and DMA’s being undetectable for the first 6 h after exposure. Throughout the study, medium MMA contents increased only slightly and DMA concentrations rose steadily until it ultimately accounted for 60% of the total arsenic within the culture medium. In contrast, exposure to arsenate under identical experimental conditions resulted in less than 6% of total arsenic being associated with cells (compared to as much as 30% with Arsenite) and the total methylation yield (both MMA and DMA’s) never exceeded 3% (Styblo et al., 1999). In addition, human fibroblast cells pre-exposed to arsenite developed an increased capacity for methylation when compared to cells having never been exposed to arsenite (Fischer et al., 1985).

In the rat, the liver has the greatest methylating capacity of all the organs. Arsenite enters the cells by diffusion and maintains its intracellular status by quickly binding to cellular components allowing extensive accumulation within the liver cells (Georis et al., 1990). Glutathione plays a critical role by helping to facilitate the diffusion of arsenite into the cell, aiding in the primary methylation reaction and in the excretion of the secondary methylation product. In fact, agents that decrease the presence of hepatic glutathione also reduce the biliary excretion of both arsenite and arsenate (Gyurasics et
al., 1991). In addition, arsenate is not extensively taken up by rat hepatocytes and is therefore poorly methylated (Georis et al., 1990). This may help explain the phenomenon seen in human liver, mice and rabbits where the in vivo methylation of arsenate requires a reduction step to the arsenite form (Vahter and Envall, 1983; Radabaugh and Aposhian, 2000). Inorganic arsenicals removed from the body through the bile are exclusively in the trivalent forms, either as arsenite or MMA (Gregus et al., 2000). However, arsenic that is excreted through the urine is found in both the tri- and pentavalent form (Gregus et al., 2000).

There is a wide degree of variation between species in the rate of arsenic methylation (Wildfang et al., 1998). In one study purification of liver methyl transferases and subsequent activity assays showed a much higher degree of MMA formation in rabbits with a Vmax (pmol/mg protein/60 min) of 39.6 compared to 14.7 in the rhesus monkey and 0.022 in the hamster. In addition, MMA activity studies showed further discrepancies between species with the rhesus monkey having a higher capacity to form DMA with a Vmax of 21.5 than the rabbit with 4.01, followed by the hamster with 0.007 (Wildfang et al., 1998). These results clearly show the differences that occur in the metabolism of arsenic within laboratory animals which may complicate comparisons with humans.

Mixtures. Elucidation of the mechanisms by which environmental contaminants exert their toxic effects is complicated by the fact that hazardous compounds are not found in the environment alone, but rather in conjunction with other compounds. The characteristics of the environment in which a toxicant such as arsenic is found may influence its solubility in water, bioavailability to plants and animals, rate of metabolism,
and metabolic pathways. One potential mechanism for the wide diversity of cellular effects caused by arsenic is its ability to influence or be influenced by other essential metals or toxicants. For example, DMA has been shown to significantly increase the release of iron from horse spleen ferritin (Ahmad et al., 2000). This remained true under both aerobic and anaerobic conditions and in the presence or absence of ascorbic acid, an agent known to aid in the absorption and release of iron (Ahmad et al., 2000). This is another pathway by which arsenic contributes to the oxidative state of an organism.

Another metalloid, selenium, has been shown to influence the metabolism of arsenite (Csanaky and Zoltan, 2003). Selenium is an essential mineral, yet highly toxic if ingested in large quantities. The interaction between selenium and arsenite in biological systems has been well established and at times both metalloids counteract the toxic affects of the other (Gregus et al., 2000). In rats, tissue levels of MMA (III and V) were lowered by administration of selenium, while DMA levels were elevated (Csanaky and Zoltan, 2003).

Furthermore, it is possible that metals/metalloids influence each other indirectly through the generation of reactive intermediates. For example, we have shown that exposure to arsenic increases the synthesis of peroxynitrite, a highly reactive isomer of the nitrate anion linked with various pathological conditions (Bunderson et al., 2002). It is known that peroxynitrite influences the release of zinc from the zinc-thiolate cluster of endothelial NOS, forming disulfide bonds between the enzyme monomers and resulting in less NO synthesis(Zou et al., 2002). This has been related to increased generation of superoxide anion in diabetic mice and may also be important in the pathogenesis of atherosclerosis (Zou et al., 2002). Therefore, an increase in peroxynitrite in response to
arsenic and arsenic mixtures would influence the normal functions of zinc and may contribute to atherogenesis. Finally, oral administration of inorganic arsenite to rats and guinea pigs has also been linked to an accumulation of copper in the kidneys, although the biological consequence of this interaction is unknown (Ademuyiwa and Elsenhans, 2000).

Environmental contaminants other than metals may also influence the biological impact of arsenic. For example, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) is a common environmental pollutant resulting from application of contaminated pesticides. TCDD is a ligand for the aromatic hydrocarbon receptor and results in induction of both phase I and phase II detoxification enzymes. Arsenite has been shown to enhance TCDD-induced levels of nicotinamide adenine dinucleotide phosphate-quinone oxidoreductase (NQO1) mRNA in mouse hepatoma Hepa-1 cells (Maier et al., 2000). However, arsenite had no impact on the levels of the TCDD-inducible cytochrome P450, Cyp1a1, suggesting that studies on the effects of environmentally relevant mixtures may be complicated by imbalances in gene expression of important detoxification enzymes (Maier et al., 2000).

**Cardiovascular Disease**

*Social Implications.* According to most recent estimates by the American Heart Association, cardiovascular disease is the number one killer in the U.S. affecting 60 million Americans with a resulting cost of 300 billion dollars a year.

*Risk Factors.* There are six risk factors that have been determined to be “causal and independent” through epidemiology, genetics, pathology and the result of clinical
trials. These risk factors are as follows: cigarette smoking, elevated blood pressure, elevated serum low-density lipoprotein-cholesterol, low serum high-density lipoprotein-cholesterol, diabetes mellitus and advanced age (Frohlich and Lear, 2002). Other factors that are considered to be “predisposing” include: physical inactivity, obesity, abdominal density, family history of premature CAD, ethnic characteristics and psychosocial variables. Further “conditional” risk factors include elevated serum triglycerides, small LDL particles, elevated serum homocysteine, elevated serum lipoprotein (a), prothrombotic factors and inflammatory markers (Frohlich and Lear, 2002).

Four of the major risk factors for cardiovascular disease share a commonality in their ability to damage the cardiovascular system. Hypercholesterolemia, hypertension, diabetes, and cigarette smoking all contribute to a state of oxidative stress that has been linked to various forms of heart disease such as ischemia, stroke and myocardial infarction (Cooper et al., 2002). In addition, these four risk factors result in an accumulation of inflammatory and thrombogenic mediators that result in a pathological blood-vessel wall interaction resulting in enhanced pathogenesis (Cooper et al., 2002).

Recent studies suggest that people who are seropositive for *Chlamydia pneumoniae* (Cp) are at increased risk for developing atherosclerosis (Neumann, 2002). In fact, Cp positive patients treated with the antibiotic roxithromycin showed a significant decrease in the progression of carotid occlusions. This is suggestive of yet another risk factor in the development of coronary artery disease (Sander et al., 2002). Furthermore, while there is a growing body of evidence linking exacerbation of atherosclerosis to certain bacterial and viral infections, several auto-antigens have been implicated in pathogenesis as well. Two of the most likely candidates for inducing an autoimmune
response that result in an increase in the severity of cardiovascular disease are oxidized LDL and heat shock proteins (Greaves and Channon, 2002). There is also evidence suggesting that measuring intima media thickness (IMT) can predict an increased risk for future probability of CAD in persons with low-to-intermediate possibility of developing the disease (Sinha et al., 2002). The presence of arsenic in the drinking water has also been shown to be a contributing factor in the prevalence of cardiovascular disease (Wang et al., 2002).

C-Reactive Protein (CRP) is a nonspecific marker for inflammation that has been correlated with the incidence of coronary heart disease and may also be an important risk factor (Pasceri et al., 2000). Addition of CRP to human umbilical vein and coronary artery endothelial cells resulted in an increase in the expression of VCAM-1 and ICAM-1 after 24 h and a significant induction of E-selectin after 6 h exposure. This effect was dependent on the presence of serum in the media. This study suggests that CRP may play a direct role in promoting the chronic inflammatory state characteristic of atherosclerosis (Pasceri et al., 2000).

Cyclooxygenase-2. Prostaglandins and other prostanoids are synthesized from arachidonic acid (AA) by prostaglandin endoperoxide H synthase, also known as cyclooxygenase (COX). AA is a polyunsaturated fatty acid that is released from membrane phospholipids through the activity of phospholipase A2 in response to chemical or mechanical stimuli (Smith and DeWitt, 1996). Following release of AA from cellular membranes, COX catalyzes its conversion to prostaglandin G2 (PGG2) through the addition of O2. PGG2 is an unstable compound and rapidly undergoes a two electron reduction forming PGH2. PGH2 is the stable precursor for all prostanoids.
These compounds include the prostaglandins, prostacyclin (PGI₂), and thromboxanes (TXA) and bind to G-protein-linked receptors resulting in altered second messenger synthesis (Smith and DeWitt, 1996).

There are two well-studied isozymes of the COX enzyme known as COX-1 and COX-2. COX-1 is considered to be the constitutive form of the enzyme and is found primarily in platelets, kidney, colon and stomach (Dannenberg and Zakim, 1999). It is known to be important in the normal physiological functions of prostaglandins such as regulation of renal blood flow and platelet aggregation (Lipsky et al., 2000). In contrast, COX-2 is undetectable in most tissues but is rapidly induced in response to cellular stress (Dannenberg and Zakim, 1999). COX-2 shares many characteristics of other stress response genes, including CCAAT/enhancer-binding protein, cyclic AMP-responsive element, pregnancy-specific protein-1 (Sp1), and an NFκB site as regulatory mechanisms of its synthesis (reviewed in Fosslien, 2000). Products of the COX-2 pathway (Figure 1) have an affect primarily at the nuclear level and are typically involved in inflammation (Smith and DeWitt, 1996).

The role that cyclooxygenase has in the development of atherosclerosis is currently a matter of debate. Under normal physiological conditions, prostaglandins serve as vasodilators or vasoconstrictors, depending on the compound and conditions, and are important for the onset of fever (reviewed in Campbell and Halushka, 1996). The various prostanoids are also known to increase glomerular filtration rate and renal blood flow as well as enhance sensitivity to pain endings and aid in fertilization (Campbell and Halushka, 1996). Perhaps the most important function of the prostaglandins with respect to cardiovascular disease is their role in inflammation. Aspirin, a nonsteroidal anti-
Figure 1. Biosynthetic pathways of cyclooxygenase and 5-Lipoxygenase

Phospholipid

Phospholipase A2

Arachidonic Acid

5-Lipoxygenase (FLAP)

5-HPETE

LTA₄

LTA₄

LTC₄

LTC₄

LTD₄

LTE₄

Cyclooxygenase

PGH₂

PGI₂

PGE₂, PGF₂α, PGD₂

Prostacyclin Synthase

TX Synthase

Isomerases

LTB₄

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inflammatory inhibitor drug (NSAID) and nonspecific COX inhibitor, is associated with a reduction in mortality among individuals being evaluated for known or suspected coronary artery disease. Patients that benefited the most were elderly patients, those with impaired exercise tolerance, and individuals with a history of coronary artery disease (Gum et al., 2001). However, there is some concern that specific COX-2 inhibitors may contribute to the severity of atherosclerosis, probably by blocking PGI₂ formation without influencing thromboxaneA₂ (TXA₂). This could result in increased platelet activation, adhesion, and aggregation thereby increasing the risk for future ischemic events.

5-Lipoxygenase. The lipoxygenases are a family of cytosolic enzymes similar to the cyclooxygenases that catalyze the oxygenation of AA and other polyenic fatty acids to lipid hydroperoxides (reviewed in Campbell and Halushka, 1996). AA is converted by the lipoxygenases to an unstable metabolite, hydroperoxyeicosatetraenoic acid (HPETE), which is then further metabolized to hydroxy fatty acids either nonenzymatically or by peroxidase activity (Campbell and Halushka, 1996).

Of particular interest is 5-lipoxygenase (5-Lo) and its biosynthetic products, the leukotrienes (Figure 1). When cells are activated by increases in intracellular Ca²⁺, 5-Lo translocates to the nucleus and associates with the 5-lipoxygenase activating protein (FLAP) resulting in activation of the enzyme (Campbell and Halushka, 1996). As a result, 5-HPETE is formed and rapidly converted to leukotriene A₄ (LTA₄) by leukotriene A synthase. Subsequent reactions lead to the various other leukotrienes, including LTB₄ and LTC₄, D₄, and E₄. The leukotrienes are primarily known for their role in asthma by causing bronchoconstriction through alterations of the airway smooth muscle cells and vascular endothelial cells (reviewed in Funk, 2001). The leukotrienes are also known to
have an important part in inflammation. In fact, LTC₄, D₄, and E₄ are known to make up the material of "slow reacting substance of anaphylaxis" (Campbell and Halushka, 1996). There has only recently been evidence emerging that suggests the importance of the 5-Lo pathway in cardiovascular disease (Mehrabian et al., 2002). Although the mechanisms are mostly unknown, it is probably a combination of an enhanced state of inflammation and vasoconstriction.

Atherosclerosis. Atherosclerosis is the initial disease state that results in the manifestation of life threatening forms of cardiovascular disease, such as myocardial infarction and stroke. Current theories on the development of atherosclerosis underscore the pathology as a "process" that is first characterized by endothelial cell dysfunction, followed by the formation of foam cells due to uptake of oxidized lipoproteins by macrophages, proliferation of vascular smooth muscle cells, and exacerbation by the inflammatory process which may eventually lead to rupture of "vulnerable" plaque (Frohlich and Lear, 2002). Specifically, inflammatory conditions within the vasculature (such as ischemia/reperfusion) have been shown to be associated with adhesion of platelets to the vascular endothelium predisposing an individual to thrombosis and occlusion of the vasculature (Greaves and Channon, 2002). There are various inflammatory mediators that have been shown to be pro-atherogenic. These include various cytokines, chemokines and proteins including: IFN-γ, IL-18, CD40-CD154, MCP-1, IL-8, IL-12, IL-1β, LTB4 and 12/15 lipoxygenase products (Greaves and Channon, 2002). Although the endothelial lining that surrounds atherosclerotic plaque has very similar characteristics to that of activated endothelial cells, they lack the presence of recruited neutrophils typical of sites of acute inflammation. However, T-cells
and monocytes are responsible for the expression of the vascular cell adhesion molecule-1 (VCAM-1) ligand—a protein that has been linked to the development of atherosclerotic plaque in genetically engineered mice (Greaves and Channon, 2002). The majority of T cells found in atherosclerotic plaque are CD4+ which may have a distinct influence on the differentiation of monocytes into macrophages. However, the absence of an adaptive immune system does not confer total resistance to the development of atherosclerosis in mice suggesting other factors, such as oxidative stress, contribute to the mechanisms of disease development.

In addition to inflammation, oxidative stress has been implicated as another important player in atherogenesis. The primary manifestations of ROS on the microcirculation are increased inflammation and increased expression of adhesion molecules on the endothelium as well as circulating blood cells (Cooper et al., 2002). This is significant due to the observation that both the endothelium and circulating blood cells can generate significant amounts of ROS under certain stimuli. It is further supported by the observation that exogenous addition of ROS results in increased leukocyte-endothelial cell adhesion which can be attenuated somewhat by the addition of antioxidants. (reviewed in Cooper et al., 2002). Human vascular endothelial cells that were induced into quiescence by inhibiting telomere function showed enhanced intercellular adhesion molecule-1 (ICAM-1) expression and decreased eNOS activity. These phenomena are both conditions that may contribute to atherogenesis (Minamino et al., 2002).

Current Treatments. Recently, inflammation has been a therapeutic target for the treatment of atherosclerosis (Greaves and Channon, 2002). One class of cholesterol
lowering agents, the statins, has been proven successful in primary and secondary
treatment of CVD in several clinical trials. Statins decrease cholesterol biosynthesis by
inhibiting the key enzyme HMG CoA reductase. The end result is not only a decrease in
total plasma cholesterol but a concomitant increase in LDL receptor expression and an
overall increase in plasma HDL cholesterol. Statin treatment is effective by increasing
the stability of the atherosclerotic plaque rather than reducing the size or number present
within the arterial wall. It has been suggested that statins may influence other aspects of
atherogenesis in addition to influencing plasma cholesterol. Improved endothelial
function, influences on NO synthesis, changes in smooth muscle cell proliferation and
migration, disruption of MCP-1 production and leukocyte adhesion and rolling on
endothelial cells have all been suggested as possible mechanisms by which statins
influence atherogenesis. Pravastatin in particular has been shown to reduce C-Reactive
Protein (CRP) levels, a systemic marker for inflammation, independently from its impact
on serum cholesterol.

Treatments aimed at increasing overall HDL levels are another potential target for
treatment of CVD. The protective effects of HDL are in part due to its ability to “reverse
cholesterol transport” from the tissue into the blood then liver. However, the positive
effects of HDL may also include effects on inflammation and oxidative stress by
inhibiting endothelial-cell activation, maintaining NO-mediated endothelial function as
well as transport of antioxidants (Greaves and Channon, 2002).

Inflammatory mediators, such as COX-2 and its products, the prostaglandins,
have been used as a potential target for the treatment of cardiovascular disease.
However, there has been some concern that selective COX-2 inhibitors contribute to the
severity of heart disease. This raises the question of whether COX-2 is an important player physiologically in cardiac performance or is a pathological component of atherosclerosis as previously assumed (Bertram et al., 2002). The primary concern is that COX-2 selective inhibitors block PGL₂ formation without influencing thromboxaneA₂ (TXA₂). This would result in increased platelet activation, adhesion, and aggregation thereby increasing the risk for future ischemic events. This theory was derived using PGI₂ receptor (IP) and TXA₂ receptor deficient mice. However, there is emerging evidence that COX-2 selective inhibitors have a beneficial effect on atherosclerosis, probably by decreasing inflammatory mediators within the vasculature. Support for this theory comes from LDLr⁻/⁻ mice fed a western diet showing a reduction in atherosclerosis after treatment with rofecoxib. This occurred despite the ability of rofecoxib to inhibit PGI₂ but not TXA₂ production (Bertram et al., 2002). Aspirin, an NSAID and nonspecific COX inhibitor, is associated with a reduction in mortality among individuals being evaluated for known or suspected coronary artery disease. Patients that benefited the most were elderly patients, those with impaired exercise tolerance, and individuals with a history of coronary artery disease (Gum et al., 2001).

In addition to COX-2, a locus on mouse chromosome 6 that supplies almost total resistance to atherogenesis has been identified as the 5-lipoxygenase (5-Lo) gene (Mehrabian et al., 2002). This was determined using a congenic mouse strain designated CON₆ that had a 5-fold reduction in 5-Lo mRNA. Furthermore, a 5-Lo knockout was bred onto an LDLr (-/-) background and heterozygous mice obtained. Both the CON₆ and the 5-Lo/LDLr (-/-) mice showed a significant decrease in aortic lesion development.
This suggests that 5-Lo plays an important part in the process of atherogenesis and could be a potential therapeutic target (Mehrabian et al., 2002).

Angiotensin II appears to play an important role in the aggravation of atherosclerosis primarily by increasing oxidative stress, and activating the nonphagocytic NAD(P)H oxidases found on endothelial cell membranes (Schiffrin, 2002). Therapeutic use of Angiotensin II receptor antagonists has been shown to retard the progression of atherosclerosis (Schiffrin, 2002).

**Arsenic and Cardiovascular Disease**

*Epidemiology.* There are numerous epidemiological studies linking arsenic to the different forms of cardiovascular disease. In a long-term study in Taiwan, there was a significant association between cumulative arsenic consumption through artesian well water and prevalence of carotid atherosclerosis (Engel and Smith, 1994). The data was consistent regardless of the duration of exposure, the average arsenic concentration within the water, or cumulative arsenic exposure and remained significant after adjusting for known risk factors in the development of atherosclerosis (Engel and Smith, 1994).

In the United States, an investigation on the relationship between population-weighted mean arsenic concentration in public drinking water and death from cardiovascular diseases (CVD) in 30 U.S. counties showed an increase in the mortality ratio for various forms of CVD (Wang et al., 2002). These included diseases of the arteries, arterioles, and capillaries as well as an elevation in the incidence of arteriosclerosis and aortic aneurysm with a mean arsenic concentration greater than 20 parts per billion (ppb). Furthermore, a cohort mortality study done in Millard County,
Utah where the median drinking water arsenic concentrations ranged from 14 to 166 ppb, showed an increased mortality from hypertensive heart disease (Lewis, 1999).

In addition, Blackfoot Disease, a peripheral arterial disease confined to an area in southwestern Taiwan, is associated with exceedingly high-arsenic concentrations in artesian well water (Tseng et al., 1995). In this area, even seemingly normal individuals are observed to have abnormal microcirculation in the extremities (Tseng et al., 1995). More recently, individuals living in this arseniasis-hyperendemic region of Taiwan were examined for signs of ischemic heart disease (IHD) that may have been overlooked in previous studies on Blackfoot Disease (Tseng et al., 2003). The authors concluded that for those individuals with a cumulative arsenic exposure of 0.1-14.9 mg/l-year, there was an increased incidence between atherosclerosis and long-term arsenic exposure as diagnosed by electrocardiogram (Tseng et al., 2003). In addition to cardiovascular toxicities, arsenic exposure associated with the use of Fowler’s solution, a “health tonic” made up of potassium arsenite, has been linked to portal hypertension, a disease of the liver characterized by increased hepatic venous pressure (Viudez et al., 2000).

Arsenic has also been implicated as an occupational hazard for pesticide applicators, primarily in vineyards. Exposure is by inhalation of contaminated fumes, dust or plant debris and ingestion of contaminated food and wine (Benowitz, 1992). Symptoms are primarily EKG abnormalities, ventricular tachycardia and red blood cell hemolysis (Benowitz, 1992). Recently, arsenic trioxide was approved for the treatment of acute promyelocytic leukemia. However, the side effects include cardiovascular toxicities such as prolongation of the QT interval and ventricular tachycardia (Li et al., 2002; Ohnishi et al., 2000).
Cellular mechanisms explaining the connection between arsenic and increased incidence of CVD are unknown. However, studies aimed at identifying involved pathways have demonstrated a link between arsenic and an increase in oxidative stress (Smith et al., 2001) and increases in inflammatory mediators (Liu et al., 2000), both of which are known contributors to the pathogenesis of atherosclerosis and other forms of CVD.

**Oxidative Stress.** Damage to the endothelial lining is one of the first steps in the onset of atherosclerosis. It has been suggested that arsenic contributes to endothelial dysfunction by initiating an increase in the synthesis of reactive oxygen species. In fact, arsenic has been shown to activate NAD(P)H oxidase on the membrane of vascular endothelial cells with as little as 5 μM sodium arsenite (Smith et al., 2001). In particular, stimulation of reactive oxygen species in vascular endothelial cells exposed to 5 μM sodium arsenite or less was observed by electron paramagnetic resonance (EPR) spectroscopy (Barchowsky et al., 1999) and may be related to activation of the transcription factor NF-κB, a common regulatory element in the promoter regions of many stress-induced genes (Barchowsky et al., 1996). Furthermore, a decrease in the expression of the Fas ligand (FasL), an important component of cytokine-induced leukocyte extravasation in endothelial cells exposed to 30 μM sodium arsenite, is dependent on an increase in the oxidative state of the cell (Tsai et al., 2001).

In addition to induction of stress-related genes, formation of superoxide anion as a result of membrane-bound oxidase activation provides an environment conducive for the formation of the extremely reactive nitrate isomer, peroxynitrite. The reaction between superoxide anion and nitric oxide is a nearly diffusion-limited reaction that occurs...
approximately three times faster than the scavenging of superoxide anion by superoxide dismutase (Beckman, 1996). The presence of peroxynitrite in a biological environment results in the nitrination of tyrosine residues which can then be measured as a biological marker for peroxynitrite as 3-nitrotyrosine (3NY) (Crow and Ischiropoulos, 1996). In fact, atherosclerosis has been characterized by extensive nitrination of protein tyrosine residues primarily within the atheroma, followed by foamy macrophage and nitrination of the endothelium (Beckmann et al., 1994). Our laboratory has demonstrated an increase in the synthesis of peroxynitrite in endothelial cells exposed to sodium arsenite, confirming a link between arsenic exposure and increased reactive oxygen and nitrogen species that could exacerbate atherosclerosis (Bunderson et al., 2002).

Generation of peroxynitrite as a result of arsenic exposure may have several cellular consequences important to the pathogenesis of atherosclerosis. Nitrination of tyrosine residues has been shown to interfere with phosphorylation cascades necessary for proper signal transduction pathways. In fact, peroxynitrite-mediated nitrination of a synthetic peptide resulted in a 51% inhibition of phosphorylation by protein tyrosine kinases (Gow et al., 1996). In addition to disrupting signal transduction pathways by interfering with protein phosphorylation, peroxynitrite has been shown to reduce the presence of soluble guanylate cyclase, the predominant intracellular nitric oxide receptor and a critical component in the formation of cGMP (Weber et al., 2001). This inhibitory effect of peroxynitrite may contribute to the oxidative stress associated with cardiovascular disease. The presence of free-3NY residues also results in severe consequences for the cardiovascular system. Rat aorta segments incubated with free-3NY resulted in a concentration-dependent impairment of acetylcholine-induced
relaxation (Mihm et al., 2000). This may be explained by the fact that 3NY is structurally similar to that of endogenous catecholamines (Figure 2) and therefore may serve as a competitive inhibitor (Kooy and Lewis, 1996). Furthermore, concentration-dependent DNA damage was observed with the TUNEL assay in rat aortic segments after incubation with 100 or 200 μM 3NY (Mihm et al., 2000).

An overall compromise in the efficiency of the cardiovascular system may also manifest itself as a result of increased peroxynitrite formation in response to arsenic exposure (Oyama et al., 1998). This was shown with an in vivo study using dogs to demonstrate an inverse relationship between left ventricular ejection fractions and the presence of myocardial 3NY residues (Oyama et al., 1998). The authors of this study hypothesized that peroxynitrite exerted its effects on the myocardium via IL-1β.

One of the primary mechanisms by which arsenic may exert its influence on the exacerbation of atherosclerosis is through the generation of reactive species. Activation
Figure 2. Chemical structures of L-Tyrosine, L-DOPA, and 3-nitro-L-Tyrosine
of membrane-bound oxidases resulting in increased oxidative stress and the formation of peroxynitrite are likely the primary components involved.

**Inflammation.** The role of the immune system in the onset and progression of atherosclerosis has been well established (reviewed in Greaves and Channon, 2002). Changes in the normal expression of inflammatory mediators as well as the adaptive and innate immune system in response to arsenic exposure would have a broad impact on the development of the disease. In the kidney, chronic arsenic exposure of 22.5 parts per million (ppm) resulted in inflammatory cell filtration and inflammatory lesions with repeated exposures (Liu et al., 2000). Furthermore, histology from liver biopsy samples obtained from individuals in Guizhou, China with a history of exposure to environmental arsenic, showed liver lesions with chronic inflammation (Lu et al., 2001). Therefore, similar events in the cardiovascular system may occur as a result of arsenic exposure.

Cyclooxygenase-2 (COX-2), the nonconstitutive form of the cyclooxygenase enzymes, is a possible candidate for influencing the inflammatory state of atherosclerosis. Cyclooxygenase 1 (the constitutively expressed form) and 2 are homodimeric, heme-containing proteins responsible for the conversion of arachidonic acid to the various prostanoids (Smith et al., 1996). Despite having very similar homology, COX-1 and COX-2 are very different functionally. COX-1 is expressed primarily in platelets, colon, and stomach and serves in the cytoprotection of the gastric mucosa, regulation of renal blood flow and platelet aggregation (Lipsky et al., 2000). In contrast, COX-2 is typically undetectable in tissues other than kidney and brain unless induced in response to cellular stress, cytokines, oncogenes, tumor promoters and various growth factors (Lipsky et al., 2000) and has been linked to ischemic heart disease in rats (Saito et al., 2000).
Arsenic exposure to human fibroblast cells resulted in an increase in COX-2 derived prostanoids, PGE\(_2\) and PGI\(_2\), at a concentration of 25 \(\mu\)M (Salzman and Bowman, 1992) and COX-2 is upregulated in aortic endothelial cells at a concentration of 10 \(\mu\)M (Bunderson \textit{et al.}, 2002). In addition, when human umbilical vein endothelial cells (HUVEC) were exposed to arsenic there was a two-fold induction of the COX-2 protein resulting in a concomitant twofold increase in the synthesis of prostaglandin E\(_2\) (PGE\(_2\)) (Tsai \textit{et al.}, 2002). This effect was attenuated by the addition of pyrrolidine dithiocarbamated (PDTC), an inhibitor of NF\(\kappa\)B, suggesting that arsenic may modulate its effects by activation of transcription factors involved in the stress response. This is a significant finding considering that an increase in the formation of proinflammatory prostanoids through induction of COX-2 contributes to experimental heart failure due to ischemic heart disease in rats (Saito \textit{et al.}, 2000).

The COX-2 protein has also been detected in atherosclerotic lesions, possibly promoting inflammation (Burleigh \textit{et al.}, 2002). Furthermore, addition of the specific COX-2 inhibitor, rofecoxib, and the nonspecific cyclooxygenase inhibitor indomethacin to male LDLr\(^{-}\) mice fed a western diet resulted in a significant reduction in the size of aortic plaque (Burleigh \textit{et al.}, 2002). In addition, platelets isolated from rats exposed to 0, 10, 25 or 50 \(\mu\)M arsenite for 60 min showed a significant increase in the percentage platelet aggregation observed versus saline controls (Lee \textit{et al.}, 2002). This phenomenon occurred simultaneously with an enhanced formation of TXA\(_2\), and expression of the adhesion molecule P-selectin (Lee \textit{et al.}, 2002). This is further evidence suggestive of an interference with activity of the cyclooxygenase pathways and inflammation upon exposure to arsenic.
Recently, another inflammatory pathway closely related to the cyclooxygenase system has been recognized as an important component of atherogenesis. Mehrabian et al., (2002), identified a locus on mouse chromosome 6 that confers almost total resistance to atherogenesis as the 5-lipoxygenase (5-Lo) gene. A dramatic decrease (>26-fold) in the development of aortic lesions was observed in 5-Lo knockout mice. The enzyme 5-Lo metabolizes arachidonic acid to leukotriene A\(_4\) (LTA\(_4\)) which is then converted to leukotriene B\(_4\) (LTB\(_4\)) and the cysteinyl-leukotrienes (C\(_4\), D\(_4\), and E\(_4\)), all of which are known to have proinflammatory effects. Furthermore, Allen et al., (1998), demonstrated an LTC\(_4\) and LTD\(_4\)-dependent state of hyperactivity in atherosclerotic coronary arteries suggesting an increase in the 5-Lo enzyme (Allen et al., 1998). Therefore, an increase in 5-Lo expression or activity in response to arsenic exposure would have a dramatic impact on the severity of atherosclerosis.

**Mouse Model**

*Available Models.* In the past, studies intended to solve the mysteries of atherosclerosis were limited to primates and LDL-deficient rabbits (Pratico, 2001). However, mice have become more popular over recent years due to ease of breeding, prolific reproductive systems, availability of inbred strains, and techniques resulting in genetically altered strains (Pratico, 2001). There is a wide array of transgenic and knockout mice that have been utilized in the study of atherogenesis (Table 1) (Reue, 2001).

One of the initiating events involved in atherosclerosis is the irreversible movement of apo B-containing lipoproteins within the vessel wall (reviewed in Sheth et
Table 1. Genetically engineered mouse models for proteins in lipid metabolism

(Reue 2001, with permission)

<table>
<thead>
<tr>
<th>Component</th>
<th>Transgenic Model</th>
<th>Knockout Model</th>
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<tr>
<td>Apolipoproteins</td>
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<td>ApoA-I</td>
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<td>A-II</td>
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<td>ApoA-V</td>
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<td>AIV</td>
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<td>ApoA-VI</td>
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<td>ApoB</td>
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<td>ApoC-I</td>
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<td>ApoC-II</td>
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<td>Apo(a)</td>
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| Lipoprotein receptors                  |                  |                |
| LDL receptor                           | +                | +             |
| LDL receptor-related protein           | +                | +             |
| VLDL receptor                          | +                | +             |
| ApoE receptor 2                        | +                | +             |
| Scavenger receptor                     | +                | +             |

| Lipoprotein remodeling enzymes         |                  |                |
| Lipoprotein Lipase                     | +                | +             |
| Hepatic lipase                         | +                | +             |
| Cholesterol ester transfer protein     | +                | +             |
| Lecithin cholesterol acyl transferase  | +                | +             |

A + in transgenic or knockout column indicates that an induced mutant mouse model has been generated for the corresponding protein.

*a The knockout model for this protein showed embryonic lethality

*b Not applicable; no mouse homolog exists.
Therefore, mice capable of synthesizing only the Apolipoprotein B100 have been used to back cross with other types of atherogenic mouse models such as the low density lipoprotein (LDL) receptor null mice (LDLr−/−) and the apolipoprotein E null mice (ApoE−/−) (Sheth et al., 2002). Due to differences in the type of lipoproteins generated between these crosses, important characteristics of the atherogenic process were elucidated (Sheth et al., 2002). For example, using this system researchers were able to determine that cholesterol packaged in small LDL particles is more atherogenic than that packaged in very low density lipoprotein (VLDL) particles. In addition, ABCA1 (Abca1−/−) null mice characterized by a virtual lack of high density lipoprotein (HDL) particles, as well as hepatic nuclear factor 1α-deficient (Hnf1α−/−) mice have been linked to problems with cholesterol metabolism and transport associated with atherosclerosis (Sheth et al., 2002). Studies using mice that are null for enzymes important in oxidative stress such as myeloperoxidase (Mpo−/−), which is involved in lipid oxidation and oxidative damage, and 12/15 lipoxygenase (12/15-Lo−/−), have demonstrated the importance of oxidation reactions in the pathogenesis of atherosclerosis (Sheth et al., 2002). Various other models have been used with varying degrees of success such as the immune deficient recombinase-activating genes 1 or 2 null mice (RAG 1 or 2 −/−), metalloproteinase deficient mice (Mmp3−/−), and vascular cell adhesion molecule-1 null mice (VCAM-1−/−) (Sheth et al., 2002).

ApoE+/LDLr−/− mice. ApoE−/− mice are currently the most widely used model for the study of atherosclerosis due to spontaneous lesion development that closely resembles complex human atherosclerotic plaque (Reardon and Godfrey, 2001). ApoE is a 34 Kd glycoprotein that is produced predominantly by the liver, followed by various other
tissues and macrophages (reviewed in Willems Van Dijk et al., 2000). There are three isoforms present in the human population including ApoE-III (most common), ApoE-IV, and ApoE-II. There is an increased incidence of dyslipidemia in healthy individuals who are positive for ApoE-II and IV, including decreased plasma LDL levels or elevated LDL levels, respectively, demonstrating a difference in the function of the different ApoE isoforms (Willems Van Dijk et al., 2000). ApoE is an important component in many steps of VLDL metabolism, including VLDL-remnant clearance, VLDL-triglyceride production and VLDL-triglyceride lipolysis (Willems Van Dijk et al., 2000). As a result, ApoE deficient mice are extremely susceptible to hyperlipidemia and accumulation of VLDL resulting in spontaneous development of atherosclerosis similar to that seen in humans (Willems Van Dijk et al., 2000).

ApoE also serves an important function in the hepatic clearance of VLDL remnants by acting as a ligand for the LDL receptor. Presence of the LDL receptor is critical for plasma clearance of lipoprotein remnants and in fact, overexpression of the receptor results in near normalization of the plasma lipid levels in mice with aberrant ApoE proteins (Willems Van Dijk et al., 2000). In general, LDLr^{−/−} mice do not develop such severe or complex lesions as the ApoE^{−/−} mice (Reardon and Godfrey, 2001). However, it is a useful model for studying the development of atherosclerosis especially when cross-bred with other genetically altered mice, such as the ApoE^{−/−} mice. The ApoE^{−/−}/LDLr^{−/−} mice have accelerated plaque formation and develop severe occlusions relatively early in life. This makes them extremely useful for studying changes in atherogenesis in response to environmental toxicants.
References


Cooper D, Stokes KY, Tailor A, and Granger DN. Oxidative stress promotes blood cell-endothelial cell interactions in the microcirculation. *Cardiovascular Toxicology* 2002;02:165-180.


Davis CD, Uthus EO, Finley JW. Dietary selenium and arsenic affect DNA methylation in vitro in Caco-2 cells and in vivo in rat liver and colon. *J Nutr* 2000;130:2903-2909.


Gum PA, Thamilarasan M, Watanabe J, Blackstone EH, Lauer MS. Aspirin use and all-cause mortality among patients being evaluated for known or suspected coronary artery disease: A propensity analysis. *JAMA* 2001;286:1187-1194.


CHAPTER ONE

Arsenic Contributes to the Oxidative and Inflammatory State of Atherosclerosis by Increasing Peroxynitrite Formation and Cyclooxygenase-2 (COX-2) Expression

Abstract

Epidemiological evidence suggests that exposure to the metalloid arsenic constitutes a risk factor for cardiovascular disease. Some of the cardiovascular effects attributed to arsenic are decreased cardiac function, peripheral vascular diseases and atherosclerosis. The purpose of this study was to determine whether arsenic could contribute to the chronic inflammatory and oxidative stress symptoms characteristic of atherosclerosis, or coronary artery disease. We found that production of peroxynitrite, a strong oxidant formed from the coupling of nitric oxide and superoxide anion, was significantly increased in bovine aortic endothelial (BAE) cells exposed to sodium arsenite. Expression of the inflammatory mediator, cyclooxygenase-2 (COX-2) was also upregulated in response to exposure, indicating an increase in the inflammatory response in BAE cells. This result was supported by an increase in the generation of prostaglandin E₂ (PGE₂) following exposure to arsenic. Since peroxynitrite is capable of nitrating protein tyrosine residues, peroxynitrite formation can be confirmed by determining the extent of nitrotyrosine formation in a protein. For this experiment, COX-2 protein was immunoprecipitated from BAE cells and submitted to Western blot analysis using an antibody to nitrotyrosine. Nitration of COX-2 was detected in arsenic-treated cells, but not in untreated control cells. The findings in this report suggest an increase in reactive species, notably peroxynitrite, in BAE cells exposed to arsenic. Furthermore, induction of
important inflammatory mediators such as COX-2 may exacerbate the inflammatory state typical of atherosclerosis.

**Introduction**

Human exposure to arsenic in drinking water has been linked to various cardiovascular diseases. In a comprehensive study involving 30 counties in the United States (Engel and Smith, 1994) an increased mortality ratio for cardiovascular disease was associated with a mean arsenic concentration in drinking water over 20 ppb. Mortality ratios were elevated for diseases of the arteries, arterioles, and capillaries including arteriosclerosis and aortic aneurysm. Furthermore, a cohort mortality study done in Millard County, Utah where the median drinking water arsenic concentrations ranged from 14 to 166 ppb, showed an increased mortality from hypertensive heart disease (Lewis et al., 1999). In addition, Blackfoot disease, a peripheral arterial disease confined to an area in southwestern Taiwan, is associated with exceedingly high-arsenic concentration in artesian well water (Tseng et al., 1995). In this area, even seemingly normal individuals are observed to have abnormal microcirculation in the extremities. Recently, arsenic trioxide was approved for the treatment of acute promyelocytic leukemia. However, the side effects include cardiovascular toxicities such as prolongation of the QT interval and ventricular tachycardia (Ohnishi et al., 2000).

Identification of the mechanisms involved in arsenic-related cardiovascular diseases could lead to strategies for prevention or treatment of those diseases.

Inorganic arsenic is the major type found in drinking water, and the oxidized form, arsenate (As[V]), is the predominant species. Arsenate is reduced *in vivo* to
arsenite (As[III]), which can be further metabolized to mono- and dimethylated
derivatives (Abernathy et al., 1999). Arsenite is considered to be the most toxic form of
arsenic (Buchet and Lison, 2000). The role of arsenic in cardiovascular disease is still
largely unknown, but it may involve generation of oxidants in or near the vessel wall.
Arsenic has been shown to activate an NAD(P)H oxidase found on the plasma membrane
of vascular endothelial cells (Smith et al., 2001) and vascular smooth muscle cells (Lynn
et al., 2000). Activation of these membrane-bound oxidases leads to increased production
of reactive oxygen species, particularly superoxide anion (O$_2^-$). Superoxide anion
combines with nitric oxide (NO') to form an extremely reactive isomer of the nitrate
anion, peroxynitrite. The reaction between superoxide anion and nitric oxide is a nearly
diffusion-limited reaction that occurs approximately three times faster than the
scavenging of superoxide anion by superoxide dismutase (Beckman, 1996). Peroxynitrite
has been implicated in the oxidation of low-density lipoprotein (LDL) to a form
recognizable by macrophage scavenger receptors, one of the putative first steps in
atherogenesis (Keaney, 2000). Peroxynitrite has also been shown to decrease the
antioxidant capacity within human blood plasma by oxidizing ascorbic acid, uric acid and
plasma sulphhydryl groups (Van der Vliet et al., 1994). There is substantial evidence
linking in vivo production of peroxynitrite with nitration of both free and protein bound
tyrosine residues (Beckman, 1996). The presence of nitrotyrosine in biological samples
has been used as a marker for the presence of peroxynitrite (Crow and Ischiropoulos,
1996). In addition, nitrotyrosine residues have been observed using immunodetection
techniques in various pathological conditions, including myocardial ischemia and
atherosclerosis (Beckman and Koppenol, 1996). Atherosclerosis is characterized by a
general, and in some cases marked, increase in the occurrence of nitrotyrosine residues (Beckmann et al., 1994; Leeuwenburgh et al., 1997).

Arsenic may also contribute to atherogenesis by increasing the inflammatory response. Cyclooxygenase-2 (COX-2) is the inducible form of a biphasic enzyme responsible for catalyzing the conversion of arachidonic acid to prostaglandin H₂ (PGH₂). PGH₂ is converted to other prostanoids, including PGE₂. The prostanoids are potent mediators of the inflammatory response and increase vascular permeability. COX-2 is found in macrophages, vascular endothelial cells and vascular smooth muscle cells (Sait et al., 2000) COX-2 is rapidly induced in macrophages and endothelial cells by proinflammatory cytokines and may be responsible for the edema and vasodilation associated with inflammation (Lipsky et al., 2000). COX-2 is induced under conditions of oxidative stress (Marrogi et al., 2000; Kumagai et al., 2000), and it has been suggested that eicosanoids produced by COX-2 contribute to oxidative stress (Nikolic and van Breemen, 2001). Since atherosclerosis is a chronic inflammatory condition (Lusis, 2000), it is likely that COX-2 is involved in the formation of atherosclerotic plaques.

The mechanisms and regulatory pathways involved in arsenic-induced cardiovascular disease are largely unknown. The purpose of this study was to determine whether arsenic could stimulate generation of factors involved in oxidative stress and inflammation, conditions associated with atherosclerosis, or coronary artery disease. In this paper we report that arsenic exposure increases production of an important oxidant, peroxynitrite, to cause the formation of nitrotyrosine residues and increased expression of a key inflammatory regulator, COX-2.
Methods

Chemicals. Peroxynitrite was synthesized according to the method described by Uppu and Pryor (1996). Sodium arsenite was purchased from GFS Chemicals (Columbus, OH), hydroethidine was obtained from Polysciences, Inc. (Warrington, PA), and IGEPAL CA-630 was purchased from Sigma (St. Louis, MO). Protease inhibitor cocktail tablets were purchased from Boehringer Mannheim (Mannheim, Germany). Superoxide dismutase and catalase were obtained from Fisher Scientific (Pittsburgh, PA). Nitrotyrosine antibody was obtained from Zymed, Inc. (San Francisco, CA), and the antimouse secondary was purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). COX-2 antibody was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), and the antirabbit secondary was purchased from Vector Laboratories, Inc. (Burlingame, CA). Actin antibody kit was obtained from Oncogene Research Products (Boston, MA). PGE$_2$ enzyme immunoassay (ELISA) was obtained from Cayman Chemical (Ann Arbor, MI). All reagents were at least analytical grade.

Cell Culture. Bovine aortic endothelial cells (BAE), originally a gift from Dr. Steve Schwartz of the University of Washington, were provided by Dr. J. Douglas Coffin of The University of Montana Department of Pharmaceutical Sciences. Cell culture components were obtained from Life Technologies, Inc. (Rockville, MD) unless otherwise noted. BAE cells were grown in Dulbecco's Modified Eagle Medium (DMEM) with penicillin/streptomycin and L-glutamine, and supplemented with 15% fetal bovine serum (HyClone Laboratories, Inc., Logan, UT). The cells were incubated at 37 °C under a humidified atmosphere containing 5% CO$_2$. BAE cells were used between passage #’s 2-6.
Cell viability assay. Cell viability was determined using an adaption of the MTT colorimetric assay (Mosmann, 1983). Cells were plated in 96-well plates at a density of 1 to 2 x 10⁴ cells/mL and allowed to attach overnight (16 h). Sodium arsenite and sodium arsenate were applied in medium for 1 h. Arsenic solutions were removed and replaced with medium alone, and the 96-well plates were incubated for 5-7 days. MTT (50 μg) was added to each well, and the cells were incubated for another 4 h. Medium/MTT solutions were removed carefully by aspiration, the MTT formazan crystals were dissolved in 100 μl DMSO, and absorbance was determined on a plate reader at 550 nm. Cell viability was plotted as percentage of control vs concentration. Cell viability was also determined for hydroethidine, l-NAME, and DPI using this assay.

Fluorimetric Determination of Peroxynitrite. To quantify peroxynitrite formation, we developed an assay based on the oxidation of hydroethidine. Hydroethidine is selectively oxidized to its fluorescent form, ethidium, by superoxide anion, hydrogen peroxide and peroxynitrite (Supinski et al., 1999; Al-Mehdi et al., 1997). This assay uses superoxide dismutase and catalase to remove extracellular superoxide and hydrogen peroxide, leaving peroxynitrite as the principal oxidant. BAE cells were grown to approximately 90% confluency then trypsinized (0.05% trypsin) and centrifuged at 2500 x g. The supernatant was removed and cells resuspended in phosphate-buffered saline (pH=7.2) containing 0, 5, 10, or 20 μM sodium arsenite. Cell numbers were calculated using a hemacytometer. Cells were allowed to incubate in a 37° C water bath for 30 min. At the end of the incubation period, 2 mL of the solution was added to a cuvette containing 25 U each of catalase and superoxide dismutase. Prior to starting each experiment, 40 μM hydroethidine was added to the cuvette. The samples were run on an
F-2000 Hitachi Fluorescence Spectrophotometer equipped with an automatic cuvette stirrer. Fluorescence was measured at 465 nm excitation and 585 nm emission over a 15 min interval. Peroxynitrite formation was calculated based on a standard curve generated by adding known concentrations of peroxynitrite to phosphate-buffered saline at timed intervals.

**COX-2 Immunoblot.** BAE cells were grown to approximately 90% confluency and treated with sodium arsenite in concentrations of 0, 5, 10 and 20 μM for 30 min. Cells were harvested with 2X Laemmli buffer, homogenized with an 18 then 25 gauge needle and boiled for 5 min. Protein concentrations were determined by a Bradford assay (Biorad, Hercules, CA), and samples (5 μg) were loaded onto a 10% SDS gel. After protein separation, the samples were transferred to a polyvinylidenedifluoride (PVDF) membrane and probed for COX-2 protein using a rabbit polyclonal COX-2 antibody and an antirabbit secondary antibody conjugated with horseradish peroxidase. COX-2 was visualized by enhanced chemiluminescence (ECL; Amersham, Buckinghamshire, England). In separate experiments, BAE cells were exposed to 20 μM sodium arsenite for 0, 0.5, 1, 12, 24 and 48 h to determine the effect of time on COX-2 expression. Blots were processed as detailed above.

**COX-2 Immunoprecipitation.** BAE cells were grown to near confluency and treated with sodium arsenite in concentrations of 0, 5, 10 and 20 μM for 30 min. Samples were solubilized in a buffer containing 50 mM tris, 1 mM ethylenediaminetetraacetic acid (EDTA), 0.1 mM butylated hydroxytoluene and a protease inhibitor cocktail tablet (pH=7.4). Cell lysates were sonicated and centrifuged at 12,000 x g for 1 h. Supernatant was collected and a final sample volume of 1 mL was attained with a buffer containing 1
mM phenylmethylsulfonyl fluoride, 150 mM NaCl, 5 mM EDTA and 0.5% IGEPAL CA-630 (pH= 8.0). Protein was assayed as before and samples equalized for protein content. Protein A sepharose CL-4B was used to preclear the solubilized protein and the samples were then centrifuged at 14,000 x g for 5 min and the supernatant collected. A polyclonal anti-COX-2 antibody (10 µg) was used to precipitate the precleared protein for 18 h at 4°C. Immune complexes were then precipitated with 50 µL protein A CL-4B for 2 h. The precipitate was washed three times with 0.5 mL SINTE (0.5% sucrose, 1% IGEPAL CA-630, 0.5 M NaCl, 50 mM tris, 5 mM EDTA; pH=7.4) followed by centrifugation. Samples were resuspended in 50 µL 2X Laemmli sample buffer and heated at 95°C for 5 min. Samples were run on a 10% tris-polyacrylamide gel at 150 V for 75 min and the protein transferred onto a PVDF membrane at 90 V for 1 h. The immunoblot was developed in the same manner as the COX-2 Western blots, substituting a mouse monoclonal antinitrotyrosine antibody and an antimouse secondary antibody.

Prostaglandin E2 Enzyme Immunoassay (ELISA). A PGE2 ELISA kit was used according to manufacturer’s instructions. BAE cells were incubated for 45 min with sodium arsenite (0, 10 and 20 µM). The media was collected and centrifuged at 2500 rpm for 5 min. The centrifuged media was added directly to the ELISA plate as directed. Data were analyzed by using a standard curve generated by plotting the percent bound divided by the maximum binding versus the log of PGE2 concentrations.

Results

Cell viability was assessed using a variation of the MTT colorimetric assay (Mosmann, 1983) that is based on the conversion of MTT to purple formazan crystals by
viable cells (Figure 3). As expected, sodium arsenite was more toxic to BAE cells than sodium arsenate after 1 h exposures at various concentrations. IC\textsubscript{50} values (concentration at which cell survival equals 50\% of control) were 54.2 +/- 8.6 \mu M for sodium arsenate. Concentrations of sodium arsenite used in subsequent experiments did not exceed 20 \mu M.

Peroxynitrite, the coupling product of nitric oxide and superoxide anion, is an oxidant that has been implicated in atherogenesis. Our approach to quantifying peroxynitrite formation in BAE cells was to first look for general evidence of oxidant production and then to use antioxidant enzymes, a general NOS inhibitor, and an oxidase inhibitor to identify peroxynitrite as the principal oxidant. Oxidant production was determined fluorimetrically by measuring the oxidation of hydroethidine to its fluorescent form, ethidium. Hydroethidine is preferentially oxidized by superoxide anion, hydrogen peroxide, and peroxynitrite (Supinski \textit{et al.}, 1999; Al-Mehdi \textit{et al.}, 1997). Total reactive species increased in a dose-dependent manner up to 10 \mu M sodium arsenite (Figure 4). Addition of SOD and CAT had little effect on levels of reactive species (Figure 5A), suggesting either that peroxynitrite was the principal oxidant generated in endothelial cells by sodium arsenite or that all of the superoxide anion and hydrogen peroxide were produced intracellularly, where added SOD and CAT would have no effect. The latter scenario seems unlikely since most superoxide generation in endothelial cells is associated with plasma membrane NAD(P)H oxidases (Barchowsky \textit{et al.}, 1999; Smith and DeWitt, 1996) the general NOS inhibitor L-NAME (Figure 5B) or the NAD(P)H oxidase inhibitor DPI (Figure 5C) reduced fluorescence to control levels, confirming that peroxynitrite is primarily responsible for the fluorescence observed in arsenic-treated cells. Cell viability studies showed no toxicity to BAE cells treated with hydroethidine,
DPI, or L-NAME at the concentrations and times used in these experiments (data not shown).

The synthesis of prostaglandins by the COX isozymes is a necessary event in normal vascular physiology and the inducible form, COX-2, is a key player in inflammation. Since atherosclerosis is an inflammatory disease, we reasoned that COX-2 might play an important role in arsenic-induced atherogenesis. Expression of COX-2 protein was measured by Western blot analysis using a polyclonal COX-2 antibody and chemiluminescence. The data show that COX-2 protein was increased after 30 min exposure to 5, 10, and 20 μM arsenite (Figure 6a-b). Furthermore, COX-2 induction was time dependent, showing a decline up to 24 h and then increasing again at 48 h (Figure 7a-b). Densitometry using COX-2/actin ratios showed a twofold increase in COX-2 protein at 20 min and an over threefold increase after 48 h. No increase in COX-2 protein was observed at sodium arsenite concentrations less than 5 μM (not shown). The presence of multiple bands is a common occurrence due to post-translational modification of COX-2 (Smith and DeWitt, 1996).

Immunoprecipitation was used to confirm the formation of peroxynitrite and to determine the extent of COX-2 nitration upon arsenic exposure. COX-2 was immunoprecipitated from endothelial cells that were treated for 30 min with 5, 10 and 20 μM sodium arsenite. Western blots were then probed with an antinitrotyrosine antibody. Nitrated COX-2 was observed in cells treated with all concentrations of sodium arsenite, whereas there was no evidence for nitration of COX-2 in the non-arsenic-treated cells. (Figure 8).
Figure 3. Cell viability for BAE cells treated with up to 500 μM sodium arsenate (As[V]) or sodium arsenite (As[III]) for 1 h.
Figure 3. Cell viability for BAE cells treated with up to 500 μM sodium arsenate (As[V]) or sodium arsenite (As[III]) for 1 h. Viability was measured using an adaptation of the MTT colorimetric assay as described under Methods. Viability is expressed as percentage of control (untreated cells); error bars represent mean +/- SD for n = 3 experiments.
Figure 4. Total oxidant formation in BAE cells treated with sodium arsenite (As[III]) at 0, 0.25, 0.5, 1.5, or 10 μM for 1 h.
Figure 4. Total oxidant formation in BAE cells treated with sodium arsenite (As[III]) at 0, 0.25, 0.5, 1.5, or 10 µM for 1 h. This assay is based on the oxidation of hydroethidine (40 µM) to its fluorescent product, ethidium (see Methods). *Significantly different from control, p<0.01. Error bars represent the mean +/- SD for n = 3 experiments.
Figure 5. Peroxynitrite formation in BAE cells treated with sodium arsenite (As[III]) at 0, 0.25, 0.5, 1,5, or 10 μM for 1 h.

A.

B.

C.
Figure 5. Peroxynitrite formation in BAE cells treated with sodium arsenite (As[III]) at 0, 0.25, 0.5, 1, 5, or 10 μM for 1 h. Same assay as in Fig. 3, except that 25 U/mL each of SOD and CAT was added with the hydroethidine. (A) No inhibitor was added to the BAE cells prior to sodium arsenite treatment. (B) General NOS inhibitor, L-NAME (30 μM), was added to the BAE cells 1 h prior to sodium arsenite treatment. (C) NAD(P)H oxidase inhibitor, DPI (10 μM), was added to the BAE cells with sodium arsenite treatment. * Significantly different from control, p<0.05. **Significantly different from control, p<0.01. Error bars represent the mean +/- SD for n = 3 experiments.
ELISA was used to measure the formation of COX-derived PGE$_2$ in endothelial cells following exposure to arsenic. Cells treated with 10 and 20 μM arsenite for 45 min showed a 14% and 80% increase, respectively, in the formation of PGE$_2$ compared to control cells (Figure 9). These data corroborate the COX-2 protein expression findings and suggest that arsenic may contribute to atherogenesis through the COX-2 pathway.

**Discussion**

Atherosclerosis is a chronic inflammatory disease exacerbated by oxidative stress. Under conditions of stress, oxidation of LDL and subsequent phagocytosis by macrophages leads to the formation of foam cells. These "fatty" macrophage cells die, leaving behind a growing mass of extracellular debris (Lusis, 2000). This will eventually cause arterial occlusion and disease. An increase in oxidative stress in response to arsenic exposure may contribute to atherosclerosis in various ways. Cytokines (Fong *et al.*, 2000) and oxidative stimuli (Fang *et al.*, 2000) can result in the upregulation of the inducible COX-2 enzyme and contribute to the inflammatory status of atherosclerosis. The formation of reactive nitrogen species seems to play a particularly important role, either by direct oxidation of LDL (Keaney, 2000), induction of inflammatory mediators (Gerszten *et al.*, 2000), alterations of protein conformation via tyrosine nitration (Gow *et al.*, 1996) or possibly through signaling pathways responsive to molecules such as peroxynitrite (Levonen *et al.*, 2001). In the case of arsenic exposure, formation of peroxynitrite as well as an increase in the inflammatory response could be contributing factors in the aggravation of arsenic-related cardiovascular disease. In the present study, a rise in peroxynitrite production was observed in arsenic-treated endothelial cells along
with an increase in expression of COX-2. This was accompanied by elevated levels of a PGE$_2$, a key eicosanoid product of COX-2 and detection of 3NY residues in COX-2.

The induction of COX-2 in response to arsenic exposure is a dynamic cellular event that is time dependent. The present results show that COX-2 protein expression increases within the first 30 min then declines over the next 24 h with continuous arsenic exposure. A significant increase in the production of PGE$_2$ at 45 min following exposure to arsenic was also observed. After 48 hours of exposure there is a second peak in the COX-2 protein. The time course of COX-2 induction may be important in both the onset and the resolution of inflammation. During an inflammatory response, induction of COX-2 converts the PGD$_2$ dominated prostaglandin synthesis seen in quiescent cells to the proinflammatory mediator PGE$_2$ (Colville-Nash and Gilroy, 2000). After the first 30 min, this event is followed by a return to PGD$_2$ synthesis and induction of anti-inflammatory stress proteins such as heat shock protein 70 (HSP70) and heme oxygenase-1 (HO-1). In agreement with the current data, the authors of this earlier report also observed a second peak in COX-2 expression at 48 h following injection of an irritant in mice (Colville-Nash and Gilroy, 2000). A recent study by Tsai et al. (2002) reported increased COX-2 expression and PGE$_2$ generation in human umbilical vein endothelial cells (HUVECs) following exposure to sodium arsenite. They showed that PGE$_2$ was elevated twofold in HUVECs after 15 h of sodium arsenite treatment (Tsai et al. 2002), suggesting a proinflammatory state at this time point.

The present results show that increased production of peroxynitrite occurs within 30 min following arsenic application, correlating to both the initial COX-2 induction and the increase in prostaglandin synthesis. The involvement of peroxynitrite in arsenic-
Figure 6. COX-2 Western blots of BAE cells treated with sodium arsenite.

A.

B.
Figure 6. COX-2 Western blots of BAE cells treated with sodium arsenite. Cell lysates were run on a polyacrylamide gel and transferred to a PVDF membrane and probed with a polyclonal anti-COX-2 antibody. Sample loading was controlled by probing the same membrane with an anti-actin antibody. Each blot is representative of two independent experiments. COX-2 positive control was from RAW 264.7 murine macrophages. (A) Cells were treated with 0, 5, 10 or 20 μM sodium arsenite (As) for 30 min. (B) Ratio between densitometry of COX-2 and actin bands from blot in (A).
Figure 7. COX-2 Western blots of BAE cells treated with sodium arsenite

A.

B.
Figure 7. COX-2 Western blots of BAE cells treated with sodium arsenite. Cell lysates were run on a polyacrylamide gel and transferred to a PVDF membrane and probed with a polyclonal anti-COX-2 antibody. Sample loading was controlled by probing the same membrane with an anti-actin antibody. Each blot is representative of two independent experiments. COX-2 positive control was from RAW 264.7 murine macrophages. (A) Cells were treated with 0, 5, 10 or 20 μM sodium arsenite (As) for 30 min. (B) Ratio between densitometry of COX-2 and actin bands from blot in (A).
Figure 8. Nitrotyrosine Western blot from COX-2 immunoprecipitations of BAE cells treated with sodium arsenite (As) at 0, 5, 10 or 20 μM for 30 min.

A.  

B.  

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Figure 8. Nitrotyrosine Western blot from COX-2 immunoprecipitations of BAE cells treated with sodium arsenite (As) at 0, 5, 10 or 20 μM for 30 min. Cell lysates were immunoprecipitated with a COX-2 polyclonal antibody and the samples loaded on a polyacrylamide gel, transferred to a PVDF membrane, then probed with a monoclonal anti-nitrotyrosine antibody. COX-2 positive control was from RAW 264.7 murine macrophages and was not nitrated. Bovine serum albumin (BSA) positive control was nitrated. Representative blot from two independent experiments. (B) Densitometry on immunoblot in (A).
Figure 9. PGE$_2$ formation in BAE cells exposed to sodium arsenite

![Graph showing PGE$_2$ formation in BAE cells exposed to sodium arsenite.](image-url)

- Control
- 10 $\mu$M As(III)
- 20 $\mu$M As(III)
Figure 9. PGE\(_2\) formation in BAE cells exposed to sodium arsenite (As) at 0, 10 or 20 μM for 45 min. Data were generated using a PGE\(_2\) ELISA. Results are from two independent experiments run on two different ELISA plates. Treatment groups marked with an asterisk (*) are significantly different from the controls (p<0.05). Error bars represent the mean +/- standard deviation for n=4 samples.
induced toxicity is supported by the work of Gurr et al. (1998). They found that increases in intracellular oxidant levels due to arsenic were inhibited by NO synthase inhibitors, but not by catalase inhibitors. They also reported that formation of arsenic-induced micronuclei was reduced by NO synthase inhibitors, superoxide dismutase and uric acid (Gurr et al., 1998). Liu and Jan (2000) demonstrated increases in nitrite levels and DNA strand breaks in BAE cells, again only at arsenic concentrations of 5 μM or more.

Peroxynitrite has been shown to be a substrate for the peroxidase activity of COX-2, and this leads to activation of the enzyme (Landino et al., 1996). In the current study, we showed that arsenic exposure and the subsequent formation of peroxynitrite leads to increased nitration of COX-2 tyrosine residues. Although peroxynitrite is an important nitrating compound, it is not the only reactive nitrogen species capable of tyrosine nitration (Ischiropoulos, 1998). In fact, there is evidence that COX-2 can be nitrated by a pathway that begins with nitric oxide reacting with a tyrosyl radical to form nitrosotyrosine. This intermediate can be oxidized to nitrotyrosine (Ischiropoulos, 1998). Therefore, we cannot rule out the possibility that nitrating species other than peroxynitrite may be involved. Nitration of tyrosine residues within the COX-2 protein has been shown to decrease enzyme activity (Boulos et al., 2000), although the net effect of increased substrate and decreased activity is unknown.

It has been suggested that reactive nitrogen species may function as signal transduction molecules (Levonen et al., 2001). In contrast, peroxynitrite may cause protein nitration that prevents phosphorylation and could target the proteins for degradative pathways (Gow et al., 1996). Perhaps after an acute arsenic exposure, the oxidative insult to the cell activates an inflammatory response by inducing the COX-2
enzyme and providing it with an initial substrate (e.g. peroxynitrite). This response could then be “turned off” by nitration of the enzyme, decreasing its activity and possibly making it more susceptible to degradation. This may then allow the cell to upregulate the necessary anti-inflammatory mediators needed for resolution, such as HSP70 and HO-1.

In chronic arsenic exposure, our data showing a second increase in COX-2 protein at 48 h may be more relevant, but more work is clearly needed in this area.

COX-2 may also play a role in the upregulation of renin, adversely affecting blood pressure homeostasis (Lipsky et al., 2000) and exacerbating cardiovascular disease. Furthermore, COX-2 overexpressing cells demonstrate an increase in endothelial migration (Tsujii et al., 1998). This may contribute to atherosclerosis by enhancing angiogenesis thereby providing an entry site for inflammatory cells (Lusis, 2000) and exacerbating plaque formation.

Our data showing an increase in reactive nitrogen species and corresponding increase in the inflammatory response suggest one way that arsenic may contribute to atherosclerosis. However, these phenomena may also contribute to other forms of cardiovascular disease. Cheng et al. (1999) demonstrated that myocardial dysfunction induced by the inflammatory mediator, IL-1β, was significantly diminished by both an inducible nitric oxide synthase inhibitor and a novel inhibitor of superoxide anion production. This suggests that peroxynitrite may not only contribute to atherosclerosis, but to cytokine-induced myocardial dysfunction as well. Furthermore, administration of the superoxide anion inhibitor abolished formation of nitrotyrosine residues within the myocardium (Cheng et al., 1999). In addition, it has been shown that there is an inverse relationship between myocardial nitrotyrosine concentrations and left ventricular ejection
fractions in dogs (Oyama et al., 1998). Treatment of cardiac myocytes with peroxynitrite results in an influx of extracellular calcium ions with a concomitant decline in cellular activity. The authors suggested that this decreased sensitivity to calcium was due to nitration of contractile proteins (Ishida et al., 1996).

Arsenic exposure leading to generation of reactive nitrogen species along with an increase in inflammatory mediators may provide an environment conducive to the onset and progression of atherosclerosis. Peroxynitrite's impact on cell signaling, protein function, and modifications should be investigated further. Also, the impact of arsenic exposure on other inflammatory mediators that may influence the onset and progression of atherosclerosis needs to be addressed.
References


Van der Vliet A, Smith D, O'Neill CA, Kaur H, Darley-Usmar V, Cross CE, Halliwell

CHAPTER TWO

Exacerbation of Atherosclerotic Plaque and Increased Nitrotyrosine Formation in Response to Arsenic Exposure by Drinking Water

Abstract

Epidemiological studies have linked arsenic to skin and bladder cancer and more recently to cardiovascular disease, although the mechanisms are primarily unknown. This is the first laboratory controlled study confirming a connection between arsenic intake and cardiovascular disease in mice. The results revealed a gross increase in the size of the atherosclerotic plaques occluding the innominate artery of ApoE^{-/-}/LDLr^{-/-} mice treated with 10 ppm (133.3 μM) arsenic in drinking water for 18 weeks. Immunohistochemistry showed nitrotyrosine formation was significantly higher within the atherosclerotic plaque of arsenic-treated mice. We conclude that amplified synthesis of reactive species, such as peroxynitrite, results in increased protein nitration in response to arsenic exposure and contributes to the pathology of arsenic-induced cardiovascular disease.

Introduction

Epidemiological studies have linked arsenic to cardiovascular disease throughout the world. Regions of Taiwan, Bangladesh, Argentina and others have exceedingly high concentrations of arsenic in the groundwater. These areas suffer from a high incidence of cancer and severe peripheral and cardiovascular disease. In a long-term study in Taiwan, there was a significant association between cumulative arsenic consumption through artesian well water and prevalence of carotid atherosclerosis (Wang et al., 2002). The data were consistent regardless of the duration of exposure, the average arsenic
concentration within the water, or cumulative arsenic exposure and remained significant after adjusting for known risk factors in the development of atherosclerosis (Wang et al., 2002). In the United States, an investigation on the relationship between population-weighted mean arsenic concentration in public drinking water and death from cardiovascular diseases (CVD) in 30 U.S. counties showed an increase in the mortality ratio for various forms of CVD (Engel and Smith, 1994). These included diseases of the arteries, arterioles, and capillaries as well as an elevation in the incidence of arteriosclerosis and aortic aneurysm with a mean arsenic concentration greater than 20 parts per billion (ppb).

Atherosclerosis is a disease characterized by chronic inflammation and arterial occlusion. Atherogenesis is a multifactorial process, making elucidation of a single mechanism for exacerbation of the disease difficult. In humans, primary risk factors include genetics, obesity, cigarette smoking, hypertension, diabetes mellitus, and increased serum cholesterol. However, the initiating event appears to be activation of the endothelial lining within the arteries leading to a cascade of changes in cell signaling and increases in oxidative stress and inflammation (Keaney, 2000).

Changes in the oxidative and inflammatory state of a system are not mutually exclusive. There is increasing evidence linking stress due to the formation of reactive oxygen and nitrogen to inflammation. In fact, peroxynitrite, an extremely reactive isomer of the nitrate anion has been linked to atherosclerosis through the detection of nitrotyrosine (3NY) residues within the plaque (Beckmann et al., 1994; Leeuwenburgh et al., 1997). In biological samples, 3NY has been used as a marker for the presence of peroxynitrite (Crow and Ischiropoulos, 1996). Recently, a link between the formation of
peroxynitrite and induction of cyclooxygenase-2 (COX-2) in rheumatoid arthritis was reported (Migita et al., 2002), and we have shown an increase in both peroxynitrite synthesis and COX-2 in endothelial cells exposed to arsenic (Bunderson et al., 2002). Oxidants have been shown to cause changes in the activation state of transcription factors and other proteins by altering phosphorylation patterns or introducing conformation changes through disulfide bonds and intramolecular disulfide bridges (Herrlich and Bohmer, 2000). Changes in these important cellular processes can lead to activation of the endothelial lining of the vasculature and recruitment of inflammatory mediators resulting in initiation and eventually, exacerbation of atherosclerosis (Libby, 2002).

While there is clear evidence linking arsenic to increases in CVD, the mechanisms behind this phenomenon are primarily unknown. Considering the tremendous impact of CVD to this country and the recently reduced standard for arsenic in the drinking water by the EPA, studies determining these mechanisms are needed. This is the first laboratory controlled study demonstrating a direct link between arsenic exposure in the drinking water and exacerbation of atherosclerosis.

Materials and Methods

Mice and dissection procedures. ApoE<sup>−/−</sup>/LDLr<sup>−/−</sup> mice were obtained from Jackson Laboratories, maintained in specific pathogen free conditions according to IACUC protocols, and treated with 133 μmol/L (10 ppm) sodium arsenite via the drinking water. Following 18 weeks of treatment, the mice were formaldehyde perfused (1%/PBS), then the heart and innominate artery were removed, fixed, sectioned and processed for Movats staining similar to published protocols (Rosenfeld et al., 2000).
Sections from each mouse were examined under a Nikon E800 microscope, the area of the lumen (L) and plaque (P) were measured using NIH ImageJ, then the L/P ratios from the water only control and As treated group were statistically compared using an unpaired t-test.

**Immunohistochemistry.** Sections (described above) from As treated and control mice were deparaffinized, rinsed in tris buffer (TB, pH= 8.0) blocked for endogenous peroxidase with 3% H$_2$O$_2$ solution/TB then blocked with 5% BSA/ 4% goat serum/TB for 30 min. Anti-3NY polyclonal serum (Upstate Biotechnology) in blocking solution was applied at 5 μg/mL overnight at 4°C. The next day, the sections were rinsed (3 X 5 min. TB), then goat anti-rabbit biotin conjugated secondary antibody (Vector Laboratories) was applied at 1:400 for 1 h. The samples were rinsed (3 X 5 min. TB), developed with Vectastain Elite ABC kit (Vector Laboratories), dehydrated and coverslipped. Sections were examined with a Nikon E800 microscope, photographed and analyzed with ImageJ for calculation of the mean grey and total plaque area.

**Results and Discussion**

This study provides laboratory-controlled evidence to support the epidemiological studies linking arsenic to CVD. We used the highly atherogenic ApoE$^{−/−}$/LDLr$^{−/−}$ mouse model. These mice develop severe occlusions early in life while fed a normal chow diet. It is well known that the ApoE lipoprotein is important for the cardioprotective effects of high-density lipoproteins (HDL) and is also a ligand for the low-density lipoprotein (LDL) receptor and vital for removal of cholesterol from the blood (Strittmatter and Bova Hill, 2002). The innominate artery has been shown to have the greatest similarities with
Figure 10. Staining and quantification of ApoE<sup>−/−</sup>/LDL<sub>R<sup>−/−</sup></sub> innominate artery

A.

B.

C.

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Figure 10. (A) Innominate artery stained with movat's from ApoE\textsuperscript{+/−}/LDLr\textsuperscript{−/−} mouse given ddH\textsubscript{2}O for 18 weeks. (B) Innominate artery stained with movat’s from ApoE\textsuperscript{+/−}/LDLr\textsuperscript{−/−} mouse given 10 ppm (133.3 μM) sodium arsenite in ddH\textsubscript{2}O for 18 weeks. (C) Changes in occlusion between control (n=9) and treated (n=7) mice measured as lumen area/internal elastic lamina area. P=.0025
the human form of atherosclerosis in an ApoE'7 mouse model (Rosenfeld et al., 2000). We observed a significant increase in the atherosclerotic occlusion of the innominate artery of apoE'7/LDLr'7 mice treated with 10 ppm (133.3 μM) arsenic for 18 weeks (Figure 10a-c). Changes in intima media thickness of the coronary artery in humans is correlated with increases in blood pressure, smoking, and high LDL cholesterol—all risk factors in the development of heart disease (Sinha et al., 2002).

Arsenic is a ubiquitous contaminant within our environment (Abernathy et al., 1999) and also merits consideration as a risk-factor for atherosclerosis. Arsenite is considered to be the most toxic form of inorganic arsenic (Buchet and Lison, 2000). The mechanisms by which arsenic contributes to atherosclerosis are not fully understood, however, our findings suggest that generation of reactive species plays a role. Arsenite in particular has been shown to activate an NAD(P)H oxidase found on the plasma membrane of vascular endothelial cells (Smith et al., 2001) and vascular smooth muscle cells (Lynn et al., 2000). Activation of these membrane-bound oxidases leads to increased production of reactive oxygen species, particularly superoxide anion (O$_2^{-}$). Superoxide anion combines with nitric oxide (NO) to form an extremely reactive isomer of the nitrate anion, peroxynitrite. There is substantial evidence suggesting that peroxynitrite is responsible for the nitration of both free and protein-bound tyrosine residues (Beckman and Koppenol, 1996). This has implications for disruption of cell-signaling cascades as well as basic cellular functions such as inadequate folding of secondary and tertiary protein conformation patterns. In addition, peroxynitrite has been shown to oxidize LDL molecules, a known component of atherogenesis (Keaney, 2000).
3NY residues in biological samples have been used as a marker for the presence of peroxynitrite (Crow and Ischiropoulos, 1996). In fact, atherosclerosis has been characterized by a general, and in some cases marked increase in the occurrence of 3NY residues (Beckmann et al., 1994; Leeuwenburgh et al., 1997). Our laboratory previously demonstrated an increase in the synthesis of peroxynitrite upon exposure to arsenic in vascular endothelial cells (Bunderson et al., 2002). We confirm this finding in the present study by demonstrating that the biomarker of peroxynitrite, 3NY, is found in greater abundance within the plaque of arsenic-treated mice than controls (Figure 11 a-d). This could provide a stimulus for endothelial cell dysfunction. Damage to the endothelial lining of the vasculature is believed to be a primary component in the pathophysiology of atherosclerosis (Callow, 2002). Vascular endothelial cells normally serve as a barrier to regulate the movement of fluid and various proteins from the blood to the interstitium (Yoshikawa, 2002). However, disruption of the normal cellular signaling pathways or mechanical injury to the artery results in a cascade of events leading to the early formation of plaque (Yoshikawa, 2002). In its simplest form, this increase in reactive species would result in modification of lipoproteins and subsequent uptake by the scavenger receptor on the macrophage leading to foam cell formation. Perhaps more importantly, an increase in the oxidative stress within the endothelial lining may result in an upregulation of inflammatory mediators such as vascular cell adhesion molecule-1 and subsequent monocyte recruitment to the injured area (Libby, 2002).

Arsenic has been shown to increase the risk of CVD, first through epidemiological studies, and now through a laboratory-controlled mouse model. This exacerbation of a highly complex disease can partially be explained by arsenic's ability
Figure 11. 3NY Immunohistochemistry of ApoE⁻/⁻/LDLr⁻/⁻ innominate artery

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Figure 11. (A) Innominate artery of ApoE<sup>−/−</sup>/LDLr<sup>−/−</sup> mouse given ddH<sub>2</sub>O for 18 weeks and stained for 3NY using immunohistochemical techniques. *Top*: 3,3′-Diaminobenzidine (DAB) staining *Bottom*: DAB stained sample converted to grayscale and a density threshold set (minimum = 40 maximum = 175). (B) Innominate artery of ApoE<sup>−/−</sup>/LDLr<sup>−/−</sup> mouse given ddH<sub>2</sub>O containing 10 ppm (133.3 μM) sodium arsenic for 18 weeks and immunostained for 3NY. *Top*: DAB staining *Bottom*: DAB stained sample converted as described above. (C) Innominate artery of a control mouse stained with antibody preincubated with a 3NY solution as a negative control. *Top*: DAB staining *Bottom*: DAB stained sample converted as described above. (D) Changes in density of 3NY staining between control (n=9) and treated (n=7) mice measured as mean gray/plaque area. P=.046.
to increase synthesis of reactive oxygen and nitrogen species resulting in oxidative stress and inflammation, hallmarks of atherosclerosis.
References


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CHAPTER THREE

Induction of 5-lipoxygenase and changes in the important inflammatory mediators, leukotrienes and prostanoids, are observed in arsenic-induced atherosclerosis.

Abstract

Numerous epidemiological studies have linked arsenic in drinking water to cancer and more recently to cardiovascular disease. Although an association between arsenic exposure and atherosclerosis has been established, the mechanisms are primarily unknown. Atherosclerosis is an inflammatory disease. Therefore, any agent that enhances vasculature inflammation may facilitate atherosclerosis. Previously, our laboratory established an induction of the cyclooxygenase-2 (COX-2) protein in bovine aortic endothelial (BAE) cells exposed to arsenic (Bunderson et al., 2002). Also, we have shown that arsenic exposure enhances atherosclerotic occlusions in the innominate artery of ApoE<sup>+</sup>/LDL<sup>−/−</sup> mice (see Chapter 2). Therefore, the goal of this study was to determine the effect of arsenic exposure on a second inflammatory mediator, 5-lipoxygenase (5-Lo), thought to be involved in the atherosclerotic process. In addition, expression of both COX-2 and 5-Lo in the atherosclerotic plaques of the ApoE<sup>+</sup>/LDL<sup>−/−</sup> atherogenic mouse model was examined as well as leukotriene and prostaglandin synthesis after exposure to arsenic. Using Western immunoblots, we show that 5-lipoxygenase is upregulated in a time-dependent manner in response to arsenic exposure in bovine aortic endothelial (BAE) cells, but no changes in the expression of 5-Lo or COX-2 within the atherosclerotic plaque were observed between control and treated mice. There was an increase in LTE<sub>4</sub> but not LTB<sub>4</sub> synthesis in response to arsenic
exposure both in vitro and in the serum of arsenic-treated mice. In addition, two products of COX-2 were measured in vivo after arsenic treatment, prostaglandin E\textsubscript{2} (PGE\textsubscript{2}) and prostacyclin (PGI\textsubscript{2}). There were no changes in the proinflammatory PGE\textsubscript{2} but a significant increase in PGI\textsubscript{2}. This study demonstrates arsenic’s ability to influence the dynamic nature of inflammatory pathways that have been associated with atherogenesis.

Introduction

Cardiovascular disease is the leading cause of morbidity and mortality in the Western World. Principle among cardiovascular diseases is atherosclerosis, which often results in the manifestation of heart attack and stroke. Epidemiological studies have linked arsenic to cardiovascular disease throughout the world, including regions of Taiwan, Bangladesh, Argentina, and others with exceedingly high concentrations of arsenic in the groundwater. In a long-term study in Taiwan there was a significant association between cumulative arsenic consumption through artesian well water and the prevalence of carotid atherosclerosis (Wang et al., 2002). In the United States, an investigation on the relationship between population-weighted mean arsenic concentration in public drinking water and death from cardiovascular diseases (CVD) in 30 U.S. counties showed an increase in the mortality ratio for various forms of CVD (Burleigh et al., 2002). These included diseases of the arteries, arterioles, and capillaries as well as an elevation in the incidence of arteriosclerosis and aortic aneurysm with a mean arsenic concentration greater than 20 parts per billion (ppb). The mechanisms behind arsenic-induced cardiovascular disease are primarily unknown, and our laboratory only recently provided the first direct laboratory-controlled evidence linking arsenic to
CVD (See chapter two). Atherosclerosis is a disease characterized by chronic inflammation and arterial occlusion and is a multifactorial process, making elucidation of a single mechanism for exacerbation of the disease difficult. However, an initiating event appears to be activation of the endothelial lining leading to a cascade of changes in cell signaling and increases in oxidative stress and inflammation (Keaney, 2000).

Our laboratory previously showed that arsenic induces the inflammatory mediator, COX-2 in aortic endothelial cells (Bunderson et al., 2002) and at high concentrations of arsenic (20 μM), increases in the COX-2 product, prostaglandin E₂ (PGE₂), were also observed. Recently, another inflammatory pathway closely related to the cyclooxygenase system has been recognized as an important component of atherogenesis. Mehrabian et al., (2002), identified a locus on mouse chromosome 6 that confers substantial resistance to atherogenesis as the 5-lipoxygenase (5-Lo) gene. A dramatic decrease (>26-fold) in the development of aortic lesions was observed in 5-Lo knockout mice. The enzyme 5-Lo metabolizes arachidonic acid to leukotriene A₄ (LTA₄) which is then converted to leukotriene B₄ (LTB₄) and the cysteinyl-leukotrienes (C₄, D₄, and E₄), all of which are known to have proinflammatory effects. Furthermore, Allen et al., (1998), demonstrated an LTC₄ and LTD₄-dependent state of hyperactivity in atherosclerotic coronary arteries suggesting an increase in the 5-Lo enzyme.

In this study we demonstrate the importance of 5-Lo as an inflammatory mediator in the pathology of arsenic-exacerbated atherosclerosis.
Materials and Methods

Mice. ApoE^+/LDLr^-/- mice were obtained from Jackson Laboratories, maintained in specific pathogen free conditions according to IACUC protocols, and treated with 133 μmol/L (10 ppm) sodium arsenite via the drinking water. Following 18 weeks of treatment, whole blood was obtained, allowed to coagulate and centrifuged for 10 min at 10,000 rpm in a TOMY TX-160 centrifuge. The serum was collected and maintained at -80°C until used for ELISA analysis.

Dissection procedures. Following 18 weeks of treatment, dissection of the innominate arteries of the ApoE^+/LDLr^-/- mice was conducted according to published procedures (Rosenfeld et al., 2000). Briefly, mice were formaldehyde perfused (1%/PBS), the heart and innominate artery removed, fixed, sectioned and stained using immunohistochemical techniques.

Immunohistochemistry. Cross-sections of the innominate arteries (described above) from arsenic treated and control mice were deparaffinized, rinsed in tris buffer (TB, pH= 8.0) blocked for endogenous peroxidase with 3% H2O2 solution/TB then blocked with 5% BSA/4% goat serum/TB for 30 min. Anti-COX-2 or 5-Lo polyclonal serum (Cayman Chemicals) in blocking solution was applied at 5 μg/mL overnight at 4°C. The next day, the sections were rinsed (3 x 5 min. TB), and goat anti-rabbit biotin conjugated secondary antibody (Vector Laboratories) applied at 1:400 for 1 h. The samples were rinsed (3 x 5 min. TB), developed with Vectastain Elite ABC kit (Vector Laboratories), dehydrated and coverslipped. Sections were examined with a Nikon E800 microscope, photographed and analyzed with the NIH program, ImageJ. DAB positive
staining was converted to grey scale for calculation of the mean grey and total plaque area. Statistics were conducted using an unpaired $t$-test $P<0.05$.

**Cells.** Bovine aortic endothelial cells (BAE), originally a gift from Dr. Steve Schwartz of the University of Washington, were provided by Dr. J. Douglas Coffin of The University of Montana Department of Pharmaceutical Sciences. Cell culture components were obtained from Life Technologies, Inc. (Rockville, MD) unless otherwise noted. BAE cells were grown in Dulbecco's Modified Eagle Medium (DMEM) with penicillin/streptomycin and L-glutamine, and supplemented with 15% fetal bovine serum (HyClone Laboratories, Inc., Logan, UT). The cells were incubated at 37°C under a humidified atmosphere containing 5% CO$_2$. BAE cells were used between passages #2-8.

**Western Blot.** BAE cells were grown to approximately 90% confluency and treated with 10 $\mu$M sodium arsenite for 0.5, 1, 24 and 48h. Cells were harvested with 2X Laemmli buffer, homogenized with an 18 and 25 gauge needle and boiled for 5 min. Protein concentrations were determined by a Bradford assay (Biorad, Hercules, CA), and samples (10 $\mu$g) were loaded onto a 10% SDS gel. After protein separation, the samples were transferred to a polyvinylidenedifluoride (PVDF) membrane and probed for 5-lipoxygenase (5-Lo) protein using a rabbit polyclonal 5-Lo antibody (Cayman Chemicals) and an anti-rabbit secondary antibody conjugated with horseradish peroxidase (Vector Laboratories). 5-Lo was visualized by enhanced chemiluminescence (ECL; Amersham, Buckinghamshire, England).

**ELISA.** PGI$_2$ was analyzed indirectly by measuring 6-keto prostaglandin F$_{1\alpha}$ using an immunoassay kit purchased from R&D Systems (Minneapolis, Minnesota) according
to manufacturers instructions. Serum PGE\(_2\), LTE\(_4\), and LTB\(_4\) was analyzed using EIA immunoassay kits purchased from Cayman Chemicals (Ann Arbor, Michigan) according to manufacturers instructions. Arsenic treated BAE cells were analyzed for LTE\(_4\) concentrations with or without the addition of the 5-lipoxygenase inhibitor AA861 (76.6 \(\mu\)M), administered concomitantly with arsenic for 48h. Statistics were conducted using an unpaired \(t\)-test \(P<.05\) for serum samples and ANOVA coupled with Dunn's multiple comparison test for BAE samples \(P<.05\).

**Results**

In addition to exacerbating atherogenesis by increasing oxidative stress (see Chapter 2), our laboratory has shown that arsenic induces the inflammatory mediator, COX-2 in aortic endothelial cells (Bunderson et al., 2002). At high concentrations of arsenic (20 \(\mu\)M), increases in the COX-2 product, prostaglandin E\(_2\) (PGE\(_2\)), were also observed. However, we were unable to detect an increase in the presence of COX-2 in the arteries of our treated mice over the controls (Figure 12). We did observe an increase in prostacyclin (PGI\(_2\)) (Figure 13), but not PGE\(_2\) (Figure 14) in the serum of the arsenic treated mice. Furthermore, we observed an increase in 5-Lo protein in bovine aortic endothelial cells treated with 10 \(\mu\)M arsenic over a period of 48 hours (Figure 15 a and b). In addition, we observed a significant increase in Leukotriene E\(_4\) (LTE\(_4\)) synthesis in these cells after exposure to 10 \(\mu\)M arsenic for 48 h (Figure 15c). We were not able to detect an increase in 5-Lo within the innominate artery of our treated mice over controls (Figure 16), however, we were able to measure a significant increase in LTE\(_4\) (Figure 17), but not LTB\(_4\) (Figure 18) in the serum of these animals.
Figure 12. COX-2 Immunohistochemistry of ApoE<sup>−/−</sup>/LDL<sub>r</sub><sup>−/−</sup> innominate artery

A.  

B.  

C.  

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Figure 12. (A) Innominate artery of ApoE^{−/−}/LDLr^{−/−} mouse given ddH₂O for 18 weeks and stained for COX-2 using DAB staining; sample converted to grayscale and a density threshold set (minimum=25 maximum=150). (B) Innominate artery of ApoE^{−/−}/LDLr^{−/−} mouse given ddH₂O containing 10 ppm (133.3 μM) sodium arsenite for 18 weeks and stained for COX-2 using DAB staining; sample converted as described above. (C) Changes in density of COX-2 staining between control and treated mice measured as mean grey/plaque area. Data not significant.
Figure 13. Prostacyclin (PGI₂) in the serum of ApoE⁻/⁻/LDLr⁻/⁻ mice.
Figure 13. Prostacyclin (PGI₂) in the serum of ApoE<sup>−/−</sup>/LDLr<sup>−/−</sup> mice given either ddH₂O or ddH₂O containing 10 ppm (133.3 μM) sodium arsenite/ddH₂O for 18 weeks. PGI₂ is estimated from the indirect measurement of the stable metabolite 6-keto prostaglandin F₁α in the mouse serum using ELISA techniques. P<0.01
Figure 14. BicycloProstaglandin E$_2$ (PGE$_2$) measured by ELISA technique in the serum of ApoE$^{+/-}$/LDLr$^{-/-}$ mice.
Figure 14. Bicycloprostaglandin E₂ (PGE₂) measured by ELISA technique in the serum of ApoE−/−/LDLr−/− mice given either ddH₂O or 10 ppm (133.3 μM) sodium arsenite/ddH₂O for 18 weeks. Data not significant.
Figure 15. BAE cells treated with 10 μM sodium arsenite

A.

B.

C.
Figure 15. (A) Western blot analysis of 5-lipoxygenase in bovine aortic endothelial cells treated with 10 μM sodium arsenite at various time points, representative of two separate experiments. (B) Ratio between densitometry of 5-Lo and actin bands from blot in “A” (C) Leukotriene E₄ measured by ELISA technique in bovine aortic endothelial cells with a range of sodium arsenite concentrations with or without AA-861, a 5-lipoxygenase inhibitor, for 48 h. P<.05
Figure 16. 5-Lo Immunohistochemistry of ApoE<sup>−/−</sup>/LDLr<sup>−/−</sup> innominate artery

A.  

B.  

C.

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Figure 16. (A) Innominate artery of ApoE\(^{+/−}\)/LDLr\(^{-}\) mouse given ddH\(_{2}\)O for 18 weeks and stained for 5-Lo using DAB staining; sample converted to grayscale and a density threshold set (minimum=25 maximum=150). (B) Innominate artery of ApoE\(^{+/−}\)/LDLr\(^{-}\) mouse given ddH\(_{2}\)O containing 10 ppm (133.3 \(\mu\)M) sodium arsenite for 18 weeks and stained for 5-Lo using DAB staining; sample converted as described above (C) Changes in density of 5-Lo staining between control and treated mice measured as mean grey/plaque area. Data not significant.
Figure 17. Leukotriene E$_4$ measured by ELISA technique in the serum of ApoE$^{+/-}$ /LDLr$^{+/-}$ mice.
Figure 17. Leukotriene E₄ measured by ELISA technique in the serum of ApoE⁺/⁻/LDLr⁺ mice given either ddH₂O or 10 ppm (133.3 μM) sodium arsenite/ddH₂O for 18 weeks. P<0.05
Figure 18. Leukotriene B₄ measured by ELISA technique in the serum of ApoE⁺/⁻/LDLr⁻/⁻ mice.
Figure 18. Leukotriene B₄ measured by ELISA technique in the serum of ApoE⁺/-/LDLr⁻/⁻ mice given either ddH₂O or 10 ppm (133.3 μM) sodium arsenite/ddH₂O for 18 weeks. Data not significant.
Discussion

We previously described the first laboratory-controlled evidence to support the epidemiological studies linking arsenic to CVD. In the ApoE\(^{-/-}\)/LDLr\(^{-/-}\) mouse model, we observed a significant increase in the degree of occlusion in the innominate artery (see chapter two). In the current study we used two models in order to examine possible mechanisms for arsenic's role in contributing to cardiovascular disease. First, we used the BAE cell line in order to establish pathways involving the endothelium that would contribute to atherosclerosis. Dysfunction of the endothelial lining of the vasculature is believed to be a primary component in the pathophysiology of atherosclerosis (Callow, 2002). Vascular endothelial cells normally serve as a barrier to regulate the movement of fluid and various proteins from the blood to the interstitium (Yoshikawa, 2002). However, disruption of the normal cellular signaling pathways or mechanical injury to the artery results in a cascade of events leading to the early formation of plaque (Yoshikawa, 2002). Our laboratory previously showed an increase in the reactive nitrogen species, peroxynitrite, in response to arsenic exposure (Bunderson et al., 2002). This could provide just such a stimulus for endothelial dysfunction. In its simplest form, this increase in reactive species would result in modification of lipoproteins and subsequent uptake by the scavenger receptor on the macrophage leading to foam cell formation. Perhaps more importantly, an increase in the oxidative stress within the endothelial lining may result in an upregulation of inflammatory mediators such as vascular cell adhesion molecule-1 (VCAM-1) and subsequent monocyte recruitment to the injured area (reviewed in Libby, 2002).
In addition to the endothelial cells, we used the highly atherogenic ApoE<sup>+</sup>/LDLr<sup>+</sup> mouse model. These mice develop severe occlusions early in life while fed a normal chow diet. It is well known that the ApoE lipoprotein is an important component of the cardioprotective effects of high-density lipoproteins (HDL) and is also a ligand for the low-density lipoprotein (LDL) receptor and vital for removal of cholesterol from the blood (Strittmatter and Bova Hill, 2002). Exposure to arsenic significantly increases the degree of occlusion within the innominate artery (See chapter two).

The mechanisms underlying arsenic's ability to exacerbate atherosclerosis are mostly unknown. There are many potential steps in the development of atherosclerosis which may be impacted by exposure to arsenic. Current theories on the development of atherosclerosis underscore the pathology as a "process" that is first characterized by endothelial cell dysfunction, followed by the formation of foam cells due to uptake of oxidized lipoproteins by macrophages, proliferation of vascular smooth muscle cells, and exacerbation by the inflammatory process which may eventually lead to rupture of "vulnerable" plaque (Frohlich and Lear, 2002). Specifically, inflammatory conditions within the vasculature (such as ischemia/reperfusion) have been shown to be associated with adhesion of platelets to the vascular endothelium predisposing an individual to thrombosis and occlusion of the vasculature, although thrombotic events have not been characterized in mouse models of atherogenesis (Greaves and Channon, 2002).

In addition to the "workhorse" proteins, such as VCAM-1, changes in inflammatory modulators such as the prostaglandins and leukotrienes contribute to the pathology of atherosclerosis (Burleigh et al., 2002; Walker et al., 2002; Mehrabian et al., 2002; Gurm et al., 2003). Prostaglandins and leukotrienes are products of the
cyclooxygenase and lipoxygenase pathways, respectively. These may be important pathways by which arsenic is a factor in the pathogenesis of atherosclerosis. Specifically, our laboratory has previously shown COX-2 to be upregulated in response to arsenic exposure in the endothelial cell line and at 20 μM arsenic there was a significant increase in PGE\(_2\) (Bunderson et al., 2002). Burleigh et al., (2002) demonstrated the importance of COX-2 in the early stages of lesion formation using LDL receptor-deficient mice. We observed an increase in the COX-2 product, PGI\(_2\), in the serum of our arsenic treated mice although we did not see an increase in PGE\(_2\) within the serum of the treated mice. This is reflected in the immunohistochemistry data that showed no increase in the COX-2 protein within the plaque of arsenic-treated versus control mice. It is possible that the increase in PGI\(_2\) is a response to changes in prostacyclin synthase, an enzyme downstream from the cyclooxygenase enzymes in the eicosanoid biosynthetic pathways. Salzman and Bowman (1992) saw a similar effect on PGI\(_2\) synthesis in fibroblast cells treated with sodium arsenite. It has been suggested that PGI\(_2\) may serve as a stabilizing response to increased platelet-vessel wall interactions (Cheng et al., 2002). It is also a potent vasodilator and may be a physiological attempt to counteract the rapid development of plaque within the arteries of the arsenic-treated mice.

Presently, we have demonstrated an increase in 5-Lo protein in the aortic endothelial cell line and observed a concurrent increase in LTE\(_4\) both in the arsenic-treated cells and the serum of the arsenic-treated mice. However, no significant changes in the presence of 5-lipoxygenase in the plaque of arsenic-treated versus control mice suggests activation of other enzymes is involved in the increased synthesis of LTE\(_4\) in response to arsenic exposure. It is possible that increases in the activation of
phospholipaseA₂ and the resulting increase in arachidonic acid cleavage from membrane phospholipids contributes to available substrate pools for 5-Lo. Therefore, the increase in LTE₄ observed would be due to increased enzyme activity rather than overall protein induction. However, no significant change in the highly vasoconstrictive and chemotactic LTB₄ suggests that an increase in LTC₄ synthase (see figure1) is a more likely explanation. In addition, induction of 5-Lo within the vascular endothelium may be sufficient to explain the increases in LTE₄ irrespective of changes in protein expression within the plaque itself.

An increase in leukotriene synthesis may contribute to atherosclerosis in various ways. The conversion of arachidonic acid to the various leukotrienes involves an oxygenation step and formation of unstable epoxides (Samuelsson et al., 1987). As a result, lipooxygenase activity may contribute to the progression of atherosclerosis simply by oxidizing LDL lipids (Cathcart and Folcik, 2000). The cysteinyl leukotrienes, including LTE₄, have also been shown to increase vascular permeability, facilitate smooth muscle cell chemotaxis, and serve as potent vasoconstrictors—all of which would exacerbate atherosclerosis.

Arsenic has been shown to increase the risk of cardiovascular disease, first through epidemiological studies, and recently in a laboratory-controlled mouse model. This exacerbation of a highly complex disease can be explained at least partially by arsenic’s ability to increase synthesis of reactive oxygen and nitrogen species resulting in oxidative stress, a hallmark of atherosclerosis. Furthermore, increases in key markers of inflammation in response to arsenic exposure demonstrate a long reaching impact on all phases of development of cardiovascular disease.
References


CHAPTER FOUR

Peroxynitrite Generation in Aortic Endothelial Cells Exposed to Arsenic is Increased by Manganese

Abstract

Long-term exposure to arsenic in drinking water has been linked to cancer and other health effects, including cardiovascular disease. Arsenic is found in combination with a range of metals that could influence the toxicity of arsenic. Manganese, in particular, is a metal that is typically found in conjunction with arsenic in contaminated groundwater. Two potential mechanisms for arsenic-induced cardiovascular disease include the formation of peroxynitrite, a highly reactive oxidant and nitrating agent with a high affinity for tyrosine residues; and an enhanced immune response. Nitrotyrosine, a biological marker for the presence of peroxynitrite, is found in high levels in the plaques of individuals with coronary heart disease—a disorder characterized by chronic inflammation. The goal of this study was to examine the effects of manganese on arsenic-induced toxicity and peroxynitrite formation in endothelial cells. The effect of manganese on arsenic toxicity was determined in bovine aortic endothelial (BAE) cells using the MTT assay. Arsenic toxicity was potentiated by the addition of manganese at manganese concentrations that were non-toxic when administered alone. BAE cells were also used to measure the generation of peroxynitrite in the presence of arsenic, manganese and arsenic-manganese mixtures. Supernatant from treated cells was also used to measure changes in the inflammatory mediator, PGE$_2$. We have previously shown that arsenic increases peroxynitrite formation at low concentrations (0.25 μM) and PGE$_2$ at high
concentrations (20 μM) using this model. BAE cells were exposed to sodium arsenite, manganese chloride or an arsenic-manganese mixture for 1 h. Peroxynitrite was measured indirectly by its ability to oxidize hydroethidine to its fluorescent form, ethidium, in the presence of superoxide dismutase and catalase. PGE$_2$ was measured using a standard Enzyme Immunoassay (EIA) kit. There was a significant increase in the amount of fluorescence emitted from arsenic, manganese, and arsenic-manganese mixture-treated cells compared to control cells. Individually, arsenic and manganese enhance peroxynitrite formation by 50% over the controls at 5 μM arsenic and 25 μM manganese. However, given together at equivalent concentrations, peroxynitrite formation was increased 2-fold versus controls. These data suggest that manganese may exacerbate the toxic effects of arsenic on the vascular system.

Introduction

Over 60 million Americans have one or more types of cardiovascular disease (CVD). CVD is the number one killer in the United States, costing over $300 billion per year. Arsenic exposure has been identified as a risk factor for CVD in a number of epidemiological studies (Engel and Smith, 1994; Chen et al., 1996; Hertz-Picciotto et al., 2000), and specific diseases that have been linked to chronic arsenic exposure include atherosclerosis (Wang et al., 2002), ischemic heart disease (Tseng et al., 2003), and hypertension (Rahman et al., 1999). A better understanding of the role for arsenic in CVD could lead to prevention or treatment strategies for CVD. Historically, arsenic has been released into the air, soil and water by human activities such as mining, smelting, manufacturing and pesticide application. An important source of arsenic exposure for
humans is drinking water. Most public water supplies have low levels (less than 10 ppb) of arsenic, but many areas of the U.S., especially the western states, are known to have much higher levels (Buchet and Lison, 2000; Engel and Smith, 1994).

Arsenic has been shown to increase levels of reactive oxygen species such as superoxide anion and hydrogen peroxide in endothelial cells (Lynn et al., 2000). Peroxynitrite is a powerful oxidant that is the product of a rapid reaction between superoxide anion and nitric oxide. We have generated evidence for peroxynitrite formation in bovine aortic endothelial cells exposed to sodium arsenite (Bunderson et al., 2002). Peroxynitrite has been implicated in the oxidation of low-density lipoprotein (LDL) to a form recognizable by macrophage scavenger receptors, one of the putative first steps in atherogenesis (Keaney, 2000). There is substantial evidence linking in vivo production of peroxynitrite with nitration of both free and protein bound tyrosine residues (Beckman, 1996). Atherosclerosis is characterized by a general, and in some cases marked, increase in the occurrence of nitrotyrosine residues (Beckmann et al., 1994; Leeuwenburgh et al., 1997).

The question of how metals impact arsenic toxicity has not been well studied. Of particular interest is the effect of manganese due to its high levels at hazardous waste sites related to large scale mining activities (Moore and Luoma, 1990). Although manganese alone does not seem to be problematic with regard to CVD (Houtman, 1996), little is known of its effect in combination with arsenic. In this study, we evaluated the effect of manganese on arsenic toxicity and peroxynitrite generation in endothelial cells, and the production of the proinflammatory prostaglandin, PGE$_2$, in cell culture media from those cells.
Materials and Methods

Cell Culture. Bovine aortic endothelial (BAE) cells from primary cultures were a gift from Dr. Steve Schwartz of the University of Washington. Cell culture components were obtained from Life Technologies, Inc. (Rockville, MD) unless otherwise noted. BAE cells were grown in Dulbecco’s Modified Eagle Medium (DMEM) with penicillin/streptomycin and L-glutamine, and supplemented with 15% fetal bovine serum (HyClone Laboratories, Inc., Logan, UT). The cells were incubated at 37°C under a humidified atmosphere containing 5% CO₂. Cells were used between passages # 2-6.

Cell Viability Assay. Cell viability was determined using an adaptation of the MTT colorimetric assay (Mosmann, 1983). Cells were plated in 96-well plates at a density of 1-2 x 10⁴ cells/mL and allowed to attach overnight (16 h). Sodium arsenite and selected metals were applied for the indicated times. Solutions were removed and replaced with medium alone, and the 96-well plates were incubated for 5-7 days. MTT (50 µg) was added to each well, and the cells were incubated for another 4 h. Medium/MTT solutions were removed carefully by aspiration, the MTT formazan crystals were dissolved in 100 µL DMSO and absorbance was determined on a plate reader at 550 nm. Cell viability was plotted as percent of control vs. concentration. Statistics were calculated using a one-way t-test with unequal variance.

Fluorimetric Determination of Peroxynitrite. Peroxynitrite formation was determined using our published assay (Bunderson et al., 2002). Briefly, BAE cells were plated at a density of 10,000 cells/well on 96-well plates and allowed to attach overnight. Sodium arsenite, manganese chloride or a mixture of the two was added to the wells at
the indicated concentrations and allowed to incubate for 1 h. The plates were rinsed twice with 100 µL of phosphate-buffered saline (PBS). Immediately following the rinses, 100 µL of 40 µM hydroethidine/PBS solution was added with 25 U/mL of catalase and superoxide dismutase. The plates were read on a fluorescence plate reader at 465 nm excitation and 585 nm emission after a 15 min incubation period during which the plates were shaken every five min with the automatic plate shaker. Peroxynitrite formation was reported as percent of control (untreated cells). Statistics were conducted using a two-way unpaired t-test. *P<.05; **P<.005.

ELISA. PGE$_2$ was analyzed using EIA immunoassay kits purchased from Cayman Chemicals (Ann Arbor, Michigan) according to manufacturers instructions.

Results

The toxicity of sodium arsenite to BAE cells was examined in mixtures containing metals (iron, copper, zinc, manganese) commonly found at waste sites related to mining activities (figure 19). Only manganese significantly increased the toxicity of arsenic at metal concentrations that were non-toxic when applied alone (Figure 20). Manganese is an essential element in mammalian systems, required for proper development and function (reviewed in Reaney et al., 2002), serving a role as an essential cofactor in glutamine synthetase, as well as various transferases, hydrolases and antioxidant pathways. Therefore, peroxynitrite and PGE$_2$ formation in BAE cells was studied with sodium arsenite, manganese chloride and arsenic/manganese mixtures. One potential mechanism for manganese toxicity includes the formation of reactive oxygen species. Since arsenic is known to activate a membrane-bound NAD(P)H oxidase on
vascular endothelial cells (Smith et al., 2001), manganese could exacerbate this increase in the oxidative state of the cell, either in a synergistic or additive fashion. Both arsenic and manganese increased peroxynitrite levels compared to controls (figure 21). Peroxynitrite formation for the arsenic/manganese mixtures was increased by a factor of two compared to arsenic and manganese alone. However, there were no significant changes in PGE$_2$ synthesis after exposure to arsenic, manganese, or arsenic/manganese mixtures at these concentrations (figure 22). It could be that the presence of arsenic and manganese in conjunction with each other compromise the electron transport chain as both are known for disrupting the normal mitochondrial functions (Reaney et al., 2002; Nemeti and Gregus, 2002). This may partially explain the increase in toxicity observed with the mixture.
Figure 19. Cell Viability of BAE Cells Exposed to Sodium Arsenite/Metal Mixtures.
Figure 19. Cell Viability of BAE Cells Exposed to Sodium Arsenite/Metal Mixtures. The metals iron, manganese, copper and zinc were applied to BAE cells for 1 h along with sodium arsenite (1-100 μM). Concentrations of Fe[II] (10 μM), Mn[II] (50 μM), Cu[II] (500 μM), and Zn[II] (500 μM) were used that were non-toxic when applied alone (data not shown). Error bars represent mean +/- standard deviation for n=3 experiments.

*P=.01 **P=.006
Figure 20. Cell Viability of BAE Cells Exposed to Sodium Arsenite/Manganese Chloride Mixtures for 24 h (A) and 48 h (B).

A.

B.
Figure 20. Cell Viability of BAE Cells Exposed to Sodium Arsenite/Manganese Chloride Mixtures. Sodium arsenite (1 μM, 5 μM, 10 μM) and manganese chloride (5 μM, 25 μM, 50 μM) were applied separately, or in the same concentrations as a mixture, to BAE cells for 24 h (A) or 48 h (B). Error bars represent mean +/- standard deviation for n=3 experiments. *P=.03 **P=.005
Figure 21. Peroxynitrite Formation in BAE Cells Exposed to Sodium Arsenite/Manganese Chloride Mixtures.
Figure 21. Peroxynitrite Formation in BAE Cells Exposed to Sodium Arsenite/Manganese Chloride Mixtures. Sodium arsenite (1 μM, 5 μM, 10 μM) and manganese chloride (5 μM, 25 μM, 50 μM) were applied separately, or in the same concentrations as a mixture, to BAE cells for 1 h. Error bars represent mean +/- standard deviation for n=3 experiments. *P=.037 **P=.011 ***P=.002
Figure 22. Prostaglandin E$_2$ Formation (% Control) in BAE Cells Exposed to Sodium Arsenite/Manganese Chloride Mixtures.
Figure 22. Prostaglandin E\textsubscript{2} formation (% control) in BAE cells exposed to sodium arsenite/manganese chloride mixtures. Sodium arsenite (1 \textmu M, 10 \textmu M) and manganese chloride (5 \textmu M, 50 \textmu M) were applied separately, or in the same concentrations as a mixture, to BAE cells for 1 h. Error bars represent mean +/- standard deviation for n=2 experiments (6-wells). Data not significant.
Discussion

Elucidation of the mechanisms by which environmental contaminants exert their toxic effects is complicated by the fact that hazardous compounds are not found in the environment alone, but rather in conjunction with other compounds. The characteristics of the environment in which a toxicant such as arsenic is found may influence its solubility in water, bioavailability to plants and animals, rate of metabolism, and metabolic pathways. One potential mechanism for the wide diversity of cellular disturbances caused by arsenic is its ability to influence or be influenced by--other metals or toxicants. For example, the trivalent methylated form of arsenic, dimethylarsenic (DMA) has been shown to significantly increase the release of iron from horse spleen ferritin (Ahmad et al., 2000). Another example is the ability of arsenic to enhance the induction of both phase I and phase II detoxification enzymes caused by 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) exposure, a common environmental pollutant resulting from application of pesticides contaminated with TCDD residues (Maier et al., 2000). These could be pathways by which arsenic contributes to increased oxidative stress within an organism. Another metalloid, selenium, has been shown to influence the metabolism of arsenic (Csanaky and Zoltan, 2003). Selenium is an essential mineral yet highly toxic if ingested in large quantities. The interaction between selenium and arsenic in biological systems has been well established and at times both metalloids counteract the toxic effects of the other (Gregus et al., 2000). Finally, oral administration of inorganic arsenic to rats and guinea pigs has been linked to an accumulation of copper in the kidneys, although the biological consequence of this interaction is unknown (Ademuyiwa and Elsenhans, 2000).
It is possible that metals/metalloids influence each other indirectly through the generation of reactive intermediates. This is relevant because atherosclerosis is a chronic inflammatory disease exacerbated by oxidative stress. Under conditions of stress, oxidation of LDL and subsequent phagocytosis by macrophages leads to the formation of foam cells. These "fatty" macrophage cells die, leaving behind a growing mass of extracellular debris (Lusis, 2000). This will eventually cause arterial occlusion and disease. An increase in oxidative stress in response to arsenic exposure may contribute to atherosclerosis in various ways and be enhanced by the presence of other contaminants either in an additive or synergistic fashion.

The formation of reactive nitrogen species seems to play a particularly important role in atherogenesis, specifically arsenic-induced atherosclerosis, either by direct oxidation of LDL (Ademuyiwa and Elsenhans, 2000), induction of inflammatory mediators (Gerszten et al., 2000), alterations of protein conformation via tyrosine nitration (Gow et al., 1996), or possibly through signaling pathways responsive to molecules such as peroxynitrite (Levonen et al., 2001). In the case of exposure to arsenic and arsenic/manganese mixtures, an increase in the formation of peroxynitrite could be a contributing factor in the aggravation of arsenic-related cardiovascular disease. In fact, peroxynitrite has been shown to influence the release of zinc from the zinc-thiolate cluster of endothelial NOS, possibly forming disulfide bonds between the enzyme monomers and decreasing NO synthesis (Zou et al., 2002). This has been related to increased generation of superoxide anion in diabetic mice and may also be important in the pathogenesis of atherosclerosis (Zou et al., 2002). Therefore, an increase in
peroxynitrite in response to arsenic and arsenic/manganese mixtures may indirectly impact atherosclerosis by disrupting the normal cellular functions of zinc.

This study demonstrates that the presence of manganese enhances the formation of peroxynitrite in response to arsenic exposure—although mechanisms are unknown. The combination of these two metals/metalloids is a common occurrence in the environment and may have serious consequences on the incidence of cardiovascular disease.
References


CONCLUSIONS

Over 60 million Americans have one or more types of cardiovascular disease (CVD). CVD is the number one killer in the United States, costing over $300 billion per year. Arsenic exposure has been identified as a risk factor for CVD in a number of epidemiological studies (Engel and Smith, 1994; Chen et al., 1996; Hertz-Picciotto et al., 2000), and specific diseases that have been linked to chronic arsenic exposure include atherosclerosis (Wang et al., 2002), ischemic heart disease (Tseng et al., 2003), and hypertension (Rahman et al., 1999).

Exposure to arsenic and the resulting health effects are an important concern in Montana, where mining has resulted in the contamination of lakes, streams and aquifers (Moore and Luoma, 1990). As a consequence, Montana is home to a dense region of EPA designated superfund sites. The Milltown Reservoir Superfund Site (MRSS) is one such location. Located near Milltown, MRSS is an artifact of the Milltown Dam, built at the confluence of the Clark Fork and Blackfoot Rivers in 1907. Acting as a repository for sediment and mining wastes, the reservoir is highly contaminated and caused the formation of a groundwater arsenic plume that impacts Milltown's drinking water supply (USEPA). EPA added the site to its National Priorities List (NPL) in September 1983 and is addressing the problem through the combined actions of federal and state agencies and the Potentially Responsible Parties (PRPs), primarily the Atlantic Richfield Company (ARCO) and the Northwestern Energy Corporation.

Arsenic contaminated drinking water has a huge impact on the health, environment, and economy of this country. Given the high incidence of cardiovascular
disease and the associated costs, research aimed at understanding how arsenic may be contributing to the incidence of CVD is needed. The research done in our laboratory has aimed at elucidating some of those basic mechanisms by: (1) Defining the changes in key atherogenic regulatory proteins upon exposure to arsenic in bovine aortic endothelial cells (2) Investigating the effects of arsenic on the formation of peroxynitrite in endothelial cells (3) Determining the effects of arsenic on atherogenesis and expression of key CVD regulatory molecules in the ApoE\(^{-/-}\)/LDL\(^{-/-}\) atherosclerotic mouse model and (4) Do preliminary experiments to determine the effects of other environmentally relevant metals on arsenic-induced cardiovascular disease.

COX-2, the inducible form of the COX protein, is a key player in inflammation and synthesis of prostaglandins by COX 1 and 2 is a necessary event in normal vascular physiology. Since atherosclerosis is an inflammatory disease, we reasoned that COX-2 might play an important role in arsenic-induced atherogenesis. Expression of COX-2 protein was measured by Western blot analysis using a polyclonal COX-2 antibody and chemiluminescence. The data show that COX-2 protein was increased after 30 min exposure to 5, 10, and 20 \(\mu\)M arsenic. Furthermore, COX-2 induction was time dependent, showing a decline up to 24 h and then increasing again at 48 h. Densitometry using COX-2/actin ratios showed a twofold increase in COX-2 protein at 20 min and an over threefold increase after 48 h. No increase in COX-2 protein was observed at sodium arsenite concentrations less than 5 \(\mu\)M.

Peroxynitrite, the coupling product of nitric oxide and superoxide anion, is an oxidant that has been implicated in atherogenesis. Oxidant production was determined fluorimetrically by measuring the oxidation of hydroethidine to its fluorescent form,
Ethidium. Hydroethidine is preferentially oxidized by superoxide anion, hydrogen peroxide, and peroxynitrite (Supinski et al., 1999; Al-Mehdi et al., 1997). Total reactive species increased in a dose-dependent manner up to 10 μM sodium arsenite. Addition of SOD and CAT had little effect on levels of reactive species, suggesting that peroxynitrite was the principal oxidant generated in endothelial cells by sodium arsenite. The general NOS inhibitor L-NAME or the NAD(P)H oxidase inhibitor DPI reduced fluorescence to control levels, confirming that peroxynitrite is primarily responsible for the fluorescence observed in arsenic-treated cells. Cell viability studies showed no toxicity to BAE cells treated with hydroethidine, DPI, or L-NAME at the concentrations and times used in these experiments.

Immunoprecipitation was used to confirm the formation of peroxynitrite and to determine the extent of COX-2 nitration upon arsenic exposure. COX-2 was immunoprecipitated from endothelial cells that were treated for 30 min with 5, 10 and 20 μM sodium arsenite. Western blots were then probed with an antinitrotyrosine antibody. Nitrated COX-2 was observed in cells treated with all concentrations of sodium arsenite, whereas there was no evidence for nitration of COX-2 in the non-arsenic-treated cells.

In addition to the in vitro model using BAE cells, we used the highly atherogenic ApoE<sup>-/-</sup>/LDLr<sup>-/-</sup> mouse model. These mice develop severe occlusions early in life while fed a normal chow diet. It is well known that the ApoE lipoprotein is important for the cardioprotective effects of high-density lipoproteins (HDL) and is also a ligand for the low-density lipoprotein (LDL) receptor and vital for removal of cholesterol from the blood (Strittmatter and Bova Hill, 2002). The innominate artery has been shown to have the greatest similarities with the human form of atherosclerosis in an ApoE<sup>-/-</sup> mouse
model (Rosenfeld et al., 2000). We observed a significant increase in the atherosclerotic occlusion of the innominate artery of ApoE−/−/LDLr−/− mice treated with 10 ppm (133.3 μM) arsenic for 18 weeks. Changes in intima media thickness of the coronary artery in humans is correlated with increases in blood pressure, smoking, and high LDL cholesterol—all risk factors in the development of heart disease (Sinha et al., 2002).

As mentioned earlier, in addition to exacerbating atherogenesis by increasing oxidative stress, our laboratory has shown arsenic to induce the inflammatory mediator, COX-2 in aortic endothelial cells (Bunderson et al., 2002). At high concentrations of arsenic (20 μM), increases in the COX-2 product, prostaglandin E2 (PGE2), were also observed. However, we were unable to detect an increase in the presence of COX-2 in the arteries of our treated mice over the controls. We did observe an increase in prostacyclin (PGI2), but not PGE2 in the serum of the arsenic treated mice. Furthermore, we observed an increase in 5-Lo protein in bovine aortic endothelial cells treated with 10 μM arsenic over a period of 48 h. In addition, we observed a significant increase in Leukotriene E4 (LTE4) synthesis in these cells after exposure to 10 μM arsenic for 48 h. We were not able to detect an increase in 5-Lo within the innominate artery of our treated mice over controls, however, we were able to measure a significant increase in LTE4, but not LTB4 in the serum of these animals. The relationship between increased synthesis of oxidants and inflammatory mediators is outlined in figure 23.
Figure 23. Schematic overview of the mechanisms of action of arsenic-induced cardiovascular disease

AsIII Activation

Arginine

LPS, Cytokines, AsIII

Arachidonate

NADPH Oxidase

NO Synthase

COX-2 & 5-Lo

PGE2 & LTE4

Superoxide Dismutase

O2 + H2O2

Oxidative Stress, Modified LDL

ATHEROSCLEROSIS!
The toxicity of sodium arsenite to BAE cells was examined in mixtures containing metals (iron, copper, zinc, manganese) commonly found at waste sites related to mining activities. Only manganese significantly increased the toxicity of arsenic at metal concentrations that were non-toxic when applied alone. Therefore, peroxynitrite and PGE$_2$ formation in BAE cells was studied with sodium arsenite, manganese chloride and arsenic/manganese mixtures. Both arsenic and manganese increased peroxynitrite levels compared to controls. Peroxynitrite formation for the arsenic/manganese mixtures was approximately additive compared to arsenic and manganese alone. However, there were no significant changes in PGE$_2$ synthesis after exposure to arsenic, manganese, or arsenic/manganese mixtures at these concentrations.

In conclusion, arsenic appears to play a primary role in endothelial cell dysfunction by activation of membrane oxidases that lead to formation of reactive species such as peroxynitrite. This also results in an overall increase in the oxidative stress experienced by the cell and probably contributes to such risk factors as modified lipoproteins and activation of intracellular monocytes and subsequent foam cell formation. Furthermore, an activation of inflammatory pathways such as COX-2 and 5-Lo would have a significant impact on the development of atherosclerosis. Particularly because these inflammatory mediators also influence vascular tone and may contribute to conditions of hypertension.

Extensive studies still need to be conducted in order to understand the impact that arsenic has on the immune system as a whole as well as cardiovascular performance. Furthermore, in depth characterization of the changes that occur within the plaque composition in response to arsenic may shed some insight into further pathways that are
being disrupted in response to arsenic—leading to an increased understanding of the
mechanisms of action of arsenic-induced cardiovascular disease.
References


