Unfolded Protein Response Signaling and MAP Kinase Pathways Underlie Pathogenesis of Arsenic-Induced Cutaneous Inflammation

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Abstract
Arsenic exposure through drinking water is a major global public health problem and is associated with an enhanced risk of various cancers including skin cancer. In human skin, arsenic induces precancerous melanosis and keratosis, which may progress to basal cell and squamous cell carcinoma. However, the mechanism by which these pathophysiologic alterations occur remains elusive. In this study, we showed that subchronic arsenic exposure to SKH-1 mice induced unfolded protein response (UPR) signaling regulated by proteins, inositol-requiring enzyme-1 (IRE1), PKR-like endoplasmic reticulum kinase (PERK) and activating transcription factor 6 (ATF6). Arsenic activated all three UPR regulatory proteins in the skin. Arsenic induced IRE1 phosphorylation which resulted in augmented splicing of X-box binding protein 1 (XBP-1) leading to its migration to the nucleus, and also enhanced transcriptional activation of downstream target proteins. Hyperphosphorylation of PERK which induces eukaryotic translation initial factor 2 alpha (eIF2α) in a phosphorylation-dependent manner enhanced translation of ATF4, in addition to augmenting proteolytic activation of ATF6 in arsenic-treated skin. A similar increase in the expression of CHOP was observed. Enhanced XBP-1s, ATF4, and ATF6 regulated downstream chaperones GRP94 and GRP78. In addition, arsenic induced inflammation-related p38/MAPKAPK-2 MAPK signaling and alterations in Th-1/Th-2/Th-17 cytokines/chemokines and their receptors. Antioxidant N-acetyl cysteine blocked arsenic-induced reactive oxygen species, with a concomitant attenuation of UPR and mitogen-activated protein kinase (MAPK) signaling and proinflammatory cytokine/chemokine signatures. Our results identify novel pathways involved in the pathogenesis of arsenic-mediated cutaneous inflammation which may also be related to enhanced cancer risk in arsenic exposed cohorts. Cancer Prev Res; 4(12); 1–9. ©2011 AACR.

Introduction
Exposure to arsenic, a highly toxic metalloid, occurs through occupational and environmental pollution. More recently, contamination of groundwater due to interaction of rocks and minerals containing soluble arsenic caused widespread chronic human arsenic exposure in geographical areas including Bangladesh, Taiwan, Mexico, Mongolia, Argentina, India, Chile, and in some parts of the United States. Approximately 100 million people are exposed to toxic concentrations of arsenic worldwide (1, 2), which is associated with enhanced morbidity and mortality (3). Enhanced risk for various cancers including bladder, kidney, lung, and skin has been associated with arsenic exposure in a dose-dependent manner (4). However, the mechanism by which arsenic causes toxic and carcinogenic manifestations remains largely unknown.

Endoplasmic reticulum (ER) is the site of biosynthesis, folding, assembly, and maturation of many secretory and membrane-bound proteins. Increased demand for folded proteins disturbs homeostasis of ER and may result in accumulation of unfolded or misfolded proteins leading to the condition known as ER stress. Unfolded protein response (UPR) signaling restores the protein-folding capacity of ER and provides a balance between protein-folding overload and impaired ER capacity. This is done in a translational- and transcriptional-dependent manner by engaging 3 ER membrane resident proteins, PERK, IRE1, and ATF6 (5). In addition to restoring protein folding capacity of ER, UPR is involved in obesity, diabetes, atherosclerosis, neurodegenerative diseases, inflammation, and cancers (6, 7). Some of these conditions have also been reported in arsenic-exposed populations (1, 3, 4).
Arsenic induces precancerous lesions, melanosis, and keratosis some of which progress to basal cell carcinoma (BCC) and squamous cell carcinoma (SCC; refs. 8, 9). We hypothesized that one mechanism by which these early changes occur in arsenic exposed populations may involve activation of UPR signaling pathways. UPR signaling may mediate cutaneous inflammation and enhance cancer risk (6, 7, 10). In this study, we used SKH-1 hairless mice as a murine model to study the pathogenesis of arsenic-induced inflammation. Our data showed that arsenic induced UPR signaling by activating 3 classic pathways conserved throughout the eukaryotic system, and concomitantly activated proinflammatory p38 mitogen-activated protein kinase (MAPK) and cytokine/chemokine signaling pathways. Interestingly, arsenic induced these signaling pathways in a reactive oxygen species (ROS)-dependent manner and antioxidant N-acetyl cysteine (NAC) treatment attenuated some of these effects. These data provide a novel mechanism by which toxic and carcinogenic effects of arsenic are mediated.

Materials and Methods

Reagents

Primary antibodies as listed in Supplementary Table S1 and horseradish peroxidase (HRP) secondary antibodies were purchased. CM-H2DCFDA was obtained from Invitrogen. Sodium arsenite and NAC were from Sigma Chemical Co. Primers were synthesized by Invitrogen Co. PCR array plates (PAMM-011A-24), RT2 First Strand kit (C-03), and RT2 qPCR Master Mix (PA-011-12) were from SABiosciences.

Animal model

In the first experiment, 25 age-matched SKH-1 hairless mice (5 mice per group) were fed ad libitum, respectively, drinking water containing arsenic at 0, 50, 100, and 200 ppm concentrations for a period of 1 month. Then all of these animals were killed, their skin excised and processed for histology/immunohistochemistry/immunofluorescence studies or Western blot/PCR analysis. The dose selection in the current experiments is based on 10-year consumption of arsenic by a human population in geographical areas with high arsenic levels in drinking water (150.1–864.0 μg/L) considering an average consumption of 15.5 L water/person/day. This population manifests various cutaneous lesions (3). We also studied the effects of arsenic at a dose level of 300 ppm. However, this dose was cytotoxic and did not follow the dose response relationship exhibited by the majority of other doses, except that related to inflammatory response assessment. Therefore, we described only the inflammatory effects related to this dose. In a separate experiment, 15 age-matched SKH-1 mice divided into 3 groups of 5 mice each received no treatment or arsenic (200 ppm) or arsenic (200 ppm) + NAC (150 mg/kg body weight, intraperitoneally). Arsenic in these groups was administered for a period of 4 weeks. However, the NAC treatment was given for 7 days once daily prior to the termination of the experiment. At the termination of the experiment, skin samples were collected for analysis as described above.

Western blot analysis

Skin tissues were homogenized in an ice-cold lysis buffer (50 mmol/L Tris, pH 7.5, 1% Triton X-100, 0.25% NaF, 10 mmol/L β-glycerolphosphate, 2 mmol/L EDTA, 5 mmol/L sodium pyrophosphate, 1 mmol/L Na3VO4, 10 mmol/L DTT, and protease inhibitor). Clear lysate was prepared by centrifugation at 10,000 × g for 10 minutes. Extracts were aliquoted in small volumes and stored at −80°C before use. Aliquots of total tissue homogenates were mixed with 4× loading buffer, boiled for 5 minutes, and subjected to SDS-PAGE. Proteins were electrophoretically transferred to polyvinylidene difluoride membranes and then nonspecific sites were blocked with 5% (W/V) nonfat dry milk in TBST (25 mmol/L Tris-HCl, pH 7.5; 150 mmol/L NaCl; 0.05% Tween-20) for 1 hour at room temperature followed by probing with primary antibody overnight at 4°C or 1 hour at room temperature. The membranes were incubated for 1 hour with HRP-conjugated secondary antibody. The blots were developed with enhanced chemiluminescence (ECL) according to the manufacturer's instructions (Amersham). In most cases, 40 μg protein was loaded. However, to detect phosphorylation of PERK, 100 μg lysates were subjected to 6% SDS-PAGE to obtain a better resolution. The membranes were probed with anti-PERK antibody and developed by ECL as described above. At least 3 independent samples from each group were used for Western blot analysis. The integrated density of bands was measured with Image J. Statistical analysis was conducted using Excel 2003.

Immunofluorescent staining

Strips (1 × 0.4 cm²) of skin were fixed in cold formalin solution overnight at 4°C. The sections were dehydrated in ethanol and xylene, rehydrated, and treated for antigen unmasking. After blocking with 2% BSA/PBS, primary antibodies were added (diluted in 2% BSA/PBS) and slides were incubated overnight at 4°C. Followed by incubation with Alexa Fluor 596–conjugated anti-goat or rabbit secondary antibody for 1 hour. After removal of antibodies, slides were rinsed with PBS and mounted with mounting medium containing 4′, 6 diamidino 2 phenylindole (DAPI; Vector). Fluorescence was immediately recorded on an Olympus EX51 microscope.

Reverse transcription PCR

Total RNA was isolated from skin according to manufacturer's protocol using TRizol reagent (Catalog No. 15596-026) extraction kit (Invitrogen). RNA concentration and purity were determined by measuring OD260 and OD260/280. A total of 1 μg of RNA was used for reverse transcription using iScript cDNA synthesis kit (Bio-Rad). Primers used in this study are described in Supplementary Table S2.
PCR array

PCR was done using SABiosciences PCR Array System. First-strand cDNA synthesis was done using RT² First Strand kit. Real-time PCR was done with Mouse Inflammatory Cytokines & Receptors PCR Arrays on the iQ5 (Bio-rad) using RT² qPCR Master Mix. The program was 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. For each group, 3 skin samples were used for PCR array analysis. Relative fold changes of gene expression were calculated according to the manufacturer’s instruction and software.

Measurement of ROS

Freshly cut OCT (optimal cutting temperature compound)-embedded cryosections (4–6 μm) were incubated with 10 μmol/L CM-H2DCFDA dissolved in ACAS buffer (127 mmol/L NaCl, 0.8 mmol/L MgCl₂, 3.8 mmol/L KCl, 1.2 mmol/L KH₂PO₄, 1.2 mmol/L CaCl₂, 5 mmol/L glucose, and 10 mmol/L HEPEs, pH = 7.4) for 1 hour at 27°C followed by two 5-minute washes with PBS in dark. Slides were mounted with mounting medium containing DAPI (Vector) and visualized under microscope. Skin sections incubated with ACAS buffer only served as negative control.

Statistical analysis

Statistical analysis was performed using the Student t test, P < 0.05 was considered to be statistically significant.

Results

Arsenic activates cutaneous UPR signaling

UPR signals through the activation of 3 distinct pathways which are mediated via PERK, IRE1α, and ATF6α as described earlier. To test whether arsenic can activate one or more of these pathways, we first examined the expression of PERK in the skin of arsenic-treated mice. A shifted band of PERK represented phosphorylated PERK. Phospho-PERK expression increased in all arsenic treatment groups (Fig. 1A). Consistently, the downstream target proteins, including p-eIF2α, ATF4, and CHOP, were enhanced following arsenic treatment. GADD34, a negative regulator of p-eIF2α, was also increased in arsenic-treated animals suggesting an intact adaptive response. Activation of the IRE1α pathway was confirmed by an increase in the expression of IRE1α. The subsequent accumulation of spliced XBP-1 at both protein and mRNA levels (Fig. 1B) suggest the activation of the IRE1α pathway. The control skin showed cytosolic expression of XBP-1 whereas arsenic-treated skin showed nuclear expression. Translocation of XBP-1 to the nucleus (Fig. 1D) also confirms activation of the IRE1α pathway. However, we could not record phosphorylated IRE1α levels.

Similarly, we observed that ATF6α p90 and ATF6α p50 were both significantly upregulated following arsenic treatment (Fig. 1A). The 2 other important UPR signaling proteins GRP78 and GRP94 were also increased significantly (Fig. 1C). We also recorded significant increase of CHOP and GRP78 in mRNA level (Fig. 1B). Immunofluorescent staining for CHOP, XBP-1, ATF6α, and GRP78 in the skin showed that in control animals low levels of these proteins were localized in the basal layer of epidermis. However, their increased expression and nuclear localization (Fig. 1D) both in basal and suprabasal epidermis confirmed activation of these UPR pathways following arsenic exposure.

Arsenic-induced ROS acts upstream of UPR signaling

Previously, we and others showed that arsenic induces ROS production in vitro in cells in culture (11, 12). To test whether arsenic also induces cutaneous ROS production, we used a fluorescent probe, CM-H2DCFDA which under oxidative stress generates a fluorescent metabolite detectable under the fluorescent microscope. Enhanced tissue fluorescence serves as a marker of ROS production. ROS is known to play an important role in UPR signaling during the pathogenesis of various cancers (7, 13). Significantly enhanced epidermal fluorescent staining was observed in arsenic-treated animals compared with vehicle-treated controls (Fig. 2A). Arsenic-enhanced fluorescent staining attenuated following pretreatment of arsenic-exposed animals with antioxidant NAC, confirming that arsenic also augments ROS production in the skin (Fig. 2A). To show that ROS production by arsenic is upstream of arsenic-mediated enhancement in UPR signaling, we tested whether NAC treatment can dampen UPR signaling. As shown in Fig. 2B, arsenic-mediated enhancement of the expression of UPR marker proteins, p-eIF2α, XBP-1s, and ATF6α was decreased significantly (P < 0.02) in the NAC treatment group which correlated with the decrease in ROS production in the epidermis of these animals. Furthermore, the relative expression levels of UPR signaling readout proteins, GRP78 and GRP94 showed identical decrease (Fig. 2B). These data show that arsenic-induced UPR signaling in the skin of SKH-1 mice was regulated by ROS production.

Arsenic enhances cutaneous inflammation via UPR signaling

Cutaneous inflammation is observed in arsenic exposed humans and is associated with enhanced keratosis and pitting of the palms and soles (8, 9, 14). We therefore tested whether arsenic mediated similar inflammatory changes in murine skin. For this, we assessed inflammatory signaling pathways, MAPK and cytokine/chemokine signaling, which were found to be associated with cutaneous inflammation (15, 16). Arsenic treatment increased expression of p-p38, and p-MAPKAPK-2. Although the basal levels of MAPKAPK-2 were significantly augmented, p38 levels remained largely unaffected (Fig. 2C). Similarly, we did not detect significant changes in the levels of total or p-ERK (data not shown). We also examined whether ROS production contributed to the induction of p38 MAPK signaling. The ability of NAC to significantly diminish the levels of arsenic-induced p-p38, p-MAPKAPK-2, and MAPKAPK-2 in this study (Fig. 2D) suggests that ROS production triggered arsenic-mediated inflammation.
Figure 1. Arsenic induces UPR signaling in the skin of SKH-1 hairless mice. A, Western blot analysis and relative expression level of PERK, p-eIF2α, GADD34, ATF4, CHOP, IRE1α, XBP-1s, and ATF6α (\( P < 0.05; ^{*}; P < 0.01 \)). B, RT-PCR showing the mRNA expression of XBP-1u, XBP-1s, CHOP, and GRP78. 18s rRNA was used as loading control. C, Western blot analysis and relative expression level of epidermal GRP78 and GRP94 (\( P < 0.05; ^{*}; P < 0.01 \)). D, immunofluorescent staining for cutaneous CHOP, XBP-1, ATF6α, and GRP78. For this, skin sections were obtained from control and arsenic (200 ppm)-treated animals. Original magnification, 200×. Insets represent magnified (×2) epidermal staining of these proteins. The region above the dotted white line represents epidermis whereas the region below this line is dermis. The larger scale bar is 50 μm and the smaller scale bar is 10 μm. Each staining is representative of 3 independent samples. Arrows in control skin indicate baseline cytoplasmic localization of these proteins whereas arrows in arsenic-treated skin indicate nuclear localization of CHOP, XBP-1, and ATF6α and increased expression of GRP78 in the cytoplasm. Skin samples in A, B, and C were from SKH-1 hairless mice fed on arsenic ad libitum in drinking water at 0, 50, 100, and 200 ppm.
Figure 2. Arsenic-induced UPR and MAPK signaling pathways are dependent on ROS production. A, images showing fluorescence of oxidized CM-H2DCFDA in the epidermis as outlined by the dotted lines (original magnification, 200×). The area above the dotted lines shows some nonspecific fluorescent staining of the stratum corneum. The area below the dotted lines represents dermis. Each staining is representative of 3 independent samples; scale bar, 50 μm. B, Western blot analysis and relative expression level of epidermal p-eIF2α, XBP-1s, ATF6α p90, ATF6α p50, β-Actin, GRP78, and GRP94 (△, P < 0.05; △△, P < 0.01). C and D, Western blot analysis and relative expression levels of epidermal p-p38, p38, p-MAPKAPK-2, and MAPKAPK-2 (*, P < 0.05; **, P < 0.01). Skin samples in A, B, and D were taken from animals in control, arsenic (200 ppm), and arsenic (200 ppm) + NAC (150 mg/kg) groups, whereas those in C were from 0, 50, 100, and 200 ppm arsenic-treated animals.
Furthermore, using a mouse inflammatory cytokine/chemokine PCR array containing 84 genes, we detected gene expression signatures characterizing Th1, Th2, and Th17 microenvironment as described in Supplementary Tables S3 and S4. Arsenic upregulated 24 genes whereas it downregulated 20 genes and the remaining 43 genes were not significantly altered (Fig. 3A and B). Among these markers, IL-1β (encoding interleukin 1 beta, a keratinocyte mitogen) and TNF (encoding tumor necrosis factor) which have been shown to induce UPR in other systems (17, 18) were and TNF (encoding tumor necrosis factor) which have been shown to induce UPR in other systems (17, 18) were significantly altered among Th2-related markers, IL-20 (encoding interleukin 20), which regulates proliferation and differentiation of keratinocytes, was dramatically reduced by 6- to 20-fold following arsenic treatment. Ccl1 [encoding chemokine (C-C motif) ligand 1] and IL-15 (encoding interleukin 15) also decreased significantly in arsenic treatment groups. Th17-related markers, Cxcl5 (encoding C-X-C motif chemokine 5) and Cxcl1 [encoding C-X-C motif chemokine 1], showed significant changes in their expression. However, at arsenic dose 300 ppm we also observed significant increase of 2-fold in the expression of Spp1 encoding secreted phosphoprotein 1, which is known to polarize immune response to Th1 type (19). Similarly, a significant decrease in the expression of Ccl22 encoding Chemokine (C-C motif) ligand 22 was observed at this dose. Concomitant

![Image](cancerpreventionresearch.aacrjournals.org)
with its effect on ROS production, UPR and MAPK signaling activation, NAC treatment also ameliorated cytokine/chemokine expression profile. Upon NAC treatment among 15 genes which were significantly upregulated, 12 genes including 3 Th1 markers (IL-1β, Ccl4, and TNF) were significantly reversed. However, of the 14 downregulated genes (including 3 Th2 markers, IL-20, Ccl1 and IL-15; and 2 Th17 markers, IL-15 and Cxcl5) only 3 (CX3cl1, Abcf1, Tollip1) showed a significant recovery (data not shown). Although we and others observed a number of biochemical changes in the skin of SKH-1 mice following arsenic exposure through drinking water, visually no pathobiologic effects were observed in these animals. Arsenic treatment by itself does not induce skin cancer or hyperkeratosis in any of the currently known murine models. The only murine model so far described for arsenic-induced cancer is the in utero transplacental treatment model (20).

Discussion

Arsenic is a known human cutaneous toxicant and carcinogen (1, 2). In this paper we describe a novel mechanism by which arsenic induces cutaneous inflammation (Fig. 4). We showed that arsenic activated all 3 known UPR signaling

![Flow diagram showing arsenic-mediated UPR, MAPK signaling, and inflammation in murine skin. Arsenic triggers ROS production in the skin and induces accumulation of unfolded proteins in ER which causes ER stress. Under stress condition, the chaperone GRP78 dissociates from ER membrane resident sensors PERK, IRE1α, and ATF6α, which leads to their phosphorylation or proteolysis-dependent activation. Activated IRE1α functions as a nuclease and splices XBP-1 mRNA. XBP-1s is translated into a potent transcription factor which activates the transcription of UPR target genes including GRP78 and GRP94. Activated PERK phosphorylates eIF2α which causes global translation attenuation and selectively upregulates the translation of ATF4 mRNA. Following migration into nucleus, ATF4 activates the transcription of its downstream UPR target genes such as CHOP indicating the activation of PERK-dependent signaling. Activated ATF6α translocates from ER to Golgi where it is cleaved by S1P or S2P proteases producing an active transcription factor ATF6α p50. ATF6α p50 also migrates into nucleus and leads to transcriptional activation of UPR target genes in the skin. In addition, arsenic-induced ROS activates p38 MAPK and its downstream protein MAPKAPK-2. Activation of UPR and MAPK signaling both contribute to the onset of inflammation in the skin. However, the antioxidant NAC treatment reduces ROS production and at least partially attenuates UPR and MAPK signaling as well as inflammatory cytokines/chemokines.](attachment://flowchart.png)
pathways, indicating that ER stress is one of the major underlying mechanisms of arsenic toxicity/carcinogenicity. These results are consistent with the current notion that ER stress is involved in the pathobiology of multiple disease conditions including inflammation and cancer (6, 7). In the skin, UPR pathway is known to alter differentiation (21) which is consistent with the frequent appearance of hyperkeratosis in arsenic-exposed humans. Skin cancer and other cutaneous manifestations are usually observed following a decade of arsenic exposure, particularly in geographical areas of high arsenic levels in water. We therefore used relatively higher arsenic levels in our animal experiments to unravel the molecular mechanisms associated with high arsenic. Our observations that arsenic activated UPR signaling which is linked to cutaneous inflammation may also partially explain the observed augmented skin cancer risk in exposed populations (10).

The demonstration in this study that NAC blocked arsenic-mediated ROS production with a concomitant attenuation of UPR, MAPK, and other cytokine/chemokine signaling pathways suggests that arsenic-mediated ROS production triggered these pathways, and that blocking ROS production in exposed populations may be beneficial in reducing inflammation and possibly cancer as predicted in Fig. 4. Our results confirmed other studies showing that NAC as well as other antioxidants protect against arsenic-associated cutaneous toxicity (22). In this regard, the results from a recent NIH/National Cancer Institute clinical trial in Bangladesh (http://projectreporter.nih.gov/project_info_ description.cfm?icde=0&aid=8009229) indicated an advantage of antioxidants in reducing toxic manifestations of arsenic in humans. Further results from this ongoing study will clarify whether identical mechanisms invoke arsenic-mediated pathogenesis of skin diseases in murine models and in humans.

Although both ROS and UPR pathways regulate inflammatory response independently (6, 15), it is not clear from our studies whether the observed cutaneous inflammatory response was attributable to crosstalk between the 2 pathways or to each pathway independently. The partial but significant recovery of MAPK signaling following NAC treatment coupled with identical cytokine/chemokine signaling gene profile suggests that UPR and/or other undefined ROS-independent factors regulate arsenic-mediated cutaneous inflammation. Defining these intricate relationships is beyond the scope of this report. However, it is known that under certain experimental settings MAPK signaling regulates both the expression of inflammation provoking and UPR signaling related proteins (23). In summary, our data indicate that UPR signaling is involved in arsenic-mediated cutaneous pathobiology and that production of ROS plays a key role in triggering this response.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed

Grant Support

This work was supported by R21ES017494 to M. Athar. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received July 5, 2011; revised August 18, 2011; accepted September 7, 2011, published OnlineFirst September 12, 2011.

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Published OnlineFirst September 12, 2011; DOI: 10.1158/1940-6207.CAPR-11-0343


Cancer Prevention Research

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Cancer Prev Res. Published OnlineFirst September 12, 2011.

Updated version
Access the most recent version of this article at:
doi:10.1158/1940-6207.CAPR-11-0343

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