Arsenic exposure exacerbates atherosclerotic plaque formation and increases nitrotyrosine and leukotriene biosynthesis

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Received 22 December 2003; accepted 19 April 2004
Available online 15 June 2004

Abstract

A correlation between arsenic and cardiovascular disease (CVD) has been established through epidemiological studies, although the mechanisms are unknown. Using a mouse model that develops atherosclerotic lesions on a normal chow diet, we have confirmed a connection between long-term arsenic intake and CVD. Our results reveal a significant increase in the degree of atherosclerotic plaque stenosis within the innominate artery of ApoE−/−/LDLr−/− mice treated with 10 ppm sodium arsenite (133 μM) in drinking water for 18 weeks compared to controls. Immunohistochemistry shows nitrotyrosine formation, a marker of reactive nitrogen species generation, is significantly higher within the atherosclerotic plaque of arsenic-treated mice. In addition, there is a significant increase in the 5-lipoxygenase (5-LO) product, leukotriene E4 (LTE4), in the serum of arsenic-treated mice. This is supported by induction of the 5-LO protein and subsequent increases in LTE4 synthesis in bovine aortic endothelial cells. This increase in LTE4 is partially inhibited by inhibitors of nitric oxide synthase, suggesting a link between reactive nitrogen species and arsenic-induced inflammation. Furthermore, there is a significant increase in prostacyclin (PGI2) in the serum of arsenic-treated mice. We conclude that changes in specific inflammatory mediators such as LTE4 and PGI2 are related to arsenic-induced atherosclerosis. In addition, amplified synthesis of reactive species such as peroxynitrite results in increased protein nitration in response to arsenic exposure. This finding is consistent with the pathology seen in human atherosclerotic plaques.

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Keywords: Arsenic; Atherosclerosis; Nitrotyrosine; Leukotriene E4; 5-Lipoxygenase

Introduction

Epidemiological studies have linked arsenic to cardiovascular disease (CVD) 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, severe peripheral vascular disease, and other forms of CVD. A long-term study in Taiwan reported 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 in the water, or cumulative arsenic exposure, and remained significant after adjusting for known risk factors in the development of atherosclerosis. In the United States, an investigation on the relationship between population-weighted mean arsenic concentration in public drinking water and death from CVD in 30 U.S. counties showed an increase in the mortality ratio for various forms of CVD (Engel and Smith, 1994). Atherosclerosis is the underlying cause of virtually all forms of CVD and develops through a multifactorial process, making elucidation of a single mechanism for exacerbation of the disease difficult. To date, primary human risk factors include genetics, obesity, cigarette smoking, hypertension, diabetes mellitus, and increased serum cholesterol (Keaney, 2000).

Atherosclerosis is a disease characterized by oxidative stress and chronic inflammation leading to arterial occlu-
sion. The initiating event appears to be activation of the endothelium (Keaney, 2000). Mechanistically, oxidative stress and inflammation are linked in the exacerbation of the disorder. Oxidants are known to cause changes in the activation state of transcription factors and other signaling molecules (Herrlich and Bohmer, 2000). Peroxynitrite in particular, an extremely reactive isomer of the nitrate anion, disrupts phosphorylation cascades and interferes with signaling pathways through the formation of 3-nitrotyrosine (3NY) (Gow et al., 1996), its biological marker (Crow and Ischiropoulos, 1996). An increase in other reactive species has been linked to the synthesis of the proinflammatory leukotrienes (Coffey et al., 2002), the biosynthetic products of the 5-lipoxygenase (5-LO) pathway (Sala and Folco, 2001). The 5-LO gene has been identified as a contributor to atherogenesis with a significant decrease in atherosclerotic aortic lesions seen in 5-LO knockout mice (Mehrabian et al., 2002).

Changes in the oxidative and inflammatory state of a system are not mutually exclusive. There is increasing evidence linking stress from the formation of reactive oxygen and nitrogen to inflammation. Peroxynitrite has been linked to atherosclerosis through the detection of 3NY residues within the plaques (Beckmann et al., 1994; Leeuwenburgh et al., 1997) and this laboratory recently demonstrated an increase in peroxynitrite synthesis in aortic endothelial cells exposed to arsenic (Bunderson et al., 2002). This has implications for disruption of phosphorylation patterns due to nitration of proteins, as well as directly interfering with other aspects of signal transduction pathways by reactive species. Two significant mediators of inflammation, the lipooxygenase (LO) and cyclooxygenase (COX) enzymes, in particular, have been shown to be affected by the generation of reactive oxygen and nitrogen species. Pathological changes in the inflammatory system from disturbances of these important cellular processes can lead to activation of the vascular endothelium, resulting in initiation and progression of atherosclerosis (Libby, 2002).

The LO and COX enzymes along with their biosynthetic products are two closely related pathways important to inflammation. In addition to 5-LO, other LO enzymes have been linked to atherosclerosis, particularly 12- and 15-LO (Keaney 2000; Lusis, 2000). The inducible form of the COX enzyme, COX-2, is rapidly upregulated in response to proinflammatory cytokines (Lipsky et al., 2000) and oxidative stress (Kumagai et al., 2000). It has been suggested that the enzymatic process leading to synthesis of the COX eicosanoids contributes to oxidative stress (Nikolic and van Breemen, 2001) and COX-2 has been implicated in the pathogenesis of atherosclerosis as well (Burleigh et al., 2002).

While there is clear evidence linking exposure of arsenic through the drinking water to increases in CVD, the mechanisms behind this phenomenon are primarily unknown. Considering the enormous impact of CVD to this country and the recently reduced standard for arsenic in the drinking water by the EPA, investigations of these mechanisms are needed. In this report, we demonstrate a direct link between arsenic 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 ad libitum via the drinking water. Mice were 10 weeks of age at the beginning of the study, and male vs. female animals were evenly distributed between treatment and control groups with an n = 12 and n = 10 for the control and arsenic treated groups, respectively. At termination of the study, hematocrit was calculated with whole blood centrifugation to ensure all mice were sufficiently hydrated. At 18 weeks of treatment, the mice were formaldehyde perfused (1%PBS), then the heart and innominate arteries were removed, fixed overnight, and paraffin embedded. The samples were sectioned 5 μm thick and stained with Movat’s pentachrome staining according to published protocols (Rosenfeld et al., 2000). This method allows identification of acid mucopolysaccharides with a blue color, nuclei and elastic fibers are stained black, muscle becomes red, collagen yellow, and fibrin is stained an intense red color. Quantitative analysis for variation in plaque composition was conducted on Movat’s-stained plaques from arsenic-treated and control mice with Image J and Adobe Photoshop. Color (red, yellow, and black) abundance and intensity were measured and compared for each group. A t test was applied to assess statistical differences.

**Immunohistochemistry.** Cross-sections (10 μm) 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% H<sub>2</sub>O<sub>2</sub> solution/TB, then blocked with 5% BSA/4% goat serum/TB for 30 min. Anti-3NY polyclonal serum (Upstate Cell Signaling Solutions, Lake Placid, NY) in blocking solution was applied at 5 μg/ml overnight at 4 °C. The negative control was prepared by incubating the primary antibody with 10 mM nitrotyrosine in PBS for 1 h at room temperature. This solution was used in place of the untreated 3NY antibody as suggested by the manufacturer. The next day, the sections were rinsed (3 × 5 min TB), and goat antirabbit biotin-conjugated secondary antibody (Vector Laboratories) was applied at 1:400 for 1 h. The samples were rinsed (3 × 5 min TB), developed with Vectastain Elite ABC kit (Vector Laboratories), dehydrated, and cover-slipped. Sections were examined with a Nikon E800 microscope, photographed, and analyzed with the NIH program, ImageJ. DAB positive staining was con-
verted to gray scale for analysis. Minimum and maximum threshold values were established to remove background staining and a mean gray value for the plaques was then calculated.

**Western immunoblot.** Bovine aortic endothelial (BAE) cells were grown to approximately 90% confluency and treated with 10 μM sodium arsenite for 0.5, 1, 24, and 48 h. Cells were harvested with 2× Laemmli buffer, homogenized with an 18- and 25-gauge needle and boiled for 5 min. Protein concentrations were determined by a Bradford assay (Bio-Rad, Hercules, CA), and samples (10 μg) 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, Ann Arbor, MI) and an antirabbit secondary antibody conjugated with horseradish peroxidase (Vector Laboratories). 5-LO was visualized by enhanced chemiluminescence (ECL; Amersham, Piscataway, NJ).

**Enzyme immunoassay.** Serum prostaglandin E₂ (PGE₂), leukotriene E₄ (LTE₄), and LTB₄ were analyzed using Enzyme immunoassay (EIA) kits purchased from Cayman Chemicals according to the manufacturer’s instructions. Serum prostacyclin (PGI₂) was indirectly analyzed by measuring 6-keto prostaglandin F₁α using an immunoassay kit purchased from R&D Systems (Minneapolis, MN) according to manufacturer’s instructions. Arsenic-treated BAE cells were grown as described above in the presence of sodium arsenite with or without the addition of the 5-LO inhibitor AA861 (0.8 μM; Biomol Research Laboratories Inc.) or the nitric oxide synthase (NOS) inhibitors, L-NAME (30 μM; Sigma Chemical Co.) or S-ethylisothiourea (ETU) (100 μM; Sigma) for 36 h. Treatment was then readministered in serum-free media for an additional 12 h and LTE₄ concentrations were determined.

**Statistical analysis.** Values for control vs. arsenic-treated means and percent stenosis are expressed as the mean ± SEM for in vivo studies and analyzed using an unpaired t test with Welch’s correction. All other statistics were conducted using one-way ANOVA coupled with Newman–Keul’s multiple comparison test. Results were considered significant if P < 0.05.

**Results**

**Atherosclerotic plaque: percent stenosis**

Innominate arteries of ApoE⁻/⁻/LDLr⁻/⁻ mice were dissected and paraffin embedded, then serial cross sections of each sample were taken for staining. The occluding plaque was visualized using the Movat’s pentachrome stain. The percent stenosis was calculated by dividing the plaque area (PA) by the lumen area (LA) plus plaque area. Sections from each mouse were examined using blinded methods under a Nikon E800 microscope. The LA and PA were both measured using NIH ImageJ. A significant increase was observed in the degree of stenosis within the innominate artery of ApoE⁻/⁻/LDLr⁻/⁻ mice after treatment with 10 ppm sodium arsenite for 18 weeks (Figs. 1A–C). Results are reported as a percentage of PA / (PA + LA) from the water only controls and arsenic-treated groups. Image analysis was used to assess variation in plaque composition between arsenic-treated and control mice. No significant differences in collagen deposition, elastin, or muscle content were found. Thus, microscopy revealed no significant differences in the composition of the plaques, but the sizes of the plaques were significantly different (P < 0.05) between the arsenic-treated and control groups.

**Reactive nitrogen species**

The hypothesis that arsenic-mediated atherogenesis is enhanced by free radical formation was supported by a significant increase in 3NY formation detected by immunochemistry in the plaques of ApoE⁻/⁻/LDLr⁻/⁻ mice exposed to sodium arsenite compared to untreated mice (Figs. 2A–D). This demonstrates that a biomarker for peroxynitrite, 3NY, is found in greater abundance within the plaque of arsenic-treated mice than controls. Analysis

![Fig. 1](image-url)

Fig. 1. (A) Innominate artery stained with Movat’s from ApoE⁻/⁻/LDLr⁻/⁻ mouse treated with ddH₂O for 18 weeks. (B) Innominate artery stained with Movat’s pentachrome from ApoE⁻/⁻/LDLr⁻/⁻ mouse given 10 ppm sodium arsenite in ddH₂O for 18 weeks. (C) Percent stenosis between control (n = 12) and treated (n = 10) mice measured as the percentage of normal lumen area occupied by lesions. Magnification = 50×. *Significantly different at P < 0.05.
was conducted by comparing the color density between arsenic-treated samples and controls. The data are presented as a ratio of the total plaque area to give a relative staining density for each sample.

5-Lipoxygenase and leukotriene biosynthesis

Inflammation is linked to both atherogenesis and free radical formation. A time-dependent increase in 5-LO protein was observed by Western blot in bovine aortic endothelial (BAE) cells treated with 10 \text{ mM} \text{arsenic} with maximum expression (fourfold increase over untreated) at 48 h (Figs. 3A,B). In addition, a significant increase in leukotriene E\textsubscript{4} (LTE\textsubscript{4}) synthesis was detected in these cells after exposure to 10 \text{ mM} \text{arsenic} for 48 h (Fig. 4), which was partially reduced by the addition of the NOS inhibitors, l-NAME and ETU (Fig. 5). Arsenic-induced LTE\textsubscript{4} synthesis was inhibited by the addition of the 5-LO inhibitor, AA861. There was no detectable increase in 5-LO within the innominate artery of the treated mice over controls with immunohistochemistry (data not shown). However, a significant increase in LTE\textsubscript{4} (Fig. 6), but not LTB\textsubscript{4} (data not shown), in the serum of the arsenic-treated mice after 18 weeks of continuous arsenic exposure was measured.

COX-2 and prostaglandin biosynthesis

Components of the cyclooxygenase pathway have been linked to atherosclerosis. A significant increase in PGI\textsubscript{2} (Fig. 7), but not PGE\textsubscript{2} (data not shown), was observed using ELISA assays in the serum of the arsenic treated mice after 18 weeks of continuous arsenic exposure. There was no detectable increase in the COX-2 protein within the
innominate artery of the treated mice over controls using immunohistochemistry (data not shown).

Discussion

Arsenic is a ubiquitous contaminant within our environment and also merits consideration as a risk-factor for atherosclerosis. In the present study, the degree of stenosis was significantly increased in the innominate artery of ApoE−/−/LDLr−/− mice treated with 10 ppm arsenite for 18 weeks compared to controls. This increase in plaque size of the innominate artery in mice is comparable to the changes seen in the coronary artery in humans with atherosclerosis. Furthermore, changes in the thickness of the coronary artery in humans correlates with increases in blood pressure, smoking, and high-low density lipoprotein (LDL) cholesterol—all risk factors in the development of heart disease (Sinha et al., 2002). The present study specifically identifies an increase in stenosis of the innominate artery after arsenic exposure in a murine model. This is supported by recent work done by Simeonova et al. (2003) showing an increase in lipid deposition in the aorta of ApoE−/− mice administered 20 and 100 ppm arsenite for up to 24 weeks.

Arsenic 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, studies suggest that generation of reactive oxygen and nitrogen species plays a role. 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, partic-

Fig. 3. (A) Western blot analysis showing a fourfold increase in 5-lipoxygenase (5-LO) in bovine aortic endothelial cells (BAE) 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.

Fig. 4. Leukotriene E4 (LTE4) measured by ELISA in BAE cells exposed to a range of sodium arsenite concentrations with or without AA-861, a 5-LO inhibitor, for 48 h. * and # are significantly different from control and each other. P < 0.05.

Fig. 5. Inhibition of arsenic-induced LTE4 synthesis by the NOS inhibitors L-NAME and ETU in BAE cells exposed to 10 μM sodium arsenite for 48 h. * and # are significantly different from control and each other. P < 0.05.
ularly 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). There are various potential consequences to the cardiovascular system in response to the increase in peroxynitrite and subsequent increase in the nitration of plaque proteins after exposure to arsenic as seen in this study. Nitration of protein tyrosine residues has been shown to disrupt cell signaling cascades (Gow et al., 1996) and peroxynitrite itself has been implicated in signal transduction pathways (Minetti et al., 2002). In addition, peroxynitrite has been shown to oxidize LDL molecules, an important factor in atherogenesis (Keeney, 2000). Perhaps more importantly, an increase in the oxidative stress within the endothelial lining contributes to an upregulation of inflammatory mediators. The present results indicate that components of the COX and 5-LO pathways are involved. However, other mediators such as vascular cell adhesion molecule-1 (VCAM-1) and subsequent monocyte recruitment to the injured area (Gow et al., 1996) may also play a role.

Increased 5-LO protein was observed in BAE cells along with a concurrent increase in LTE$_4$, both in the arsenic-treated cells and the serum of the arsenic-treated ApoE$^{-/-}$/LDLr$^{-/-}$ mice. This arsenic-induced increase in LTE$_4$ synthesis was inhibited by the addition of the 5-LO inhibitor, AA861, in the BAE cells. However, no significant changes in the presence of 5-LO in the plaque of arsenic-treated vs. control mice suggests activation of other enzymes may be involved in the increased synthesis of LTE$_4$ in response to arsenic exposure. It is possible that increases in the activation of phospholipase A$_2$ (PLA$_2$) and the resulting increase in arachidonic acid cleavage from membrane phospholipids contributes to available substrate pools for 5-LO. As a result, the increase in LTE$_4$ observed would be due to increased enzyme activity rather than overall protein induction within the plaque. Furthermore, no significant change in the highly vasoconstrictive and chemotactic LTB$_4$ suggests that an increase in LTC$_4$ synthase could occur. In addition, induction of 5-LO within the vascular endothelium may be sufficient to explain the increases in LTE$_4$ irrespective of changes in protein expression within the plaque itself.

To determine the potential role for reactive nitrogen species in the elevation of LTE$_4$ levels in vitro, the NOS inhibitors, L-NAME and ETU, were added to BAE cells along with sodium arsenite. Both inhibitors significantly reduced the arsenic-induced increase in LTE$_4$ synthesis. We have measured nitric oxide production in these cells and found no significant changes with 1–10 μM sodium arsenite over time periods of 1–48 h (unpublished data). This is consistent with the findings of others (Barchowsky et al., 1999) and suggests that peroxynitrite may be at least partially responsible for the elevated LTE$_4$. Increased superoxide anion generated due to arsenic exposure could react with available pools of nitric oxide leading to production of peroxynitrite. Therefore, any increase in nitric oxide would likely be rapidly scavenged for the formation of peroxynitrite, and it is not surprising that no measurable differences in nitric oxide were detected. We previously reported an increase in peroxynitrite synthesis in BAE cells treated with sodium arsenite (Bunderson et al., 2002).

There is evidence linking the 5-LO and inducible NOS (iNOS) pathways, whereby use of the leukotriene receptor antagonist, montelukast, decreased iNOS expression in ovalbumin-challenged rats (Offer et al., 2003) and reduced exhaled nitric oxide in patients with mild asthma (Sandrini et al., 2003). Recently, addition of nitric oxide donors to peritoneal macrophage cells resulted in attenuation of LTB$_4$ synthesis (Brock et al., 2003). However, these investigators did not measure LTE$_4$ levels. In addition to direct links between the NOS and 5-LO pathways, there is...
arsenite, there was a significant increase in PGE2 synthesis in aortic endothelial (BAE) cells, and at 20 μM arsenite, an increase in COX-2 is upregulated in response to arsenic exposure in bovine endothelial cells. Previously, it was shown that COX-2 is closely related to the inflammatory pathway that may be involved in atherogenesis of the disease.

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, 5-LO activity may contribute to the progression of atherosclerosis simply by oxidizing LDL lipids (Cathcart and Folcik, 2000). The loss of a functional ApoE component, as seen in this mouse model, results in aberrant cholesterol transport contributing to the progression of atherosclerosis. Therefore, an increase in modified LDL resulting from increased 5-LO activity or reactive nitrogen species would exacerbate the condition. In addition, LDLr−/− mice alone do not develop severe stenosis, possibly due to a secondary LDL-related receptor that acts as a compensation mechanism (Daugherty, 2002). However, modified LDL may be a poor ligand for these receptors resulting in a shift to LDL uptake by macrophage scavenger receptors and increased foam cell formation. Therefore, the combination of a genetically atherogenic mouse model exposed to conditions of increased inflammation enhanced by oxidative stress due to arsenic exposure results in exacerbation of the disease.

In addition to changes in leukotriene biosynthesis, a closely related pathway that may be involved in atherogenesis is the COX system. Previously, it was shown that COX-2 is upregulated in response to arsenic exposure in bovine aortic endothelial (BAE) cells, and at 20 μM sodium arsenite, there was a significant increase in PGE2 synthesis (Bunderson et al., 2002). Burleigh et al. (2002) demonstrated an increase in COX-2 in the early stages of lesion formation using LDL-receptor-deficient mice. In the present study, a significant increase in the COX product, PGL2, but not PGE2 was detected in the serum of arsenic-treated mice compared to untreated mice. Salzman and Bowman (1992) saw a similar effect on PGI2 synthesis in fibroblast cells treated with sodium arsenite. An increase in COX-2 protein within the plaque of arsenic-treated mice was not seen in the present study. Similarly, chronic treatment with celecoxib, a COX-2 inhibitor, had no effect on lesion progression or composition in older Apo E−/− mice (Bea et al., 2003). However, induction of COX-2 in response to arsenic may primarily occur in the endothelium and therefore may not be detectable with immunohistochemistry in the plaque. Alternatively, the increase in PGI2 observed in this model may be a product of the constitutively expressed COX-1 protein.

PGI2 is a potent vasodilator and may be a physiological attempt to counteract the rapid development of plaque within the arteries of the arsenic-treated mice. A recent study by Goya et al. (2003) demonstrated that an orally active analog of PGL2 administered to patients with type II diabetes mellitus resulted in a reduction of VCAM-1. Other studies have suggested PGL2 is an important regulator of airway inflammation (Nagao et al., 2003). Therefore, PGL2 could be a physiological mechanism for reducing vascular inflammation. However, this “compensation” mechanism may be overwhelmed by the enhanced proinflammatory effects of LTE4 and increased reactive nitrogen species seen after arsenic exposure.

Arsenic has been shown to increase the risk of CVD, first through epidemiological studies and now through a laboratory-controlled mouse model, specifically within the innominate artery and at a concentration that is lower than those reported elsewhere (Simeonova et al., 2003). This exacerbation of a highly complex disease can partially be explained by arsenic’s ability to increase synthesis of reactive oxygen and nitrogen species resulting in oxidative stress and inflammation, hallmarks of atherosclerosis.

References


