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

Changzhao Li¹,², Jianmin Xu¹, Fugui Li¹, Sandeep C. Chaudhary¹, Zhiping Weng¹, Jianming Wen², Craig A. Elmets¹, Habibul Ahsan³ and Mohammad Athar¹

¹Department of Dermatology and Skin Diseases Research Center, University of Alabama at Birmingham, Birmingham, Alabama, USA.
²Department of Pathology, The First Affiliated Hospital, Sun Yat-Sen University, Guangzhou, Guangdong, China.
³Center for Cancer Epidemiology and Prevention, University of Chicago, Chicago, IL, USA.

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Address all correspondence to: Mohammad Athar, Department of Dermatology, The University of Alabama at Birmingham, Volker Hall, Room 509, 1530 3rd Avenue South, Birmingham, Alabama 35294-0019, USA. Phone: (205) 934-7554; Fax: (205) 934-7500; E-mail: mathar@uab.edu.

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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 pathophysiological alterations occur remains elusive. In this study, we showed that sub-chronic 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 α (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. Additionally, 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 MAPK signaling and pro-inflammatory 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.

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 USA. 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 secretary 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 three 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 (BCC) and squamous cell carcinoma (SCC) (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 employed 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 three classic pathways conserved throughout the eukaryotic system, and concomitantly activated pro-inflammatory p38 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 supplemental Table 1 and HRP-secondary antibodies were purchased. CM-H2DCFDA was obtained from Invitrogen Co.
(Carlsbad, CA). Sodium arsenite and NAC were from Sigma Chemical Co. (St. Louis, MO). Primers were synthesized by Invitrogen Co. (Carlsbad, CA). PCR array plates (PAMM-011A-24), RT² First Strand kit (C-03) and RT² qPCR Master Mix (PA-011-12) were from SABiosciences (Frederick, MD).

Animal model

In the first experiment, twenty-five age-matched SKH-1 hairless mice (5 mice/group) were fed ad libitum respectively drinking water containing arsenic at 0ppm, 50ppm, 100ppm and 200ppm 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 ten year consumption of arsenic by a human population in geographical areas with high arsenic levels in drinking water (150.1-864.0µg/L) considering an average consumption of 1.5L 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 three groups of 5 mice each received either no treatment or arsenic (200ppm) or arsenic (200ppm) + NAC (150mg/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 (50mM Tris pH 7.5, 1% Triton X-100, 0.25% NaF, 10mM β-glycerolphosphate, 2mM EDTA, 5mM Sodium pyrophosphate, 1mM Na3VO4, 10mM 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 4X loading buffer, boiled for 5 minutes and subjected to SDS-PAGE. Proteins were electrophoretically transferred to PVDF membranes and then nonspecific sites were blocked with 5% (W/V) nonfat-dry milk in TBST (25mM Tris-HCl, pH 7.5; 150mM NaCl; 0.05% Tween-20) for 1h at RT followed by probing with primary antibody overnight at 4°C or 1h at RT. The membranes were incubated for 1h with HRP-conjugated secondary antibody. The blots were developed with ECL according to the manufacture’s instructions (Amersham, IL). In most cases 40µg protein was loaded. However, to detect phosphorylation of PERK, 100µg 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 three independent samples from each group were used for Western blot analysis. The integrated density of bands was measured with Image J. Statistical analysis was performed using Excel 2003.
Immunofluorescent staining

1 X 0.4 cm strips of skin were fixed in cold formalin solution overnight at 4°C. The sections were dehydrated passing through the gradient of 70% ethanol, 95% ethanol and 100% ethanol and were embedded in paraffin wax and sectioned onto slides. The slides were deparaffinized in 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 1h. After removal of antibodies, slides were rinsed with PBS and mounted with mounting medium containing DAPI (Vector). Fluorescence was immediately recorded on an Olympus EX51 microscope.

RT-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 OD 260/280. 1μg RNA was used for reverse transcription using iScript cDNA synthesis kit (Bio-Rad). Primers used in this study are described in Supplementary Table 2.

PCR Array

PCR Array was performed using SABiosciences PCR Array System. First strand cDNA synthesis was performed using RT² First Strand kit. Real-Time PCR was performed with Mouse Inflammatory Cytokines & Receptors PCR Arrays on the iQ5 (Bio-rad) using RT² qPCR Master Mix. The program was 95 °C for 10min,
followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. For each group, three 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 cryo-sections (4-6 μm) were incubated with 10 μM CM-H2DCFDA dissolved in ACAS buffer (127 mM NaCl, 0.8 mM MgCl2, 3.8 mM KCl, 1.2 mM KH2PO4, 1.2 mM CaCl2, 5 mM glucose & 10 mM HEPES PH 7.4) for 1 hr at 27 ºC followed by 2 X 5 minutes wash 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 Student’s t test, \( p < 0.05 \) was considered to be statistically significant.

**Results**

**Arsenic activates cutaneous UPR signaling:** UPR signals through the activation of three 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 (Figure 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 (Figure 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 (Figure 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 (Figure 1A). The two other important UPR signaling proteins GRP78 and GRP94 were also increased significantly (Figure 1C). We also recorded significant increase of CHOP and GRP78 in mRNA level (Figure1B). 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 (Figure 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,
To test whether arsenic also induces cutaneous ROS production we employed 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 to vehicle-treated controls (Figure 2A). Arsenic-enhanced fluorescent staining attenuated following pre-treatment of arsenic exposed animals with antioxidant NAC, confirming that arsenic also augments ROS production in the skin (Figure 2A). To demonstrate 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 Figure 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 (Figure 2B). These data demonstrate 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, MAP Kinase 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 (Figure 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 MAP kinase signaling. The ability of NAC to significantly diminish the levels of arsenic-induced p-p38, p-MAPKAPK-2 and MAPKAPK-2 in this study (Figure 2D) suggests that ROS production triggered arsenic-mediated inflammation.

Furthermore, employing 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 3 and 4. Arsenic upregulated 24 genes whereas it downregulated 20 genes and the remaining 43 genes were not significantly altered (Figure 3A and B). Among these markers, Il1b (encoding interleukin 1 beta, a keratinocyte mitogen) and TNF (encoding tumor necrosis factor) which have been demonstrated to induce UPR in other systems (17, 18) were enhanced significantly (1.8-2.0 fold and 1.3-1.6 fold respectively). Ifng (encoding interferon gamma) which is mainly expressed by Th1 cells did not alter significantly. Among Th2-related markers, Il20 (encoding interleukin 20), which regulates proliferation and differentiation of keratinocytes, was dramatically reduced by 6-20 fold following arsenic treatment. Ccl1 (encoding Chemokine (C-C motif) ligand 1) and Il15 (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 with its effect on ROS production, UPR and MAP kinase 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 (Il1b, Ccl4 and Tnf) were significantly reversed. However, out of the 14 downregulated genes (including 3 Th2 markers, Il20, Ccl1 and Il15; and 2 Th17 markers, Il15 and Cxcl5) only three (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 pathobiological effects were observed in these animals. Arsenic treatment by itself does not induce skin cancer or hyperkeratosis in any of the 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 (Figure 4). We demonstrated that arsenic activated all three known UPR signaling 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 utilized 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, MAP kinase 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 Figure 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/NCI 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 two pathways or to each pathway independently. The partial but significant recovery of MAP kinase 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.
References


Figure Legends

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 (200ppm)-treated animals. Original magnification, 200X. Insets represent magnified (x2) 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 50µm and the smaller scale bar is 10µm. Each staining is representative of three 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 0ppm, 50ppm, 100ppm and 200ppm.

Figure 2. Arsenic-induced UPR and MAP Kinase signaling pathways are dependent on ROS production. A, Pictures showing fluorescence of oxidized CM-H2DCFDA in the epidermis as outlined by the dotted lines (original magnification, 200X). The area above the dotted lines shows some non-specific fluorescent staining of the stratum corneum. The area below the dotted lines
represents dermis. Each staining is representative of three independent samples; scale bar, 50 µm. B, Western blot analysis and relative expression level of epidermal p-eIF2α, XBP-1s, ATF6α, 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 (200ppm) and arsenic (200ppm) + NAC (150mg/kg) groups, whereas those in C were from 0ppm, 50ppm, 100ppm and 200ppm arsenic-treated animals.

**Figure 3. Arsenic mediates inflammatory response in the skin of SKH-1 hairless mice.** A, Graphs showing relative gene expression levels of Th1, Th2 and Th17 associated inflammatory cytokines/chemokines and their receptors (*p<0.05); B, Clustering analysis of the expression of 84 genes related to inflammatory cytokines/chemokines and their receptors in the skin of control and arsenic-treated animals. Clustering pattern is indicated at the top of the diagram. Using the SABiosciences online RT profiler PCR array data analysis software, the cluster diagram of inflammatory genes was developed. The clustering patterns of the samples fell into two groups. 0ppm and 50ppm clustered in one group and 100ppm and 200ppm clustered in another group. Genes with higher correlation coefficients across different samples are clustered together by rows. Thus, genes within the same cluster represent closer expression patterns than genes in different clusters. Each row represents a single gene labeled with the gene name while each column represents an independent skin sample treated with the corresponding arsenic concentration. The color in each cell reflects the gene expression level of the
corresponding sample. The color scale at the bottom indicates the magnitude of gene expression. Expression levels greater than the mean are shaded in red and those below the mean are shaded in green.

**Figure 4. 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 up-regulates 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 MAP kinase and its downstream protein MAPKAPK-2. Activation of UPR and MAP kinase 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.
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