



Low-level arsenic causes proteotoxic stress and not oxidative stress

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ABSTRACT

Prolonged exposure to arsenic has been shown to increase the risk of developing a number of diseases, including cancer and type II diabetes. Arsenic is present throughout the environment in its inorganic forms, and the level of exposure varies greatly by geographical location. The current recommended maximum level of arsenic exposure by the EPA is 10 µg/L, but levels > 50–1000 µg/L have been detected in some parts of Asia, the Middle East, and the Southwestern United States. One of the most important steps in developing treatment options for arsenic-linked pathologies is to understand the cellular pathways affected by low levels of arsenic. Here, we show that acute exposure to non-lethal, low-level arsenite, an environmentally relevant arsenical, inhibits the autophagy pathway. Furthermore, arsenite-induced autophagy inhibition initiates a transient, but moderate ER stress response. Significantly, low-level arsenite exposure does not exhibit an increase in oxidative stress. These findings indicate that compromised autophagy, and not enhanced oxidative stress occurs early during arsenite exposure, and that restoring the autophagy pathway and proper proteostasis could be a viable option for treating arsenic-linked diseases. As such, our study challenges the existing paradigm that oxidative stress is the main underlying cause of pathologies associated with environmental arsenic exposure.

1. Introduction

Arsenic is a metalloid found ubiquitously in the environment. Exposure to unsafe levels of arsenic typically occurs as a result of consuming contaminated food or drinking water, particularly over a prolonged period of time. The current World Health Organization (WHO) and Environmental Protection Agency (EPA) recommended limit for arsenic levels in the drinking water is 10 µg/L (10 ppb); however, many countries including Bangladesh, India, Mexico, Chile, Taiwan, China, and even some parts of the United States and Canada, have reported concentrations that significantly exceed this limit, ranging anywhere from 50 to > 1000 ppb (µg/L) (Chowdhury et al., 2000; Harvey et al., 2002; Yoshida, Yamauchi, and Fan Sun, 2004; Wang and Mulligan, 2006; Buschmann et al., 2008; Sorg, Chen, and Wang, 2014; Karagas, Gossai, Pierce, and Ahsan, 2015; Mendez et al., 2016). Furthermore, populations chronically exposed to arsenic in this range are at an increased risk of developing a number of diseases, including skin, lung, and bladder cancer, cardiovascular, respiratory, and kidney

disease, as well as type II diabetes (Tseng et al., 2003; Navas-Acien, Silbergeld, Pastor-Barriuso, and Guallar, 2008; Parvez et al., 2008; Maull et al., 2012; Steinmaus et al., 2013; Steinmaus et al., 2014; Cheng et al., 2017).

The carcinogenic potential of arsenic has been well established in both epidemiological studies, as well as in a host of experimental models (Morales, Ryan, Kuo, Wu, and Chen, 2000; Chen et al., 2001; Eblin, Bredfeldt, Buffington, and Gandolfi, 2007; Sun et al., 2009; Martinez, Vucic, Becker-Santos, Gil, and Lam, 2011). Arsenic is classified as a Group 1 human carcinogen by the International Agency for Research on Cancer (IARC), and currently ranks number 1 on the substance priority list put forth by the Agency for Toxic Substance and Disease Registry (ATSDR). The toxic effects associated with arsenic exposure depend on the arsenical species, as well as the concentration and time of exposure. Intracellularly, arsenic can exist in its inorganic forms, arsenate [As(V)] and arsenite [As(III)], as well as in its methylated forms, methylarsonic acid/methylarsonous acid [MMA(V/III)] or dimethylarsinic acid/dimethylarsinous acid [DMA(V/III)]. Most in

Abbreviations: ATF6, activating transcription factor 6; DCF, 2',7'-dichlorofluorescein; H₂DCFDA, 2',7'-dichlorodihydrofluorescein diacetate; EIF2S1/eIF2α, eukaryotic initiation factor 2, subunit 1 alpha; IRE-1, inositol-requiring enzyme 1; LC3, microtubule-associated protein 1A/1B-light chain 3; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide; PERK, eukaryotic translation initiation factor 2 alpha kinase 3; ROS, reactive oxygen species; SQSTM1/p62, sequestosome 1; XBP1s, X-box-binding protein 1, spliced

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vitro and in vivo studies utilize either sodium arsenite (NaAsO_2) or arsenic trioxide (As_2O_3), producing As(III) which can be oxidized to form As(V), or further metabolized by arsenite 3-methyl transferase into MMA or DMA inside the cell (Thomas, Styblo, and Lin, 2001). As such, the pleiotropic effects attributed to arsenic toxicity are a result of the diversity of arsenical species and their subsequent effects on a variety of intracellular targets.

Interestingly, many of the studies investigating the pathogenic effects of arsenic exposure use concentrations of arsenite in the $> 5 \mu\text{M}$ micromolar range [375–750 ppb As(III)], reflecting concentrations that might be observed only in the most severely affected areas. Many of the observed effects in this range, such as increased reactive oxygen species (ROS) production (Alarifi, Ali, Alkahtani, Siddiqui, and Ali, 2013; Jiang, Chen, Zhao, and Zhang, 2013; Kumar, Yedjou, and Tchounwou, 2014), DNA damage (Andrew et al., 2006; Hughes, Beck, Chen, Lewis, and Thomas, 2011; Prakash, Soni, and Kumar, 2016), mitochondrial dysfunction (Liu et al., 2005), glutathione depletion (Li and Chen, 2016), and protein modifications (Shen, Li, Cullen, Weinfeld, and Le, 2013), might not occur at lower, more relevant concentrations. Furthermore, the compound, cell type, and time of treatment matters, with As_2O_3 exhibiting a more consistent increase in ROS production at acute time points (Barchowsky, Klei, Dudek, Swartz, and James, 1999; Yen et al., 2012; Lu et al., 2014), whereas chronic NaAsO_2 treatment (i.e. 8–24 weeks) has been shown to increase or decrease ROS levels depending on the cell type and length of exposure (Chang et al., 2010; Padmaja Divya et al., 2015). It is important to note that As_2O_3 is not a common environmental contaminant, and is mainly utilized as a cancer treatment, making it not suitable for determining the cellular pathways affected by environmental exposure to more common arsenicals.

To gain a better understanding of the pathological effects associated with lower arsenic concentrations, we examined the immediate cellular stress responses to low levels of arsenic exposure [sodium arsenite in the 0.5–2 μM , which is equivalent to 37.5–150 ppb As(III)]. ROS production, autophagosome accumulation, and activation of the ER stress response, at early time points (0.5–4 h), as well as cell viability (24 to 48 h), were measured in a variety of mouse and human cell lines. Interestingly, low-level arsenite treatment up to 72 h did not result in any detectable levels of ROS as measured by electron paramagnetic resonance spectroscopy (EPR) using the 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine (CMH) spin probe. Instead, acute arsenite treatment results in inhibition of the autophagy pathway, as we reported previously (Lau et al., 2013), as well as a transient moderate ER stress response. No toxicity was observed at any concentration of arsenite up to 48 h in all four of the cell types tested. These results demonstrate that lower concentrations of arsenite significantly alter proteostasis without inducing oxidative stress, at least at early time points, challenging the existing paradigm that ROS are the main underlying cause of pathologies associated with environmental arsenic exposure.

2. Materials and methods

2.1. Chemicals, antibodies, reagents, and cell culture conditions

Sodium arsenite (S7400), 2',7'-dichlorofluorescein diacetate (H_2DCFDA ; D6883), MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide; M5655], bafilomycin A1 (B1793), and the primary antibody against LC3 (L7543) were purchased from Sigma. 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine (CMH; NOX-0.2) and Krebs-HEPES buffer (NOX-07.6.1) were purchased from Noxygen. Primary antibodies against GAPDH (sc-32233), as well as horseradish peroxidase (HRP)-conjugated secondary antibodies (goat anti-rabbit, sc-2004; goat anti-mouse, sc-2005) were from Santa Cruz. The primary antibody against SQSTM1 (89-015-843) was obtained from Abnova. The primary antibodies against EIF2S1 (9722),

phosphorylated EIF2S1 (9721), and XBP1s (12782) were from Cell Signaling Technologies. The antibody against ATF6 (ab122897) was from Abcam. NIH 3T3, HeLa, and HEK 293 cell lines were purchased from the American Type Culture Collection (ATCC). NIH 3T3 and HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Corning, MT10014CV) supplemented with 10% fetal bovine serum (FBS; Atlanta Biological, S11150H), 1% L-glutamine (Gibco, 25030081), and 1% penicillin-streptomycin (15140722). HEK 293 cells were cultured in minimum essential medium (MEM; Corning, 10-010-CV) supplemented with 10% FBS, 1% L-glutamine, 0.1 mM nonessential amino acids (Hyclone, SH30238.01), 0.1 mM sodium pyruvate (Gibco, 11360070), and 0.01% gentamicin (Omega Scientific, 1350). HEMn-LP were purchased from Thermo Fisher Scientific and were cultured in medium 254 supplemented with 0.06 mM calcium chloride (Gibco, M254CF500), $1 \times$ human melanocyte growth supplement (HMGS; Thermo Fisher Scientific, S0025), and $1 \times$ gentamicin/amphotericin (Thermo Fisher Scientific, R01510). All cells were incubated at 37 °C in a humidified incubator with 5% CO_2 .

2.2. Transfection and live cell fluorescent imaging

For imaging, 7.5×10^4 NIH 3T3 cells were seeded in quad chamber glass bottom 35 mm dishes. Cells were transfected with 1 μg RFP-GFP-LC3 using Lipofectamine 3000 (Thermo Fisher Scientific, L3000075) according to the manufacturer's instructions. 24 h later, cells were either left untreated, treated with sodium arsenite (0.25, 0.5, 1, or 2 μM) or bafilomycin (0.1 μM), or starved with Hank's balanced salt solution (HBSS, $1 \times$, Hyclone, SH30268.01) for the indicated time points. Prior to imaging, cells were gently washed with $1 \times$ phosphate buffered saline (PBS) and DMEM without phenol red was added. Images were taken with a Zeiss Observer.Z1 microscope using the Slidebook 4.2.0.11 software (Intelligent Imaging Innovations, Inc.).

2.3. Immunoblot analysis

To detect protein expression, 1.5×10^5 cells were seeded in 12-well plates, and 24 h later were either left untreated, treated with sodium arsenite or bafilomycin, or starved in HBSS for the indicated time points. Cells were washed in $1 \times$ PBS, harvested in $1 \times$ Laemmli or $1 \times$ NuPAGE LDS Sample Buffer with $1 \times$ NuPAGE Reducing Agent (Thermo Fisher Scientific, NP0007), and boiled for 5 min. Cell lysates were resolved by SDS-polyacrylamide gel electrophoresis and subjected to immunoblot analysis with the indicated antibodies.

2.4. Cell toxicity assay

To measure cell toxicity, 1×10^4 cells were seeded in 96-well plates, and 24 h later were either left untreated or treated with the indicated concentrations of sodium arsenite for 24 and 48 h. 20 μL of MTT (2 mg/mL) were added to each well and the plates were incubated for 2 h at 37 °C. Medium was removed and formazan salts were diluted by adding 100 μL of isopropanol/HCl. The plates were shaken at room temperature for 10 min and absorbance was measured at 570 nm using a BioTek plate reader. Cells were seeded in quadruplicate.

2.5. Reactive oxygen species measurements

To measure the generation of reactive oxygen species using EPR, 7.5×10^4 cells were seeded in 24-well plates, and 24 h later were either left untreated, or treated with sodium arsenite (0.5, 1, 2 or 10 μM), or with 250–500 μM hydrogen peroxide (H_2O_2), or 25–50 μM FCCP for the indicated time points. Following treatment, cells were incubated in 20 mM Krebs-HEPES buffer (pH 7.4) containing 200 μM CMH for 30 min. Buffer was then collected, and changes in CMH oxidation were measured for 15 min using the e-scanM Multipurpose Bench-top EPR

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