Inhibition of Liver X Receptor/Retinoid X Receptor–Mediated Transcription Contributes to the Proatherogenic Effects of Arsenic in Macrophages In Vitro

Alessandra M.S. Padovani, Manuel Flores Molina, Koren K. Mann

Objective—To determine whether arsenic inhibits transcriptional activation of the liver X receptor (LXR)/retinoid X receptor (RXR) heterodimers, thereby impairing cholesterol efflux from macrophages and potentially contributing to a proatherogenic phenotype.

Methods and Results—Arsenic is an important environmental contaminant and has been linked to an increased incidence of atherosclerosis. Previous findings showed that arsenic inhibits transcriptional activation of type 2 nuclear receptors, known to heterodimerize with RXR. Environmentally relevant arsenic doses decrease the LXR/RXR ligand-induced expression of the LXR target genes (ABCA1 and SREBP-1c). Arsenic failed to decrease cAMP-induced ABCA1 expression, suggesting a selective LXR/RXR effect. This selectivity correlated with the ability of arsenic to decrease LXR/RXR ligand-induced, but not cAMP-induced, cholesterol efflux. By using chromatin immunoprecipitation assays, we found that arsenic inhibits the ability of LXR/RXR ligands to induce activation markers on the ABCA1 and SREBP-1c promoters and blocks ligand-induced release of the nuclear receptor coexpressor (NCoR) from the promoter. Arsenic did not alter the ability of LXR to transrepress inflammatory gene transcription, further supporting our hypothesis that RXR is the target for arsenic inhibition.

Conclusion—Exposure to arsenic enhances the risk of atherosclerosis. We present data that arsenic inhibits the transcriptional activity of the liver X receptor, resulting in decreased cholesterol-induced gene expression and efflux from macrophages. Therefore, arsenic may promote an atherosclerotic environment by decreasing the ability of macrophages to efflux excess cholesterol, thereby favoring increased plaque formation. (Arterioscler Thromb Vasc Biol. 2010;30:1228-1236.)

Key Words: atherosclerosis ■ macrophages ■ receptors ■ arsenic ■ liver X receptor

Arsenic has been widely known as a carcinogen; however, recent evidence suggests that those exposed to arsenic also have an increased risk for diabetes mellitus and cardiovascular diseases. In humans, a consequence of arsenic exposure is Blackfoot disease, which is characterized by severe peripheral arteriosclerosis.1–3 Copper smelt workers exposed to arsenic were reported to have increased mortality from ischemic heart disease.4–5 A significant correlation was found in an area of Taiwan between length of exposure to arsenic in the ground water and the risk of ischemic heart disease.6 In the United States, higher levels in the drinking water correlated with an increased risk of death from vascular disease.7 More recently, arsenic accelerated carotid atherosclerosis, with a multivariate-adjusted odds ratio of 3.1.8

In vivo and in vitro data support and correlate with these epidemiological data, although the mechanism by which arsenic increases atherosclerosis is unclear. Inflammation is a key component of the generation of atherosclerotic lesions, and increased inflammatory molecule expression after arsenic exposure has been reported.9 Cells treated in vitro with arsenic have increased levels of 5-lipoxygenase, leukotriene E4, and cyclooxygenase (COX) 2, molecules that are important in inflammation and have been linked to atherosclerosis.10,11 Interleukin (IL) 8, a key chemokine for the infiltration of leukocytes into atherosclerotic lesions, is increased upon arsenic treatment.12 In addition, lymphocytes from arsenic-exposed individuals have increased inflammatory molecule expression, as assessed by cDNA microarray.9 Arsenic has been shown to increase coagulation and decrease fibrinolysis, which could contribute to endothelial cell dysfunction and atherogenesis.13 In APOE−/− and APOE−/−/LDLR−/− mice, which have a genetic predisposition to atherosclerotic plaque formation, arsenic treatment exacerbates these pathological features.10,12,14 In a study10 of APOE−/−/LDLR−/− mice, arsenic exposure significantly increased atherosclerotic plaques; this correlated with an increase in inflammatory mediators in the

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From the Segal Cancer Centre, Lady Davis Institute for Medical Research, Jewish General Hospital, Montréal, QC, Canada (A.M.S.P., M.F., and K.K.M.); and the Department of Oncology, McGill University, Montréal, QC, Canada (K.K.M.).
Correspondence to Koren K. Mann, PhD. Lady Davis Institute for Medical Research, 3755 Côte Ste Catherine Rd, Room E503, Montréal, Québec, Canada H3T 1E2. E-mail koren.mann@mcgill.ca
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1228
As2O3 does not inhibit the transpressive effects of LXR, leading to proatherogenic changes in cholesterol efflux. Evidence that arsenic alters LXR target gene promoters, induced inhibitory signaling. We hypothesized that arsenic very LDL levels and an increased LDL level.21 Mice deficient in LXR became acetylated.18,19 LXR target genes are involved in cholesterol catabolism and efflux from macrophages to the serum in which high-density lipoproteins act as acceptors.20 Although mice that are deficient in LXRα and LXRβ do not exhibit changes in total serum cholesterol levels, they have decreased serum triglyceride, high-density lipoprotein, and very LDL levels and an increased LDL level.21 Mice deficient in both LXRα and ApoE have significant peripheral lipid accumulation, which can be somewhat reversed by specific LXR ligand administration.22 LXR is clearly important for the maintenance of cholesterol homeostasis. In addition, ligand-activated LXR has a transrepressive function on inflammatory gene promoters, such as COX-2 and IL-1β. This function is independent of RXR and LXRE sequences and acts to recruit NCoR to inflammatory gene promoters in a small ubiquitin-related modifier (SUMO)-dependent manner.23

It was previously shown that arsenic trioxide (As2O3) inhibits type II nuclear receptor function, including retinoic acid receptor, vitamin D3 receptor, and peroxisome proliferator–activated receptor in Cos-1 and leukemic cell lines.24 As2O3 does not inhibit estrogen or glucocorticoid receptor transactivation, suggesting that RXR is the target of As2O3–induced inhibitory signaling. We hypothesized that arsenic could inhibit LXR/RXR activation and, thus, alter the expression of genes involved in cholesterol homeostasis. We present evidence that arsenic alters LXR target gene promoters, leading to proatherogenic changes in cholesterol efflux. As2O3 does not inhibit the transpressive effects of LXR, supporting the hypothesis that RXR is the target of As2O3–induced signaling.

Methods

Cell Lines and Chemicals

RAW 264.7 murine macrophages and THP-1 human macrophages were grown in RPMI medium 1640 containing 10% fetal bovine serum and a combination of penicillin and streptomycin. As2O3 was purchased from Sigma-Aldrich and used to make a 0.1-mol/L stock in 0.1 N sodium hydroxide, with subsequent dilutions in PBS. (22R-OHC), 9-cis retinoic acid (9-cis-RA), chloroprenehydrothio (CPT)-cAMP, fatty acid–free BSA, and purified human Apo-AI were also purchased from Sigma-Aldrich. Long-term cultures were passed twice weekly with continuous culture in media, with or without 50 or 100 mmol/L As2O3. At the first pass of the week, aliquots of cells were used for mRNA analyses after induction with LXR/RXR ligands 22R-OHC/9-cis-RA.

Isolation of Primary Macrophages

Primary SV129 murine macrophages were elicited by intraperitoneal injection of 2 mL of a 10% thioglycollate solution in PBS. After 3 to 5 days, macrophages were harvested by peritoneal lavage with PBS. Adherent cell populations were cultured for 3 days and subsequently treated with LXR/RXR ligands, with or without As2O3. Human macrophages were isolated from healthy donor peripheral blood mononuclear cells after informed consent in accordance with the Jewish General Hospital Ethics Committee–approved protocol. In brief, 50 mL of blood was centrifuged through a Ficoll gradient, and monocytes were isolated after a 2-hour adhesion step. CD14 staining confirmed the monocytic nature of adherent cells. Macrophages were obtained by culturing monocytic cells in 800 IU/mL of granulocyte/macrophage colony-stimulating factor for an additional 6 days.

Reverse Cholesterol Efflux

RAW 264.7 murine macrophages (5×104 cells per well) were labeled in RPMI medium 1640 containing 4 µCi/mL of 1,2,3-N-[3H]cholesterol (PerkinElmer) for 24 hours. Unincorporated 3H was then removed by extensive washing in PBS containing 0.1% fatty acid–free BSA. Cells were subsequently stimulated with 22R-OHC/9-cis-RA or CPT-cAMP, with or without As2O3 in RPMI medium 1640 containing 0.2% fatty acid–free BSA for 6 hours. Cells were washed in PBS containing 0.1% fatty acid–free BSA, and reverse cholesterol efflux was performed by incubating the cells for 18 hours in RPMI medium 1640 containing 0.1% fatty acid–free BSA and 10 µg/mL of purified human Apo-AI. Media were harvested, and cells were extensively washed before lysis in 0.5 mL of 0.1 N sodium hydroxide. 3H was measured in both media and cell extracts, and the percentage of total cholesterol efflux was calculated as follows: percentage cholesterol efflux=([3H]in media/[3H in media + [3H in extracts])×100.

Quantitative Real-Time PCR

RNA was isolated using TRizol reagent (Invitrogen), followed by chloroform extraction and nucleic acid precipitation in isopropyl alcohol.24 RNA was quantitated on a spectrophotometer (NanoDrop ND-1000, Thermo Scientific, Wilmington, Del). cDNA was synthesized from 5 µg of RNA by priming with random primers, followed by reverse transcription in 160 U of enzyme (SSII RT; Invitrogen). Quantitative real-time PCR was performed using a commercially available system (7500 Fast PCR; Applied Biosystems). SYBR green chemistry was used with specific primers (listed in Supplemental Table I; available online at http://atvb.ahajournals.org) for all genes except human GAPDH, which was assessed using a VIC-labeled probe (Taqmam; Applied Biosystems).

Immunoblotting

Protein analysis was performed as previously described.25 Briefly, whole cell extracts were separated using sodium dodecyl sulfate–polyacrylamide gels. Proteins were blotted onto nitrocellulose membranes, which were blocked and hybridized overnight at 4°C with rabbit-α-human ABCA1 (Novus Biologicals, Littleton, CO), rabbit-α-SREBP-1 (Santa Cruz Biotechnology), rabbit-α-LXRα/β (Santa Cruz), or mouse-α-β-actin antibody (Sigma). Membranes were washed and incubated with appropriate secondary antibodies, followed by visualization with enhanced chemiluminescence (GE Healthcare Lifescience, Baie d’Urfé, QC).

Transient Transfection Assay

RAW 264.7 macrophages were transfected using media (OPTI-MEM) and fugene–6 reagent (Roche Applied Science) at a 3:1 ratio of fugene plus DNA following the manufacturer’s instructions. Cells were transfected with 0.5 µg of receptor expression plasmid, pKC-CMV-LXRx or β and 0.5 µg of the reporter plasmid, LXRE(3)-tk-Luc. Cells were treated as indicated and harvested by
passive lysis. Luminescence was measured on a luminometer (GloMax; Promega), and luciferase units were normalized against protein content.

**Mammalian 2 Hybrid**

Cos-1 cells were transfected for 24 hours, as previously described, with pACT-LXRα or β, pBIND-RXRα, and pG5-luc, a plasmid containing 5 copies of the Gal4 binding sites. Cells were then treated for 6 hours, as described. Plates were harvested, and luciferase activity was assessed using the SteadyGlo reagent (Promega).

**Chromatin Immunoprecipitation Assay**

The chromatin immunoprecipitation assays were performed as previously described.26,27 After treatment, protein and DNA complexes were immunoprecipitated overnight at 4°C with antibodies against acetylated histone H3 (Upstate Biotechnology, Lake Placid, NY); RXRα, LXRα/β, NCoR, and SRC-1 (Santa Cruz Biotechnology); and phosphorylated RNA Polymerase II (Abcam, Cambridge, Mass). Immune complexes were precipitated for 4 hours with 60 μL of salmon sperm DNA-protein A agarose slurry (Upstate Biotechnology). DNA-protein complexes were eluted from the beads overnight at 65°C with buffer containing 200-mmol/L sodium chloride to reverse the formaldehyde-induced cross-linking. DNA was recovered and purified the next day using PCR purification columns (Qiagen, Valencia, CA). Primers (Supplemental Table I) were used with SYBR green chemistry to amplify ABCA1 and SREBP-1c promoter LXREs or corresponding chromosome-matched noncoding sequences to control for amplification specificity. Samples were normalized against corresponding inputs.

**Statistical Analysis**

Results were analyzed using computer software (GraphPad Prism 4, GraphPad, San Diego, Calif). Significance was determined using a 1-way analysis of variance and, when possible, a multiple comparison test (Newman-Keuls or Tukey test).

**Results**

**Arsenic Inhibits LXR Transactivation, Gene Expression, and Physiological Function**

We hypothesized that inhibition of LXR transactivation by arsenic could lead to decreased cholesterol efflux and, therefore, favor a proatherogenic state within macrophages. Previously, we reported that As₂O₃ inhibits the activation of nuclear receptors that bind their cognate DNA response elements as heterodimers of the RXR24 in Cos-1 and leukemic cell lines. We first extended these findings to macrophages using transient transfection assays with LXRα or LXRβ expression vectors and the (LXRE)₃-tk-Luc reporter plasmid. RAW 264.7 murine macrophages were transfected, then treated with 4 μg/mL 22R-OHC and 10 μmol/L 9-cis-RA in the presence or absence of varying As₂O₃ concentrations for 6 hours. In these macrophages, as in other cell types, As₂O₃ inhibited LXRα- or LXRβ-dependent transactivation of the LXR responsive promoter (Figure 1A). We then extended these transactivation data to regulation of specific target gene expression. As₂O₃ treatment also inhibited LXR-mediated mRNA expression of 2 target genes, ABCA1 and SREBP-1c, in RAW 264.7 macrophages and the THP-1 human monocytic leukemia cell line, in a dose-dependent manner (Figure 1B). This decrease in mRNA expression correlated with a decrease in protein levels, as assessed by immunoblotting (Figure 1C and data not shown). To determine whether As₂O₃ inhibited LXR/RXR activation from either heterodimeric partner, we treated RAW 264.7 cells with either the LXR-specific ligand, GW3935, or the RXR-specific ligand, LG1268, in the presence or absence of arsenic and assessed ABCA1 and SREBP-1c gene expression. No matter which ligand was used to activate the heterodimer, As₂O₃ could inhibit gene expression in a dose-dependent manner (Figure 1D). To demonstrate the specificity of these ligands, small and interfering RNA was used to knock down expression of LXRα, LXRβ, or RXRα in RAW 264.7 cells. In our hands, we achieved an average knockdown of 64%, 45%, and 67%, respectively, as assessed by quantitative PCR (data not shown). We used small and interfering RNA against GAPDH (75% knockdown) as a control for the nonspecific effects of small and interfering RNA. Knockdown of LXRβ decreased GW3935 and 22R-OHC/9-cis-RA-induced SREBP-1c gene expression, whereas knockdown of RXRα decreased LG1268-induced SREBP-1c gene expression (Supplemental Figure 1A–C; available online at http://atvb.ahajournals.org). As₂O₃ inhibited the remaining transcriptional activation. Interestingly, knockdown of LXRα in our cells did not alter the response to GW3935 (Supplemental Figure 1D), suggesting that either LXRβ is the more important LXR isoform in our cells or we achieved insufficient knockdown of LXRα to inhibit signaling.

To extend our studies beyond transformed cell lines, we tested the ability of As₂O₃ to inhibit LXR target gene expression in primary macrophages. Thiglycollate-elicted murine or human macrophages, differentiated from peripheral blood mononuclear cells, were treated for 6 hours with 22R-OHC/9-cis-RA, with or without 1 μmol/L As₂O₃. ABCA1 and SREBP-1c expression levels were analyzed by quantitative PCR; these had a decreased response to LXR ligands after in vitro exposure to As₂O₃ (Figure 1E).

**Arsenic Does Not Alter cAMP-Induced ABCA1 Expression**

cAMP-mediated activation of protein kinase A (PKA) is also known to induce transcription of ABCA1 in an LXR-independent manner. To determine whether As₂O₃ treatment generally inhibits ABCA1 expression or specifically affects LXR-induced gene expression, we treated RAW 264.7 macrophages with 100 μmol/L CPT-cAMP, which upregulated ABCA1 mRNA and protein expression (Figure 2). As₂O₃ was not able to inhibit cAMP-induced ABCA1 mRNA (Figure 2A) or ABCA1 protein (Figure 2B). Thus, As₂O₃ does not affect the metabolic machinery nonspecifically; rather, it specifically inhibits activation of LXR target genes.

**Arsenic Inhibits LXR-Induced ApoA1-Specific Cholesterol Efflux**

Given the well-established role of LXR nuclear receptors in lipid metabolism and homeostasis, we hypothesized that decreased activation of LXR target genes, such as ABCA1, would have significant physiological effects on the ability of macrophages to efflux excess cholesterol. To address this question, we measured the ApoA1-specific efflux of
radiolabeled cholesterol induced by LXR/RXR ligands in the presence or absence of As$_2$O$_3$. As shown in Figure 3A, LXR ligand treatment for 6 hours induced a 3-fold increase of total effluxed cholesterol, which was significantly inhibited by As$_2$O$_3$ cotreatment, 5 μmol/L. Next, we tested whether As$_2$O$_3$ could inhibit cAMP-induced cholesterol efflux. cAMP induces significantly less cholesterol efflux relative to LXR ligands, consistent with lower ABCA1 mRNA induction and previous publications. In contrast to its interference with LXR ligands, As$_2$O$_3$ had no effect on cAMP-induced ApoA1-specific cholesterol efflux (Figure 3B), thereby confirming the specificity of As$_2$O$_3$ for activation of LXR target genes. In addition, we compared the effects of arsenic on ApoA1-specific efflux and passive nonspecific cholesterol efflux in RAW 264.7 macrophages. LXR/RXR ligands induce some passive efflux from the macrophage, although significantly less than when ApoA1 is added to the assay (Figure 3C). Arsenic did not alter background efflux and did not significantly alter the LXR/RXR ligand-induced and ApoA1-independent cholesterol efflux.

**Environmentally Relevant As$_2$O$_3$ Concentrations Inhibit LXR-Induced Gene Expression**

The previous experiments show a rapid and substantial inhibition of LXR ligand-induced cholesterol efflux at relatively high doses of arsenic, which exceed the levels to which many populations are exposed. Therefore, we inves-
tigated whether the effects on LXR-mediated transcription at these high short-term doses were also observed after longer treatment with doses less than or slightly higher than the Environmental Protection Agency maximum allowable limit (10 ppb) for arsenic in drinking water. We cultured RAW 264.7 macrophages over 25 weeks in 0, 50, or 100 nmol/L As$_2$O$_3$, corresponding to 0, 7.5, and 15 ppb arsenic, respectively. These doses of As$_2$O$_3$ did not induce apoptosis in short-term experiments; however, they did alter the growth rate of the cells after 22 weeks in culture. Although no change in doubling time was seen after weeks 0, 4, or 13, the doubling time increased from 1.64 to 2.83 days when cultured in 100 nmol/L As$_2$O$_3$ for 22 weeks. After the indicated weeks in culture, cells were challenged with 22R-OHC/9-cis-RA for 6 hours, and relative mRNA levels were assessed by quantitative PCR. Both As$_2$O$_3$ concentrations significantly decreased LXR-mediated response to ligands, as reflected by their impaired ability to upregulate ABCA1 and $SREBP$-1c mRNA after just 2 weeks of As$_2$O$_3$ exposure. This effect persisted to at least 25 weeks of arsenic exposure (Figure 4), suggesting that long-term/low-dose exposure may alter the ability of macrophages to maintain proper cholesterol levels.

As$_2$O$_3$ Does Not Alter Nuclear Receptor Expression or Heterodimerization

To determine how As$_2$O$_3$ inhibits LXR/RXR ligand-induced transactivation, we assessed whether As$_2$O$_3$ affected the level of LXR or RXR expression. As$_2$O$_3$ does not significantly alter the level of $LXR{\alpha}$ or $LXR{\beta}$ mRNA in RAW 264.7 cells in response to 22R-OHC/9-cis-RA (Supplemental Figure IIA). Many nuclear receptors are regulated by modulation of protein expression. Therefore, we also investigated whether As$_2$O$_3$ decreased $LXR{\alpha}/LXR{\beta}$ protein expression. Immunoblotting experiments, using extracts from RAW 264.7 and THP-1 cells treated with 22R-OHC/9-cis-RA in the absence or presence of 1 to 5 $\mu$mol/L of As$_2$O$_3$, showed no significant alteration of $LXR{\alpha}/LXR{\beta}$ protein levels (Supplemental Figure IIB). We confirmed the previously reported result that As$_2$O$_3$ does not alter RXR on either the mRNA or protein level and extended this to show that RXR and RXR on the mRNA expression is not significantly altered by As$_2$O$_3$ treatment (Supplemental Figure III, some data not shown).

Then, we assessed whether As$_2$O$_3$ affected the ability of LXR and RXR to form heterodimers, using mammalian 2-hybrid and coimmunoprecipitation assays. In mammalian 2-hybrid assays, LXR and LXR interacted with RXR in the absence of ligands. This association was not significantly altered by ligands with or without As$_2$O$_3$ (Supplemental Figure IVA). RXR was also coimmunoprecipitated from Cos-1 cells transfected with FLAG-tagged LXR or LXR. LXR/RXR binding was not affected by ligands or As$_2$O$_3$ treatment (Supplemental Figure IVB). Together, these data show that As$_2$O$_3$ does not inhibit LXR/RXR transactivation via decreased nuclear receptor expression or heterodimerization.

**Figure 2.** As$_2$O$_3$ inhibits ABCA1 through a cAMP-independent pathway. A, RAW 264.7 murine macrophages were induced with 100 $\mu$mol/L CPT-cAMP, with or without 1, 2.5, or 5 $\mu$mol/L As$_2$O$_3$ for 6 hours. RNA was isolated and converted to cDNA, and $ABCA1$ and GAPDH gene expression was analyzed by quantitative PCR. B, RAW 264.7 macrophages were treated as previously stated, and whole cell extracts were analyzed by Western blot for ABCA1 protein levels. $\beta$-Actin was used as a loading control. Blots are representative of 2 independent experiments.
As$_2$O$_3$ Alters Epigenetic Markers and Protein Occupancy on LXR Target Gene Promoters

Chromatin immunoprecipitation was performed to determine whether As$_2$O$_3$ affects ABCA1 and SREBP-1c expression by modulating changes in protein occupancy and chromatin structure at their respective promoters. Initially, we assessed the presence of endogenous LXR and RXR at the ABCA1 and SREBP-1c promoters. DNA/protein complexes were immunoprecipitated with either LXR/H$_9$251 or RXR/H$_9$252-specific antibodies, and the DNA was amplified using primers specific for the LXR response element (LXRE) on each promoter or chromosome-matched random sequences. LXR/H$_9$251 was recruited to both promoters after 30 minutes with LXR/RXR ligands; this was not altered by the addition of As$_2$O$_3$ (Figure 5A). Constitutive RXR/H$_9$251 binding to the ABCA1 promoter was not altered by As$_2$O$_3$ in the presence or absence of LXR/RXR ligands, and RXR/H$_9$251 recruitment to the SREBP-1c promoter by ligands was not affected by As$_2$O$_3$ (Figure 5A). This confirms and extends our data showing that As$_2$O$_3$ does not modulate LXR activity by altering the expression or heterodimerization of LXRs and RXR/H$_9$251.

Next, we immunoprecipitated DNA/protein complexes using an antibody specific for acetylation of histone H3, a marker for transcriptionally active chromatin. LXR/RXR ligands induced increased acetylation of histone H3 at both promoters, confirming an “active” epigenetic conformation (Figure 5B). As$_2$O$_3$ cotreatment blocked the ligand-induced increase in histone H3 acetylation at both promoters (Figure 5B), indicating that As$_2$O$_3$ can modulate the effects of LXR/RXR ligands on target promoter epigenetic markers. We confirmed these results by assessing the recruitment of phosphorylated RNA polymerase II (Pol II) to each promoter as a marker of active transcriptional machinery. As$_2$O$_3$ cotreatment significantly decreased the LXR/RXR ligand-induced recruitment of phosphorylated Pol II to each promoter (Figure 5B).

Ligand-dependent nuclear receptor activation is associated with a release of corepressor molecules from and a recruitment of coactivators to the transcriptional machinery. Therefore, we tested whether As$_2$O$_3$ altered the release of corepressors induced by ligand treatment. In Figure 5C, ligand treatment for 30 minutes resulted in a complete loss of NCoR binding at the promoters, correlating with increased transcription and markers of activation. As$_2$O$_3$ cotreatment inhibited the ligand-induced release of NCoR at both promoters (Fig-
Furthermore, the lack of NCoR release from the promoters correlated with a lack of recruitment of the SRC-1 coactivator (Figure 5C). Thus, As$_2$O$_3$ can inhibit the transcription of LXR target genes by modulating changes in their promoter structures and occupancy.

**As$_2$O$_3$ Does Not Inhibit the Transrepression Function of LXR**

Previously, we showed that As$_2$O$_3$ inhibits nuclear receptor function via effects on the RXR heterodimer. If the inhibition of LXR/RXR transactivation presented herein is the result of As$_2$O$_3$ effects on RXR, we postulate that LXR transrepression of inflammatory target genes, an RXR-independent process, would not be altered by As$_2$O$_3$ cotreatment. Therefore, we assessed the expression of 2 inflammatory genes (COX-2 and IL-1β) after induction with lipopolysaccharide, with or without LXR/RXR ligands, in the absence or presence of arsenic. As$_2$O$_3$ can induce proinflammatory signaling$^{10,11}$; therefore, it is not surprising that As$_2$O$_3$ enhanced lipopolysaccharide-induced expression of COX-2 and IL-1β mRNA (Figure 6). As shown previously,$^{23}$ activation of LXR inhibited lipopolysaccharide-induced inflammatory gene expression (Figure 6). More important, the addition of As$_2$O$_3$ does not inhibit LXR transrepression, suggesting that inhibition of
LXR transactivation by As$_2$O$_3$ is the result of inhibitory effects on RXR.

**Discussion**

Exposure to arsenic is increasingly linked to adverse effects on cardiovascular health, including increased atherosclerosis, hypotension, ischemic heart disease, and arteriosclerosis. Despite this mounting evidence, the molecular mechanisms involved remain unresolved. We show that macrophages are targets of arsenic signaling in a manner that may result in lipid accumulation and atherogenesis. Reverse cholesterol efflux removes lipids from the periphery, including macrophages, and transports them to the liver for excretion. This process is controlled, in part, by LXR-targeted gene expression. We show that arsenic inhibits LXR-dependent gene expression in both mouse and human cell lines and primary macrophages (Figure 1). The biological significance of this decrease in transcriptional activation of LXR promoters was confirmed by decreased mRNA and protein expression of the targets ABCA1 and SREBP-1c (Figure 1C). Impairment of these promoters was reflected in decreased cellular cholesterol efflux (Figure 2). Arsenic inhibition of LXR target gene transcription correlated with decreased epigenetic markers of active chromatin (Figure 5B) and increased binding of the corepressor NCoR (Figure 5C).

Arsenic impairs nuclear receptor signaling by inhibiting RXR. As$_2$O$_3$ inhibits target gene induction by both LXR- and RXR-specific ligands (Figure 1D), as expected for the permissive LXR/RXR heterodimer. However, As$_2$O$_3$ co-treatment does not alter the RXR-independent transrepressive function of LXR (Figure 6). These data support the conclusion that RXR, rather than the heterodimeric partner, is the target for As$_2$O$_3$. Based on these data, we propose that As$_2$O$_3$ can inhibit peroxisome proliferator-activated receptor (PPAR) activation, but not transrepression. PPARγ binds to the promoter and induces expression of LXRα in macrophages; therefore, an inhibition of PPARγ could account for a decrease in LXR transactivation. However, when we treated cells with a PPARγ antagonist, no changes in As$_2$O$_3$ inhibition of LXR target genes were observed (Supplemental Figure V). Because of the permissive nature of the LXR/RXR heterodimer, we cannot exclude the direct effects on both nuclear receptors; however, in the context of previous data, RXR is a likely target.

Many of the previously published studies linking arsenic with atherogenesis were performed in either endothelial or vascular smooth muscle cells (VSMCs). Endothelial cells upregulate nuclear factor-κB in response to arsenic, which results in increased tumor necrosis factor α and IL-8 production. With arsenic treatment, VSMCs exhibit increased reactive oxygen species and increased monocyte chemoattractant protein 1 and IL-6, which can increase macrophage/monocyte attachment and migration to VSMCs. Despite this, relatively little is known about the effects of arsenic on macrophages. Human macrophages upregulate NADPH oxidase in response to arsenic exposure in vitro, thereby causing increased oxidative stress and inflammation, both of which are known to be proatherogenic. Our findings that arsenic inhibits LXR-induced reverse cholesterol efflux in macrophages add another mechanism by which arsenic exposure may lead to increased atherosclerosis.

We show that low arsenic concentrations (50 to 100 nmol/L) significantly affect LXR-mediated gene expression and function after extended in vitro exposure. The maximum contaminant level of arsenic is set at 10 ppb by the Environmental Protection Agency; however, many people are exposed to much higher arsenic levels via contaminated well water (50 to 250 ppb). Our data show that even arsenic levels less than the maximum contaminant level have profound effects on gene expression. This correlates with data from other laboratories that show that 50 ppb arsenic significantly affects VSMC remodeling in vitro, endothelial cell proliferation, and fenestration in vivo. In fact, roxarsone, an organic arsenical used extensively in the poultry industry, stimulates growth of endothelial cells at 10 nmol/L concentrations. These data suggest that arsenic has an adverse effect on cardiovascular health, even at doses at which carcinogenic effects may not be seen, suggesting that the cardiovascular system may be more sensitive to the toxic effects of arsenic. Finally, the understanding of molecular changes induced by arsenic at low doses may lead to the identification of appropriate biomarkers and potential interventions.

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**Disclosures**

None.

**References**


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Supplementary Figure I. Knock-down of LXRβ and RXRα inhibits ligand-induced gene expression, but does not alter inhibition by As$_2$O$_3$. RAW 264.7 macrophages were transfected with 115 pmol siRNA from Ambion against LXRα (s75783), LXRβ (s75786), RXRα (s73217), or GAPDH (4390849) using the Amaxa nucleofector. After 24 hours, cells were treated with 1 μM GW3935 (A &D), 1 μM LG1268 (C), or 4 μg/ml 22R-OHC and 10 μM 9cisRA (B) +/- 5 μM As$_2$O$_3$ for 6 hours. RNA was isolated, converted to cDNA, and gene expression analyzed by qPCR. *** corresponds to statistically significant decrease as compared to siGAPDH control p < 0.001.
Supplementary Figure II. As$_2$O$_3$ does not affect nuclear receptor mRNA and protein levels. A) RAW 264.7 murine macrophages were induced with 4 µg/ml 22R-OHC and 10 µM 9cisRA +/- 1, 2.5, or 5 µM As$_2$O$_3$ for 2 hours. RNA was isolated and converted to cDNA, and LXR$\alpha$, LXR$\beta$, and GAPDH gene expression was analyzed by qPCR. * p < 0.001, as compared to control. B) RAW 264.7 and THP-1 macrophages were treated as above, and whole cell extracts analyzed by western blot for LXR$\alpha$ and LXR$\beta$. β-actin was used as a loading controls. Blots and densitometry are representative of two independent experiments.

Supplementary Figure III. As$_2$O$_3$ does not affect RXR isoform mRNA expression in RAW 264.7 cells. Cells were treated for 6 hours with media, 22R-OHC/9cisRA, 2.5 µM As$_2$O$_3$, or the combination. RNA was harvested and converted to cDNA. Isoform specific primers were used to determine expression of RXR$\alpha$, β, and γ. RXRγ expression was very low, leading to significantly higher Ct values, and therefore, data are shown with murine heart RXRγ expression.

Supplementary Figure IV. As$_2$O$_3$ does not affect LXR/RXR heterodimerization. A) Cos-1 cells were transfected with FLAG-LXR$\alpha$ (top) or FLAG-LXR$\beta$ (bottom) and induced with 4 µg/ml 22R-OHC and 10 µM 9cisRA +/- 5 µM As$_2$O$_3$ for 1 and 2 hours. Whole cell extracts were immunoprecipitated with an anti-Flag antibody, and complexes visualized by western blot of RXR$\alpha$. B) Cos-1 cells were transfected with VP16-RXR$\alpha$, Gal4-LXR$\alpha$, or Gal4-LXR$\beta$, and pG5-Luc reporter vector. Cells were subsequently treated overnight with 4 µg/ml 22R-OHC and 10 µM 9cisRA +/- 5 µM As$_2$O$_3$. Cells were harvested and binding analyzed by luciferase assay. Luciferase values were
normalized to protein concentrations. Graph is representative of two independent experiments.

**Supplementary Figure V. Inhibition of PPAR does not affect As2O3-induced inhibition of LXR transactivation.** A) RAW 264.7 cells were transfected with PPRE(3)-tk-luc and PPARγ plasmids and treated with ciglitazone (CG) in the presence and absence of 1-100 nM T0070907, a PPARγ antagonist. After 24 hours, cells were harvested and luciferase measured. Results are normalized to protein. B) RAW 264.7 cells were treated with 4 µg/ml 22R-OHC and 10 µM 9cisRA +/- 5 µM As2O3 in the presence and absence of the PPARγ antagonist T0070907 (100 nM). RNA was harvested after 6 hours and converted to cDNA. SREBP-1c and 36B4 gene expression was determined.
Supplementary Data I
Supplementary Data II

A

LXRα

LXRβ

Media

1 µM As₂O₃

2.5 µM As₂O₃

5 µM As₂O₃

B

22(R)/9c

As₂O₃ (µM)

WB: LXRα/β

WB: β-Actin

RAW

WB: LXRα/β

WB: β-Actin

THP-1

RAW

THP-1
Supplementary Data IV

**A**

![Bar chart showing luciferase activity per mg protein](chart.png)

**B**

<table>
<thead>
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<th>Condition</th>
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<tbody>
<tr>
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<td>--</td>
<td>+</td>
</tr>
<tr>
<td>22(R)/9c</td>
<td>--</td>
<td>+</td>
</tr>
<tr>
<td>As$_2$O$_3$ (5 μM)</td>
<td>--</td>
<td>+</td>
</tr>
</tbody>
</table>

**IP:** α-Flag-LXRα

**WB:** RXRα

**WB:** Flag (5% input)

**Mock**

<table>
<thead>
<tr>
<th>1 hr</th>
<th>2 hr</th>
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<tbody>
<tr>
<td>--</td>
<td>+</td>
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**IP:** α-Flag-LXRβ

**WB:** RXRα

**WB:** Flag (5% input)