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ARSENITE-INDUCED AUTOPHAGY IS ASSOCIATED WITH PROTEOTOXICITY IN HUMAN LYMPHOBLASTOID CELLS

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Abstract

Epidemiological studies of arsenic-exposed populations have provided evidence that arsenic exposure in humans is associated with immunosuppression. Previously, we have reported that arsenite-induced toxicity is associated with the induction of autophagy in human lymphoblastoid cell lines (LCL). Autophagy is a cellular process that functions in the degradation of damaged cellular components, including protein aggregates formed by misfolded or damaged proteins. Accumulation of misfolded or damaged proteins in the endoplasmic reticulum (ER) lumen causes ER stress and activates the unfolded protein response (UPR). In an effort to investigate the mechanism of autophagy induction by arsenite in the LCL model, we examined the potential contribution of ER stress and activation of the UPR. LCL exposed to sodium arsenite for 8-days induced expression of UPR-activated genes, including CHOP and GRP78, at the RNA and the protein level. Evidence for activation of the three arms of the UPR was observed. The arsenite-induced activation of the UPR was associated with an accumulation of protein aggregates containing p62 and LC3, proteins with established roles in the sequestration and autophagic clearance of protein aggregates. Taken together, these data provide evidence that arsenite-induced autophagy is associated with the generation of ER stress, activation of the UPR, and formation of protein aggregates that may be targeted to the lysosome for degradation.

Keywords

ER stress; Arsenite; Autophagy; Proteotoxicity; Lymphoblastoid cell lines

Introduction

Autophagy is a cellular process by which damaged or superfluous cellular components are degraded in the lysosome to maintain homeostasis or respond to cellular stress. Diverse stressors can induce autophagy as an adaptive response to cellular damage. Nutrient deprivation is a prototypical stimulus that activates autophagy in order to degrade cellular proteins, generating free pools of amino acids needed for new protein synthesis (Onodera and Ohsumi, 2005). In addition to starvation, the broad scope of cellular perturbation capable of inducing autophagy includes endoplasmic reticulum (ER) stress, oxidative stress,

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hypoxia, pathogen infection and DNA damage (Kiffin *et al.*, 2004; Nakagawa *et al.*, 2004; Ogata *et al.*, 2006; Abedin *et al.*, 2007; Bellot *et al.*, 2009).

The ER is a cellular organelle in which the synthesis, folding, and quality control of nascent proteins occurs (Gregersen and Bross, 2010). The unfolded protein response (UPR) describes a coordinated cellular response to ER stress. At a molecular level, the UPR is activated by a hallmark of ER stress, the accumulation of misfolded proteins in the ER. ER stress triggers the UPR as a mechanism to limit cellular damage, to initiate repair of misfolded proteins, to dispose of irreparable proteins, and to initiate programmed cell death in the context of extreme ER stress. In mammalian cells the three sensor pathways of the UPR are protein kinase-like endoplasmic reticulum kinase (PERK), inositol-requiring enzyme 1 (IRE1), and activating transcription factor 6 (ATF6) (Gregersen and Bross, 2010). In the unstressed ER, PERK, IRE1, and ATF6 are ER membrane proteins that, on their ER-luminal aspect, physically associate with the chaperone, glucose-regulated protein 78kDa (GRP78/BiP). When unfolded/misfolded proteins accumulate in the ER, GRP78 is released from the sensor proteins, enabling activation of their three pathways (Kaufman *et al.*, 2010). PERK activation leads to phosphorylation of the α subunit of eukaryotic translation initiation factor 2 (eIF2 α), which results in inhibition of cap-dependent translation (Lerner and Nicchitta, 2006). The phospho-eIF2 α -mediated translational repression is not genome-wide, and activating transcription factor 4 (ATF4), a principal trans-activating protein in the UPR, is induced at the protein level during PERK activation (Harding *et al.*, 2000). Upon dissociation of GRP78, IRE1 splices X-box binding protein 1 (XBP1u) mRNA into mature, spliced XBP1 mRNA (XBP1s). The resulting XBP1s protein is another UPR trans-activating factor that transcriptionally induces a coordinated set of UPR genes. After dissociation of GRP78, ATF6 is cleaved by site 1 and site 2 proteases (S1P and S2P) in the golgi apparatus. The cleaved ATF6 is the third trans-activating protein in the generation of the UPR. A large number of target genes are induced in the UPR, including the chaperone protein GRP78/BiP and the pro-apoptotic gene, DNA-damage-inducible transcript 3 (DDIT3/CHOP) (Haze *et al.*, 1999; Harding *et al.*, 2000). The three arms of the UPR work in concert to reduce the ER load through attenuation of protein translation, to activate transcription of compensatory protein repair and protein degradation genes, or to activate pro-apoptotic genes when the compensatory response fails (Costa *et al.*, 2011).

Degradation of unfolded or misfolded proteins is required to prevent the accumulation of damaged cellular proteins that cannot be successfully re-folded. Monomeric, improperly folded proteins in the ER can be ubiquitinated, transported to the cytosol, and degraded through the ubiquitin proteasome system (Hiller *et al.*, 1996). Alternatively, damaged cellular proteins and protein aggregates (large complexes of physically associated proteins) can be degraded in the lysosome. Protein aggregates are potentially cytotoxic. Thus, the autophagic sequestration and removal of aggregated proteins is a compensatory, cytoprotective response (Yao, 2010). One mechanism through which autophagy participates in protein aggregate clearance involves sequestosome 1 (p62), a scaffold protein with distinct domains that interact with polyubiquitin, as well as with the autophagosome membrane protein, microtubule-associated protein 1 light chain 3B (LC3)-II (Yao, 2010; Pankiv *et al.*, 2007).

Using LCL as an *in vitro* model to investigate arsenite-induced targeting of the immune system, we have found that arsenite causes inhibition of cell proliferation in several LCL (Bolt *et al.*, 2010b). This proliferative inhibition is associated with the induction of autophagy markers, including the presence of autophagic vesicles (autophagosomes and autolysosomes), increased protein levels of the autophagosome marker LC3-II, and induction of lysosomal gene expression regulated by the transcription factor TFEB (Bolt *et al.*, 2010a; Bolt *et al.*, 2010b). Despite the association between arsenite-induced proliferative

inhibition and the induction of autophagy, the cellular stress responsible for autophagy induction in LCL is not known. Arsenite has been shown to bind protein thiol groups, disrupting normal protein folding and function (Cline *et al.*, 2003; Ramadan *et al.*, 2009). Arsenite has also been shown to activate the UPR (Zhang *et al.*, 2009). Based upon these observations, together with the recognition that UPR activation can induce autophagy, the objective of this study was to determine if arsenite-induced autophagy in LCL is associated with ER stress and proteotoxicity (here defined by evidence of increased mis-folded proteins, protein aggregates, or their disposal).

Materials and Methods

Reagents

Sodium arsenite (dissolved in MilliQ H₂O) and tunicamycin (dissolved in dimethyl sulfoxide (DMSO)) were purchased from Sigma Aldrich (St. Louis, MO). Bafilomycin A1 (BafA1) was purchased from Enzo Life Sciences (Plymouth Meeting, PA).

Cell Culture and Exposure Conditions

The human B lymphoblastoid cell line, Priess, was a gift from Dr. Janice Blum (Department of Microbiology and Immunology, Indiana University School of Medicine). Priess cells were cultured in suspension in Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% fetal bovine serum (FBS) and 1% Penicillin/ Streptomycin (Penn/ Strep). Cell cultures were maintained between the cell concentrations of 200,000–1,000,000 cells/ml at 5% CO₂ and 37 °C. Cultures were seeded to 200,000 cells/ml in supplemented media and maintained either under control conditions, exposure to 1.5 µM sodium arsenite for 8 days, or exposure to 5 µg/ml tunicamycin for 24 hours.

Doubling Time Measurements

In order to measure the effect of arsenite on cell proliferation, total cell counts were determined using a Scepter Automatic Cell Counter (Millipore, Billerica, MA). Cell population doubling time was calculated from total cell counts using the following equation: $D_t = ((\text{total days of growth (D)}) \times \text{Log}2 / \text{Log} (\# \text{ of cells Time } D / \# \text{ of cells Time } 0)) \times 24$. Mean doubling time $\pm$ standard deviation of the mean (S.D.) was calculated for each sample from 6 independent replicates.

Lysotracker Red Staining

Control, arsenite, or tunicamycin-exposed cells (1×10^6) were harvested, centrifuged, resuspended in 20 nM lysotracker red dye (LRD) (Invitrogen, Carlsbad, CA) and incubated at 5% CO₂ and 37 °C for 30 minutes. Cells were washed once with 1× phosphate buffered saline (PBS) and resuspended in 500 µl PBS to a final concentration of 2×10^6 cells/ml. Samples were analyzed using a LSR II Flow Cytometer (BD Biosciences, Sparks, MD). Fluorescence of LRD was collected through the 610/20 nm bandpass filter. Data were analyzed using FacsDiva (BD Biosciences, Sparks, MD) software. Mean relative fluorescence units (RFU) of LRD $\pm$ the S.D. was calculated for each sample from 6 independent replicates for the arsenite treatment group and 3 independent replicates for the tunicamycin treatment group.

Antibodies and immunoblot analysis

The following primary antibodies were used: LC3, 1:250 (Nano Tools, San Diego, CA); GRP78, 1:1000 (Cell Signaling Technology, Danvers, MA); XBP1s, 1:250 (BioLegend, San Diego, CA); ATF6, 1:1000 (Imgenex, San Diego, CA); α -tubulin (Sigma Aldrich, St. Louis, MO); eIF2 α , 1:1000 and eIF2 α [pS52], 1:1000 (Life Technologies, Grand Island, NY);

CHOP, 1:250 and ATF4, 1:1000 (Santa Cruz Biotechnology, Santa Cruz, CA). Goat anti-rabbit and anti-mouse IgG-HRP were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA). Vehicle control and arsenite (8-days, 1.5 μ M) exposed cells were harvested with or without co-treatment with 100 nM BafA1 for the last 8 and 4 hours of the exposure duration. Vehicle control and tunicamycin (5 μ g/ml) exposed cells were harvested after 24 hours of treatment. Cell pellets were lysed in sample buffer [2% SDS, 100 mM DTT, 10% glycerol, 50 mM Tris-HCl (pH 6.8), 0.1% bromophenol blue], samples were heated to 90 °C for 10 minutes. After sonication, equal amounts of whole cell lysates were subjected to SDS-polyacrylamide gel electrophoresis and immunoblot analysis. Immunoblots were visualized by chemiluminescence detection (Thermo Scientific, Rockford, IL) and imaged using the GeneGnome5 imager (Syngene, Frederick, MD).

RNA Isolation

RNA was isolated from LCL: GM18564, GM18504, GM18550, GM18532, GM19209, GM18561 and GM18853 after exposure to 0.75 μ M sodium arsenite for 0, 1, 2, 4, 6, or 8 days as previously described (Bolt *et al.*, 2010b).

Microarray Gene Expression Analysis

RNA quality was evaluated using A260/A280 ratio (> 2.0) and RNA 6000 Nano Chips in the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). The expression profiles were generated using the Affymetrix GeneChip Human Gene 1.0 ST oligonucleotide arrays according to manufacturer's protocol (Affymetrix, Santa Clara, CA), which simultaneously measures expression of 28,869 genes across the entire genome as described previously (Bolt *et al.*, 2010b).

Filter Trap Assay

To detect protein aggregates, cell lysates were vacuum-aspirated through a 0.2 μ m pore-size, nitrocellulose membrane to preferentially retain aggregated proteins as previously described (Myeku and Figueiredo-Pereira, 2011). Briefly, Priess cells were cultured under either control conditions or exposed to 1.5 μ M sodium arsenite for 8 days. Equal number of cells (8×10^6) were harvested and lysed in RIPA buffer (1% NP-40, 1% w/v sodium deoxycholate, 0.1% SDS, 0.15 M sodium chloride, 0.01 M sodium phosphate, 2 mM ethylene diamine tetracetic acid). Samples were centrifuged at 19,000 RCF for 30 minutes at 4 °C. Cell pellets containing the insoluble material were removed and placed into new centrifuge tubes. Cell pellets were treated with DNase I (in DNase buffer) for 1 hour at 37 °C. Reactions were terminated by the addition of EDTA buffer containing a final concentration of 2% SDS. Cell lysates were diluted 1:6 in MilliQ H₂O. 100 μ l of each sample was trapped by filtration through a pre-wet Trans-Blot 0.2 μ m pore-size nitrocellulose membrane adapted to a 96-well dot blot apparatus (Bio-Rad, Hercules, CA) as previously described (Wanker *et al.*, 1999). Membranes were stained with ponceau red stain to ensure equal protein loading. To analyze aggregate composition, the membranes were subjected to immunoblot analysis with the following antibodies: p62 (1:1000; MBL-BION, Des Plaines, IL), LC3 (1:1000; Cell Signaling Technologies, Beverly, MA), α -tubulin (1:1000) and goat anti-rabbit or anti-mouse IgG-HRP. Membranes were visualized by chemiluminescence and imaged using the GeneGnome5 imager. Densitometry was determined for each dot using GeneTools analysis software (Syngene, Frederick, MD).

Statistical analyses

Group mean comparison—Student T-test (unpaired) was used to compare mean differences in cell population doubling time, LRD fluorescence and immunoblot

densitometry between control and 8-day arsenite exposed or tunicamycin-exposed samples at a significance threshold of $P < 0.05$.

Microarray Analysis—Statistical analyses were performed using BRB-ArrayTools, version 4.2. (Simon *et al.*, 2007; <http://linus.nci.nih.gov/BRB-ArrayTools.html>). In order to determine if the generation of ER stress and activation of the UPR in LCL resulted from arsenite exposure, genome-wide gene expression was evaluated after arsenite exposure for gene expression changes that were indicative of the UPR. To do this, a set of 275 literature-validated genes was derived from published data (Taylor *et al.*, 2011). In LCL derived from two donors, the expression of these genes was shown to be modulated by thapsigargin (TG), an established ER stress/UPR - inducing agent. This set of genes represents a “reference” gene expression profile of the UPR in LCL. In addition, a set of 224 lysosomal genes comprising the “lysosome” gene ontology (GO) category was generated as previously described (Bolt *et al.*, 2010b) to compare UPR gene expression with lysosomal gene expression throughout the 8-day time course of arsenite exposure in Priess cells.

Gene Set Enrichment Analysis—To determine if there was an enrichment of UPR or lysosomal genes after arsenite exposure, gene set expression comparison analysis was performed separately for each reference gene set to identify if either set was overrepresented within the genes modulated by arsenite in comparison to alternative, similarly sized gene sets generated randomly from the microarray. The analysis compared the gene expression levels for all day-0 samples versus all day-8 samples at a significance threshold of $P < 0.05$. Paired tests were used for the same cell line on day-0 (control) and day-8.

ANOVA Analysis—To evaluate the time-dependent evolution of gene expression of UPR and lysosomal genes throughout the 8-days of arsenic exposure, ANOVA analyses were performed for each gene set. Separately, microarray genes available for analysis were restricted to include only the UPR reference gene set or the lysosome reference gene set. An ANOVA analysis was performed under a fixed effects model comparing groups defined by the duration of arsenic exposure (Days 0,1,2,4,6,8). Type-I error adjustment was relaxed to a false discovery rate (FDR) of 0.10 to increase inclusion of arsenite-modulated genes that may change over time. Tukey’s post-hoc tests were used to identify differentially expressed ($P < 0.05$) genes between day-0 (control) and each subsequent time-point of arsenite exposure.

Results

Prototypical ER stress in the Priess cell line results in autophagy induction

To establish that canonical UPR is inducible in Priess, cells were treated with 5 $\mu\text{g/ml}$ of the prototypical ER stress-inducing agent, tunicamycin, for 24 hours. Cell lysates were subjected to SDS page and immunoblot analysis. Tunicamycin exposure resulted in activation of two of the three arms of the UPR (Figure 1A). Within the PERK/eIF2 α pathway an increase in the level of phosphorylated eIF2 α and in ATF4 protein was observed. In the IRE1/XBP1 pathway, XBP1s protein levels increased in the tunicamycin treatment group. Based on the absence of detectable, cleaved ATF6 protein, there was no apparent activation of the ATF6 pathway after tunicamycin treatment. Interestingly, there was a decrease in the ATF6 cleavage product. Protein levels of UPR target genes, GRP78 and CHOP, were increased by tunicamycin exposure, consistent with UPR activation.

To examine the effect of tunicamycin-induced activation of the UPR on autophagy, autophagy markers were evaluated after tunicamycin exposure. LC3-II steady state levels ($P < 0.05$, data not shown) and LRD fluorescence levels ($P < 0.05$) were both increased in the

tunicamycin treatment group (Figure 1A and 1B, respectively), suggesting that autophagy and ER stress were both induced by tunicamycin in Priess cells.

Arsenite exposure in Priess induces the UPR as well as autophagy

Arsenite-induced proliferative inhibition is associated with hallmarks of autophagy—Following 8 days of exposure to arsenite (1.5 μ M), Priess cells were evaluated for proliferation and acidic vesicle content. Priess population doubling time increased by 25 hours from a mean doubling time of 50 hours to 75 hours (Figure 2A). This was comparable to the degree of proliferative inhibition previously reported in other arsenite-exposed LCL (Bolt *et al.*, 2010b). Also consistent with previous results, the decrease in cell proliferation was associated with an induction of autophagy markers. Arsenite-exposed cells had an expansion of acidic vesicles as detected by flow cytometry (Figure 2B). The mean LRD fluorescence increased 2.6 fold from 500 RFU to 1300 RFU, indicating an accumulation of acidic vesicles (autolysosomes or lysosomes) after arsenite exposure. Arsenite exposure increased the steady-state protein levels of LC3-I and LC3-II, as well as the LC3-II flux (rate of accumulation of LC3-II in the autophagosome/autolysosome during co-exposure to the lysosome alkalinizing agent, BafA1), consistent with an induction of autophagy (Figure 2C).

Arsenite exposure leads to the UPR and proteotoxicity—Previously we reported genome-wide RNA analysis in 7 LCL (lymphoblastoid cell lines derived from 7 human donors) exposed to 0.75 μ M sodium arsenite for 8-days (Bolt *et al.*, 2010b). In that study, arsenite-induced autophagy was associated with a global induction of lysosomal genes. To evaluate the generation of ER stress and activation of the UPR as a potential mechanism of cellular damage contributing to arsenite-induced autophagy, that data set was re-evaluated for evidence of gene expression changes indicating an activation of the UPR. The arsenite-induced gene expression profile was compared to the UPR “reference” set of genes (described in Materials and Methods). Gene expression data from the 7 LCL on day-8 versus day-0 of arsenite exposure were compared. Testing of the data with gene set expression comparison analysis (Xu *et al.*, 2008) suggested that the day-8 arsenite-modulated genes represented a significant enrichment for the UPR reference set of genes ($P < 0.00001$). Of the 275 genes in the reference gene set, the expression of 115 genes was significantly induced or repressed after 8 days of arsenite exposure (Supplemental Data Table 1). Among the day-8 arsenite-induced LCL genes, 96% were also induced by TG in the reference gene set. This LCL gene expression profile overlap between arsenite and TG suggests that ER stress and the consequent UPR are activated by arsenite in LCL.

Arsenite-induced activation of the UPR in LCL was also evaluated at the protein level by immunoblot analysis. Phosphorylated eIF2 α and ATF4 protein levels were elevated after the 8-day arsenite exposure (Figure 3), consistent with activation of the PERK/eIF2 α arm of the UPR. In the IRE1/XBP1 arm, arsenite exposure resulted in increased XBP1s protein levels. In contrast to tunicamycin-exposed Priess cells, ATF6 cleavage was induced by arsenite exposure. UPR target genes, GRP78 and CHOP, were both induced by arsenite. These data indicate that arsenite leads to activation of the UPR and is associated with the induction of autophagy in Priess cells.

One consequence of a cellular accumulation of misfolded or damaged cellular proteins is the formation of protein aggregates. In order to determine if arsenite exposure resulted in protein aggregate formation, we utilized the “filter-trap” assay, an established method of capturing protein aggregates onto a nitrocellulose membrane for subsequent processing similar to Western immunoblot analysis (Myeku and Figueiredo-Pereira, 2011). In this assay, the RIPA buffer-insoluble pellet from Priess lysate was DNase digested, then

partially denatured in SDS (unheated) prior to vacuum filtration through a 0.2 μ m pore nitrocellulose filter to preferentially capture larger protein complexes. The aggregate-retained membranes were probed for the scaffold protein, p62, the autophagy marker, LC3, and tubulin. Increased levels of both p62 and LC3 were retained in the nitrocellulose filters following arsenite exposure in Priess, suggesting that arsenite-induced aggregates may be targeted to the lysosome for degradation through autophagy (Figure 4).

In an effort to temporally order activation of the UPR and the induction of autophagy after arsenite exposure the RNA microarray data were evaluated throughout the 8-day time course. One measure of autophagy induction is the coordinated induction of lysosomal genes driven by the transcription factor, TFEB (Bolt *et al.*, 2010b; Settembre *et al.*, 2011). We verified this by using gene set expression comparison analysis to evaluate lysosomal gene expression, on day-8 in comparison to day-0. Arsenite exposure resulted in a significant enrichment of lysosomal genes ($P < 0.00001$).

To compare the time-course of the induction of lysosomal gene expression to that of the UPR genes, each reference gene set was analyzed separately by ANOVA analysis, with arsenite exposure duration (day-0,1,2,4,6,8) defining the comparison groups. Pairwise Tukey's post-hoc testing allowed the enumeration of genes with statistically significant gene expression differences between each day of arsenite exposure and control (day-0) samples. Within the UPR reference genes, the expression of 52 genes was significantly different between days of exposure in the ANOVA test (Supplemental Data Table 2). For the lysosomal genes, the expression of 56 genes was significantly different between days of exposure (Supplemental Data Table 3). Pairwise, post-hoc tests ($P < 0.05$) of LCL on days 1,2,4,6 and 8 of arsenite exposure against control LCL (day-0) were performed to evaluate the evolution of the lysosomal and UPR gene expression differences over the 8 days of arsenite exposure (Time Course column, Supplemental Data Tables 2 and 3). One of the 52 UPR genes, LMNB1, underwent a change in expression within the first 48 hours of arsenite exposure. In contrast, 12 of the 56 lysosomal genes were modulated by arsenite in the first 48 hours, suggesting that the induction of autophagy may precede the induction of the UPR.

Discussion

This study establishes that proliferative inhibition and autophagy induced by arsenite exposure are associated with activation of the unfolded protein response, together with the appearance of proteins associated with protein aggregate removal within filter-trapped fractions typically used to identify protein aggregates. Taken together these data suggest that arsenite induces protein misfolding, as well as protein aggregate clearance in the Priess LCL, a pattern suggestive of arsenite-induced proteotoxicity.

The UPR is a consequence of ER stress-related accumulation of improperly folded proteins. Arsenite exposure in Priess cells resulted in a genome-wide transcriptional gene expression profile that substantially overlapped that of LCL exposed to thapsigargin, a compound typically used as a positive control in ER stress studies. The robustness of the overlap between arsenite-exposed Priess and the TG "reference" gene set is underscored by the fact that the two data sets were generated in unrelated laboratories and studies. The RNA-based evidence of the UPR in Priess was corroborated by protein measurements of UPR pathway proteins that demonstrated activation of two arms of the UPR by the prototypical ER stress-inducing agent, tunicamycin, and activation of all three arms of the UPR by arsenite. In the case of both xenobiotics, expression of the downstream, trans-activated UPR gene targets, CHOP and GRP78, was induced. Together with evidence of protein damage in the endoplasmic reticulum, arsenite-exposed Priess cells also accumulated protein aggregates

that contained LC3 and p62 protein, two recognized components of an autophagy pathway that identifies and targets protein aggregates to the lysosome for degradation.

Arsenite-induced proteotoxicity has also been observed in other experimental systems. Arsenite can directly bind to thiol groups in proteins, altering folding (Cline *et al.*, 2003; Ramadan *et al.*, 2009; Zhou *et al.*, 2011). Arsenite has been shown to cause an accumulation of high molecular weight ubiquitin-conjugated proteins in human embryonic kidney (HEK) 293 cells as well as rabbit renal-cortical slices, suggesting arsenite exposure increased protein targeting to degradative pathways (Kirkpatrick *et al.*, 2003). While monomeric, polyubiquitinated proteins targeted for degradation can be processed by the proteasome, aggregated protein complexes are size-excluded from this structure. Protein aggregates occur as a protective mechanism to sequester misfolded proteins that have accumulated within the cell and facilitate their degradation using the autophagic machinery (Bjorkoy *et al.*, 2005). The scaffold protein, p62, can bind ubiquitin-conjugated aggregates and target them to the forming autophagosome, where p62 binds to LC3-II on the autophagosome membrane, anchoring the aggregated protein to the autophagic structure. Thus, the co-isolation of protein aggregates, p62, and LC3 in arsenite-exposed LCL is consistent with autophagic clearance of damaged proteins.

The occurrence of ER stress and proteotoxicity in arsenite-exposed Priess are significant, in light of studies identifying them as sources of autophagy induction. In both yeast and mammalian cells, treatment with ER stress inducing agents has been shown to be associated with the formation of autophagic vesicles, indicative of an induction of autophagy (Ogata *et al.*, 2006; Yorimitsu *et al.*, 2006; Ding *et al.*, 2007; Kouroku *et al.*, 2007). Further studies revealed that chemical inhibition of autophagy through treatment with 3-methyladenine resulted in an increase in ER-stress mediated cell death, suggesting that the induction of autophagy is a protective mechanism (Ogata *et al.*, 2006). Thus, one explanation of the co-occurrence of autophagy and the UPR in arsenite-exposed LCL is that autophagy is induced as a compensatory mechanism to clear damaged proteins and organelles.

An alternative explanation of our data worth considering is that failed or impaired autophagic clearance of arsenite-damaged macromolecules and organelles results in secondary ER-stress and the UPR. In canine kidney cells, treatment with chemical proteasome inhibitors led to an induction of mRNA encoding both ER chaperone and heat-shock proteins (Bush *et al.*, 1997). In human neuroblastoma SK-N-SH cells, autophagy inhibition resulted in an increased quantity of p62 and LC3-II in protein aggregates (Myeku and Figueiredo-Pereira, 2011). Thus, inhibition of proteasomal or autophagic degradation pathways required for the removal of damaged or misfolded proteins could result in a secondary accumulation of misfolded proteins in the ER, leading to the UPR. It is possible that the load of arsenite-damaged cellular components may compromise lysosomal clearance rates. Studies aimed at measuring the flux of damaged/aggregated proteins over time will be necessary to resolve this mechanistic distinction in LCL. Noteworthy in this regard is our evidence in LCL for an earlier induction of lysosomal biogenesis genes, compared to UPR-related genes in the RNA-microarray study. While not conclusive, this finding suggests that the UPR might be a secondary response to lysosomal loading with damaged cellular components.

This study provides evidence that arsenite-induced autophagy in the LCL model is associated with proteotoxicity, leading to accumulation of misfolded and damaged proteins, to protein aggregation and to the generation of ER stress. Whether this proteotoxic damage targets specific cellular proteins or is a more generalized effect awaits further study. In addition, a more precise analysis of molecular events leading to the induction of autophagy after arsenite exposure could provide insight into how protein damage, ER stress and the

autophagy process contribute to arsenite-induced toxicity in the LCL model. Disrupting protein quality control and homeostasis has the potential to impact many cellular processes, depending on the protein targets of arsenite. This mechanism could provide insight into arsenite-induced perturbation of autophagy in immune cells, and its contribution to arsenic toxicity in exposed individuals.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations Used

LCL	Lymphoblastoid cell lines
LRD	Lysotracker red dye
UPR	Unfolded protein response
BafA1	Bafilomycin A1
TG	Thapsigargin

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Highlights

- Arsenite induces endoplasmic reticulum stress and the unfolded protein response.
- Arsenite induces the formation of protein aggregates that contain p62 and LC3-II.
- Time-course data suggests that arsenite-induced autophagy precedes ER stress.

Figure 1.

Activation of the UPR and autophagy by tunicamycin. A) Representative (3 independent experiments) immunoblot of whole cell lysates of Priess cells exposed to tunicamycin or vehicle. "FL"- Full Length, "CL" - Cleaved. B) Mean LRD fluorescence (RFU, +/- S.D., from 3 independent replicates). "Tub" is α -tubulin. * $P < 0.05$.

Figure 2.

Arsenite-induced proliferative inhibition and induction of autophagy markers in LCL. A) Mean LCL doubling time (hours, $\pm$ S.D. from 6 independent replicates). B) Mean LRD fluorescence (RFU, $\pm$ S.D., from 6 independent replicates). C) Representative (3 independent experiments) LC3 immunoblot of whole cell lysates of LCL exposed to arsenite or vehicle, with and without bafilomycin A1 exposure prior to cell lysis. "Tub" is α -tubulin.

* $P < 0.05$.

Figure 3.

Activation of the UPR by arsenite. Representative (3 independent experiments) immunoblot of whole cell lysates of LCL exposed to arsenite or vehicle. "FL" is full length, "CL" is cleaved. "Tub" is α -tubulin. * $P < 0.05$.

Figure 4. Arsenite increases the quantity of p62- and LC3-containing protein aggregates. A) Representative (3 independent experiments) filter trap assay dot blot image from arsenite-exposed and vehicle-exposed samples. B) Densitometry from the dot blot images in part A. Mean densitometry (Chemiluminescence Intensity, $\pm$ S.D. from 3 independent experiments). "Tub" is α -tubulin. * $P < 0.05$.