HEME-REGULATED INHIBITOR (HRI) KINASE-MEDIATED PHOSPHORYLATION OF EUKARYOTIC TRANSLATION INITIATION FACTOR 2 (EIF2) INHIBITS TRANSLATION, INDUCES STRESS GRANULE FORMATION, AND MEDIATES SURVIVAL UPON ARSENITE EXPOSURE.

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Running Title: HRI mediates translational control in response to arsenite.

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Exposure to arsenite inhibits protein synthesis and activates multiple stress-signaling pathways. Although arsenite has diverse effects on cell metabolism, we demonstrate phosphorylation of eukaryotic translation initiation factor 2 at Ser 51 on the alpha subunit is necessary to inhibit protein synthesis initiation in arsenite-treated cells and is essential for stress granule formation. Of the four protein kinases known to phosphorylate eIF2α, only the heme-regulated inhibitor kinase (HRI) is required for the translational inhibition in response to arsenite treatment in mouse embryonic fibroblasts. In addition, HRI expression is required for stress granule formation and cellular survival after arsenite treatment. In-vivo studies elucidated a fundamental requirement for HRI in murine survival upon acute arsenite exposure. The results demonstrate an essential role for HRI in mediating arsenite stress-induced phosphorylation of eIF2α, inhibition of protein synthesis, stress granule formation, and survival.

Introduction

The aqueous form of the trivalent arsenical is a potent environmental toxin that significantly contributes to human pathogenesis. Exposure to the arsenite anion (AsO₃⁻) results in skin disease, cancer, peripheral neuropathy, and cardiovascular disease [1]. Arsenite exposure is also causative of diabetes [2]. Unfortunately, chronic exposure to arsenite through contaminated water occurs frequently and contributes to carcinogenicity as well as neurological and renal pathology [3]. Based upon incidence, toxicity and potential for human exposure, this toxin is ranked as the number one hazardous substance [4]. Higher doses of arsenite (>5 μM) damage different tissues by induction of apoptosis and necrosis. The dose-dependent and tissue-dependent apoptosis induced by arsenite has led to the use of this compound for the treatment of certain types of cancer including acute promyelocytic leukemia and multiple myeloma [5, 6]. As an issue of major public health concern and its potential application to treat cancer, it is essential to obtain an understanding of the mechanisms mediating arsenite toxicity as well as those affording protection against arsenite exposure.
The physiologically important biological target(s) of arsenite exposure is unknown. Arsenite-induced stress is thought to be the chemical paradigm of heat stress. It is believed that acute arsenite toxicity is due to oxidation of cysteine residues in target proteins that directly alter their conformation and/or activity [7]. Therefore, many cellular responses to arsenite exposure may be a consequence of protein misfolding due to arsenite modification. Compounding the production of unfolded protein, the ubiquitin-proteosome pathway responsible for degrading misfolded and damaged proteins is impaired upon arsenite exposure [8]. Alternatively, arsenite may interact with cysteine-rich, redox-sensitive molecules, such as thioredoxin and glutathione, to generate oxidative stress and interfere with specific biological processes. Exposure to arsenite influences numerous signal transduction pathways that include activation of the stress-activated, mitogen-activated protein (MAP) kinases, extracellular regulated kinase (ERK), c-jun amino-terminal kinase (JNK) and p38 to induce AP-1-dependent transcription [9-11]. In addition, arsenite inactivates proinflammatory NF-κB signaling pathway through inhibition of IkB kinase β [12], inhibits of the STAT family of transcription factors [13], and activates heat shock transcription factor [14]. As a consequence, arsenite suppresses expression of genes encoding anti-apoptotic functions and induces transcription of genes encoding pro-apoptotic functions, cytosolic protein chaperones and other stress-response genes, such as CHOP/GADD153 and metallothionein [15-18]. One of the most immediate cellular responses to arsenite exposure is inhibition of protein synthesis that correlates with phosphorylation of the eukaryotic translation initiation factor 2 at Ser51 on the alpha subunit (eIF2α) [19-21]. Phosphorylation of eIF2α is a fundamental regulatory mechanism that controls global rates of protein synthesis in all eukaryotic cells [20]. Eukaryotic translation initiation factor 2 is a heteromeric GTP-binding protein that binds initiator tRNA_{Met} to form a ternary complex required for 40S ribosomal subunit recognition of the AUG initiation codon within mRNAs. Prior to 60S ribosomal subunit joining, GTP is hydrolyzed to GDP. In order to catalyze another round of initiation, it is necessary to exchange GDP for GTP. As this spontaneous exchange rate is slow, this reaction requires catalysis by the guanine nucleotide exchange factor eIF2B.

Since the cellular amount of eIF2B is limiting over the amount of eIF2 [22], continued protein translation initiation requires catalytic recycling of eIF2B. The recycling of eIF2B is regulated by phosphorylation of eIF2α at residue Ser51. Phosphorylation at this site increases the affinity of eIF2-GDP for eIF2B by 150-fold [23]. As a consequence, eIF2B is sequestered in a nonproductive complex with eIF2-GDP and prevents further initiation events. Therefore, small increases in the level of phosphorylated eIF2α profoundly inhibit the cellular protein synthetic rate. Such a drastic reduction in the rate of translation upon arsenite exposure may be an adaptive mechanism to conserve cellular energy and resources and to prevent the further accumulation of unfolded protein.

It was previously demonstrated that arsenite treatment induces phosphorylation of eIF2α [21]. There are four protein kinases known to phosphorylate eIF2α at Ser 51 in mammalian cells: the double-stranded RNA activated protein kinase PKR [24], the mammalian homologue of yeast GCN2 [25], the endoplasmic reticulum (ER) localized eIF2α kinase PERK/PEK [26, 27], and the heme-regulated inhibitor kinase HRI [28]. Although these protein kinases are homologous in their kinase catalytic domains, they each have different regulatory domains that respond to different stress stimuli. PKR gene expression is induced by class 1 interferons and the protein is translated in a latent form that requires activation by binding to highly-structured or double-stranded RNA. GCN2 is activated by amino acid starvation and mediates translational and transcriptional adaptation to amino acid limitation. PERK is activated by the accumulation of unfolded proteins in the ER. HRI is expressed most abundantly in erythroid cells. HRI is the most important eIF2α kinase in erythroid cells where it is activated by deficiency in heme, the prosthetic group of hemoglobin, through two heme-binding domains. In this manner, HRI coordinates globin mRNA translation with available iron [29]. HRI is required to prevent accumulation of misfolded globin chains in the absence of heme. Previous studies demonstrated that arsenite mediates
phosphorylation of eIF2α through HRI in erythroid cells [30]. However, it is not known which, if any, of these eIF2α kinases mediates the translational inhibition by arsenite in non-erythroid cells.

Environmental stress initiates the appearance of phase-dense particles termed stress granules (SGs) that were first identified upon heat treatment of tomato cells in culture [31]. SG assembly occurs when translation initiation is aborted. When translation is initiated in the absence of functional eIF2-GTP-tRNAiMet, the 40S ribosome stalls on the mRNA to form a 48S preinitiation complex. These complexes of 40S ribosomes, initiation factors, and their associated mRNA transcripts accumulate to form SGs. SG formation requires TIA-1 (T-cell internal antigen 1) and TIAR (TIA-1-related protein) proteins [32, 33]. The mRNAs in SGs are in dynamic equilibrium with the polysomes [34]. Stress granule formation in response to ER stress requires the ER stress kinase PERK [35]. Although depletion of the eIF2-GTP-tRNAiMet ternary complex correlates with SG formation, it is not known whether eIF2 inactivation through eIF2α phosphorylation is required for SG formation [36].

In this study we demonstrate the inhibitory effect of arsenite on protein synthesis is mediated through phosphorylation at Ser51 on eIF2α. Phosphorylation of eIF2α was necessary for arsenite-mediated inhibition of protein synthesis and SG formation. We demonstrate that HRI is expressed and is required for eIF2α phosphorylation to inhibit protein synthesis in response to arsenite-induced stress in murine embryonic fibroblasts (MEFs). The significance of arsenite-induced protein synthesis inhibition was corroborated in vivo as HRI was required for survival when cells or mice were exposed acutely to arsenite.

Experimental Procedures

**Generation of kinase-null mice and MEFs.**

Homozygous HRI+/− mutant mice [37] and mice with the Ser51Ala allele of eIF2α [38] were generated as previously described. PKR−/− mutant mice were kindly provided by Brian Williams [39]. MEFs were generated using standard isolation procedures [40]. PERK−/− mutant GCN2−/− mutant, and control MEFs were kindly provided by David Ron [27, 53]. MEFs were maintained in Dulbecco’s modified essential medium (DMEM) with high glucose (Gibco Corp.) with the addition of 10% fetal bovine serum (HyClone, Logan, Utah), glutamine, penicillin/streptomycin, essential and non-essential amino acids (Gibco Corp.).

**HRI mRNA expression analysis.**

RNA was extracted from wild-type and HRI+/− mutant MEFs using the RNAqueous kit (Ambion, Austin, TX) as per the manufacturer’s protocol. The reverse-transcriptase (RT) reaction mixture was assembled as follows: 2 pmol oligo dT15 (Promega, Madison, WI); 4 μl 5x AMV-RT buffer and 10 units AMV-RT (Promega); 40 units rRNAsin recombinant RNAse inhibitor (Promega); 2 μg RNA; 1.25 mM dNTP mix (Qiogene, Montreal, Quebec); purified water to a final reaction volume of 20 μl. The reaction was heated to 50°C for 1 h, then 75°C for 10 min. The resulting cDNA was purified using a PCR purification column (Qiagen, Valencia, CA) and eluted in 30 μL water. cDNA eluate (5 μL) or HRI cDNA (5 ng) was used as template for the PCR reaction. The PCR was performed using the Advantage cDNA PCR kit (BD Biosciences Clontech, Palo Alto, CA) per the manufacturer’s protocol plus 5% dimethylsulfoxide. The specific primers for HRI were 5’-ATGCTGGGGGGCAGCTCC-’3 and 5’-TCATCTCTTCATCCCTCTG-3. The reaction was cycled at 95°C for 1 min, then 50°C for 1 min, then 72°C for 2 min for 32 cycles, followed by a final extension of 72°C for 2 min. The expected product size for the HRI+/− MEFs was 1549 base pairs and for the HRI−/− MEFs was 1145 base pairs. The PCR products were electrophoresed on a 0.9% agarose gel for analysis. For Northern blot analysis, total RNA was extracted using the RNAqueous kit(Ambion). RNA was electrophoresed on a formaldehyde gel and then transferred onto a nylon membrane for hybridization to a digoxigenin-labeled DNA probe specific to HRI. Detection was performed using anti-digoxigenin alkaline phosphatase-complexed antibody.

**Protein synthesis analysis.**

MEF cultures were grown overnight after passage and incubated for 30 min at 37°C in media
containing 0-200 μM arsenite or 1 μM thapsigargin. Cells were rinsed with phosphate-buffered saline (PBS) and then radiolabeled with 200 μCi/ml [35S]-methionine and cysteine (Specific activity = 1000 Ci/mmol; Amersham Corp., Piscataway, NJ) in methionine- and cysteine-free media (Gibco Corp.) for 10 min. After washing with ice-cold PBS, cell extracts were prepared. Radioactivity incorporated was measured by precipitation with 10% trichloroacetic acid and scintillation counting. Some cell extracts were analyzed by SDS-PAGE and prepared for autoradiography using EnHance (NEN Life Science Products, Inc., Boston, MA).

**Western blot analysis.**
MEFs were cultured and treated as indicated. Cell extracts were prepared with lysis buffer (50 mM Tris pH=7.4, 150 mM NaCl, 10 mM β-glycerolphosphate, 50 mM NaF, 1 mM orthovanadate, 0.1 mM EDTA, 10% glycerol, 1% Triton X-100 and Boehringer Mannheim Complete mini-protease inhibitors). Proteins were analyzed by SDS-PAGE on reducing gels and the phosphorylated and total forms of eIF2α were detected by Western blot analysis as previously described [41]. Antibody for phosphorylated eIF2α was obtained from Biosource (Camarillo, CA) and mouse monoclonal antibody was used for detection of total eIF2α [41]. Anti-phospho-p38 antibody was obtained from Cell Signaling Technology, Beverly, MA. Densitometry scanning was performed and quantified with Image J software obtained from the NIH website.

**Polysome profile analysis.**
Cells in log phase growth were treated with cycloheximide (20 μg/mL for 10 min) prior to being scrape-harvested and processed for polysome analysis essentially as described [42]. The fractionated polysomes were eluted using an ISCO fractionator, with continual monitoring at OD254.

**Immunofluorescence of stress granules.**
Cells were treated with different stress treatments including graded concentrations of sodium arsenite for 45 min, heat shock (44°C for 1 h), puromycin (20 μg/mL for 2 h), and glucose starvation. After treatment, the cells were fixed and stained for goat polyclonal antibodies against TIA-1 (T cell intracellular antigen-1) (# sc-1751, Santa Cruz Biotech.), eIF3 (# sc-16378, Santa Cruz Biotech.), and other SG markers as described previously [33]. MEFs were transfected using QIAGEN reagent as previously described [32].

**Cell viability assay.**
MEF cells were cultured in 96 well plates overnight before exposure to media containing sodium arsenite. Cell viability was measured with a ProCheck Cell Viability Assay Kit (Serologicals Corp., Norcross, Ga.). The assay is based on the conversion of an oxidized tetrazole to a reduced form by mitochondria in metabolically active cells. The absorbance at 480 nm is proportional to the number of viable cells.

**In vivo response to arsenite injection.**
The survival of wild-type, HRI−/−, PKR−/−, and HRI−/−PKR−/− mutant mice upon acute arsenite exposure was assessed 24 h after subcutaneous injection of freshly prepared, sterile sodium arsenite (0-20 mg/kg body weight, as indicated) or phenylhydrazine (45 mg/kg body weight). The University of Michigan Committee for the Care and Use of Animals (UCUCA) approved all animal procedures. Blood samples were collected in EDTA by tail bleed before and at 1 h and 4.5 - 5 h after injection. Reticulocyte counts were analyzed by Coulter counting based on size. Liver samples were taken at 4 h after injection and extracts prepared by homogenization in NP-40 lysis buffer (1% NP-40, 50 mM Tris HCl pH 8.0, 150 mM NaCl) containing protease inhibitors (Boehinger Mannheim), NaF (150 mM), orthovanadate (0.5 mM), β-glycerolphosphate (50 mM), and phenylmethylsulfonyl fluoride (100 μg/ml).

**Results.**

**Arsenite-mediated inhibition of protein synthesis requires phosphorylation of eIF2α.**
To evaluate the role of eIF2α phosphorylation in the cellular stress response to arsenite, we studied MEFs that harbor a Ser51Ala knock-in mutation into the eIF2α gene [38]. Cells were treated with increasing concentrations of sodium arsenite and then pulse-labeled with [35S]-methionine and cysteine to measure the rates of protein synthesis. Where increasing concentrations of sodium arsenite inhibited protein synthesis greater than 90% in wild-type MEFs, the inhibition was significantly less in the homozygous eIF2α A/A mutant MEFs (Fig.)
1A,B). Although the spectrum of abundant polypeptides synthesized was similar under control and arsenite-treatment conditions, the homozygous eIF2\(\alpha\) A/A mutant cells were more resistant to the inhibition mediated by arsenite (Fig. 1A). In three independent experiments, 50 \(\mu\)M sodium arsenite reproducibly inhibited protein synthesis in wild-type MEFs with little change in the homozygous eIF2\(\alpha\) A/A mutant MEFs (Fig. 1C). Immunoblot analysis using antibodies that specifically recognize phosphorylated eIF2\(\alpha\) and total eIF2\(\alpha\) demonstrated a 3-fold increase in eIF2\(\alpha\) phosphorylation in wild-type (S/S) MEFs, where phosphorylation was not detected in the homozygous eIF2 A/A mutant MEFs (Fig. 1D).

Treatment of cells with sodium arsenite activates p38 MAP kinase [7]. To determine whether arsenite actually induces a stress response in the homozygous eIF2\(\alpha\) A/A mutant MEFs, we studied the activation of the p38 MAP kinase cascade by Western blot analysis using an antibody that is specific to phosphorylated p38. The results demonstrated that sodium arsenite activated p38 to a similar level in the wild-type (S/S) and homozygous eIF2\(\alpha\) A/A mutant cell lines by comparison to the total eIF2\(\alpha\) loading control (Fig. 1D). Therefore, although arsenite induced a stress response, protein synthesis was not inhibited in the eIF2\(\alpha\) A/A mutant cells. In addition, p38 activation did not require eIF2\(\alpha\) phosphorylation or translational attenuation.

**Arsenite-mediated eIF2\(\alpha\) phosphorylation and inhibition of protein synthesis requires HRI.**

To evaluate the requirement for the four different eIF2\(\alpha\) kinases in arsenite-mediated eIF2\(\alpha\) phosphorylation and translational inhibition, we studied arsenite responses in MEFs isolated from mice that harbor gene deletions in the different eIF2\(\alpha\) kinases: HRI, PKR, GCN2, and PERK. Homozygous deletion of PKR, GCN2, or PERK had no effect on the arsenite-induced phosphorylation of eIF2\(\alpha\) and inhibition of protein synthesis (Fig. 3A,B). In contrast, deletion of HRI alone prevented both arsenite-mediated eIF2\(\alpha\) phosphorylation and translational inhibition. Although there was a background level of eIF2\(\alpha\) phosphorylation in the HRI\(^{+/+}\) mutant MEFs, this phosphorylation was not increased upon sodium arsenite treatment (Fig. 3A; lanes 7,8). Treatment of the MEFs with thapsigargin, an agent that induces ER stress, significantly inhibited protein synthesis in all cell lines except the PERK\(^{-/-}\) mutant MEFs (Fig. 3B), consistent with the role of PERK in translational attenuation on ER stress [26, 27].

Phosphorylation of eIF2\(\alpha\) inhibits translation at the level of initiation and causes dissociation of polyribosomes and accumulation of monoribosomes and ribosomal subunits. Therefore, we analyzed polysome profiles for control and arsenite-treated MEFs (Fig. 4). These results were quantified by measuring the ratio of polysomes/80S species observed for treated cells relative to cells untreated (values presented in Fig. 4). Comparison of these ratios between wild-type and mutant MEFs shows the area of polysomes/80S upon arsenite treatment was approximately 2-fold greater in the HRI\(^{-/-}\) and eIF2\(\alpha\) A/A mutant MEFs compared to their wild-type controls. In addition, this ratio between the PKR\(^{-/-}\) MEFs and wild-type MEFs did not significantly differ (0.9) (Fig. 4). Thus, less polysome dissociation occurred upon arsenite treatment of homozygous eIF2\(\alpha\) A/A mutant MEFs and HRI\(^{-/-}\) mutant MEFs (Fig. 4). These differences in polysome profiles are consistent with the effects of arsenite on protein synthesis in these cells.

Our data support the conclusion that HRI is the dedicated eIF2\(\alpha\) kinase that mediates translational control in response to sodium arsenite in MEFs. Since previous studies questioned the significance of HRI detected in non-erythroid cells [43, 44], we characterized HRI expression.
Unfortunately, immunoblot analysis with several anti-HRI antibodies did not detect HRI in extracts of wild-type MEFs. Therefore, we performed RT-PCR as a more sensitive means to detect whether HRI mRNA is expressed in MEFs. This analysis demonstrated the presence of a 1.5 kb product in wild-type MEFs that was absent in the HRI-/- mutant MEFs (Fig. 5A). HRI-/- mutant MEFs displayed a 1.1 kb product, consistent with deletion of 404 bases in the mRNA from the HRI-targeted allele [37]. In addition, Northern blot analysis detected a minor RNA species of the expected molecular sizes in both the wild-type and HRI-/- cells (Fig. 5B). Therefore, we conclude that HRI is expressed at a low, but physiologically significant level to mediate arsenite-induced translational attenuation in MEFs.

**Phosphorylation of eIF2α is necessary for stress granule formation.**

The phosphorylation of eIF2α correlates with the assembly of stress granules (SGs) [32], discrete cytoplasmic foci at which untranslated mRNAs and stalled preinitiation complexes accumulate in cells subjected to environmental stress [33, 35]. In order to form SGs, it is necessary to inhibit protein synthesis by greater than 90% [34]. Transient over-expression of a non-phosphorylatable mutant of eIF2α (S51A) inhibited the assembly of SGs in cells subjected to arsenite-induced oxidative stress [32], suggesting that phosphorylated eIF2α is required for SG assembly. Consistent with this hypothesis, we found that wild-type S/S MEFs assembled SGs in response to arsenite, whereas homozygous eIF2α mutant A/A MEFs did not (Fig. 6A, C). Similar results were observed in cells subjected to heat shock or FCCP (carbonyl cyanide p-trifluoromethoxyphenylhydrazone) -induced energy deprivation (data not shown) indicating that phosphorylation of eIF2α is absolutely required for SG assembly. In addition, the HRI-/- mutant MEFs exhibited a severely impaired ability to form SGs in response to sodium arsenite treatment (Fig. 6B, C), although their ability to form SGs (and to phosphorylate eIF2α) in response to heat shock was indistinguishable from that of wild-type MEFs (data not shown). We conclude that arsenite treatment activates HRI, which in turn phosphorylates eIF2α, and thus drives SG assembly. In contrast, heat shock likely activates other eIF2α kinases in addition to HRI.

To further evaluate the requirement for eIF2α phosphorylation to promote stress granule formation in the Ser51Ala eIF2α mutant MEFs, we transfected these MEFs with expression vectors encoding a Ser51Asp mutant eIF2α to mimic a phosphorylated serine that was previously shown to act as a dominant inhibitor of translation [45]. Cells were co-transfected with a β-galactosidase expression vector in order to identify the transfected cells. Analysis of either eIF3 or TIA-1 stress granule markers demonstrated that the homozygous A/A mutant MEFs formed stress granules in the presence of the Ser51Asp mutant eIF2α without any exogenous stress (Fig. 6D). In contrast, transfection with Ser51Ala mutant eIF2α did not induce the formation of stress granules in these cells (data not shown). Therefore, the homozygous A/A mutant MEFs are able to form stress granules in the presence of a phospho-mimetic form of eIF2α. We conclude that phosphorylated eIF2α is both necessary and sufficient for stress granule formation.

**The HRI response to arsenite promotes cellular and whole animal survival.**

To determine whether HRI serves a protective role in response to arsenite, we measured cell survival after sodium arsenite treatment. After 2 h acute exposure to 100µM arsenite, the survival of HRI-/- mutant MEF cells was significantly lower than wild-type or heterozygous HRI +/- mutant MEFs (Fig. 7A). However, all cell lines were sensitive to arsenite-induced cell death upon longer periods of treatment. Similar analysis of the homozygous eIF2α A/A MEFs did not yield consistent results, possibly due to their slow growth rate and/or the inability to grow at low density (D. Scheuner and R.J. Kaufman, unpublished) which may reflect an increased sensitivity to oxidative stress, as described for PERK-/- MEFs [50].

To further investigate the role of HRI in survival upon arsenite exposure, we evaluated the toxicity of sodium arsenite in vivo in adult wild-type and HRI-/- mutant mice. In vivo, arsenite is toxic to target tissues such as liver, kidney, and red blood cells [46-48]. Wild-type HRI+/- mice were injected with a single dose of arsenite at
concentrations of 5-20 mg/kg body weight (Fig. 7B). The 24 h survival was 58% (7/12 mice) after a dosage of 15 mg/kg, consistent with the reported LD$_{50}$ for acute arsenite exposure [49]. The sub-lethal dosage of 15 mg/kg was used for comparison of survival for wild-type and HRI$^+$ mutant mice derived from littermates (Fig. 7C). Where greater than 55% of the wild-type mice survived arsenite injection, only 3% of the HRI$^+$ mutant mice survived (Fig. 7C). The body mass and arsenite dosages were comparable between both groups of mice. There was no significant difference in survival after arsenite exposure observed between male and female mice (not shown).

Previously, PKR was suggested to be the eIF2$\alpha$ kinase that is activated in response to arsenite treatment [19]. Therefore, to evaluate the relative roles of PKR and HRI in arsenite-mediated toxicity, we crossed the PKR-null mice with the HRI-null mice. The HRI$^+$ PKR$^+$ double mutant mice displayed no obvious significant phenotype. MEFs prepared from the HRI$^+$ PKR$^+$ double knock-out mice were resistant to translational inhibition in response to arsenite, similar to the HRI$^+$ single knock-out MEFs (data now shown). Upon injection of arsenite, the survival of PKR$^+$ mice was similar to the wild-type mice, where the survival of HRI$^+$ PKR$^+$ double knock-out mice was reduced to a level comparable to the survival of the HRI$^+$ single knock-out mice (Fig. 7C). Therefore, we conclude that PKR, in contrast to HRI, does not provide significant protection to arsenite exposure under these conditions in mice.

Analysis of red blood cells from HRI$^+$ mice indicated they are more sensitive to oxidative stress, such as that induced by the administration of phenylhydrazine [37]. The survival of phenylhydrazine-treated HRI$^+$ mutant mice was dramatically reduced and this correlated with red blood cell hemolysis. Therefore, we examined whether the reduced survival of HRI$^+$ mutant mice in response to arsenite treatment correlated with increased red blood cell hemolysis. Whereas phenylhydrazine significantly reduced the number of red blood cells in the wild-type mice, acute arsenite treatment actually slightly increased the red blood cell count in the HRI$^+$ mice (Fig. 7D). Therefore, we conclude that arsenite toxicity of the HRI$^+$ mice is likely due to defects in other organs that require HRI to protect against arsenite toxicity. Analysis of liver samples from arsenite-treated mice demonstrated a significant increase in phosphorylated eIF2$\alpha$ in wild-type mice, where there was a 2-fold lesser increase in HRI$^+$ mice (Fig. 7E). Therefore, upon arsenite treatment, HRI contributes significantly to eIF2$\alpha$ phosphorylation in the liver, however, there are additional kinase(s) that also mediate eIF2$\alpha$ phosphorylation under these conditions. It is possible that another eIF2$\alpha$ kinase may be activated indirectly as a consequence of arsenite treatment, e.g. amino acid starvation may activate PERK. Thus, these studies establish that the HRI kinase not only mediates control of translation in response to arsenite stress, but also identify a mechanism necessary for cellular and whole animal survival upon acute arsenite exposure.

**Discussion**

Treatment of cells with agents that are known to modify protein structure, such as heat shock or sodium arsenite, produce high levels of phosphorylated eIF2$\alpha$ and inhibit protein translation. However, there is no direct data to support that eIF2$\alpha$ phosphorylation is necessary and/or sufficient to inhibit protein synthesis in arsenite-treated cells, nor has the responsible kinase(s) been identified. Recent studies demonstrate PERK can mediate eIF2$\alpha$ phosphorylation in response to oxidative stress [50]. Although arsenite induces formation of reactive oxygen species, it is not known whether arsenite treatment can activate PERK. Previous studies also suggested that PKR is the eIF2$\alpha$ kinase activated by arsenite [19, 20]. However, since these studies were performed by expression of a trans-dominant negative mutant PKR, it is not known whether the dominant-negative acts through inhibiting PKR activity or interfering with some additional regulator of eIF2$\alpha$ phosphorylation. Another study demonstrated that arsenite induces activation of the PKR activator PACT that was suggested to mediate eIF2$\alpha$ phosphorylation [51]. However, the studies that implicate PKR in the arsenite translational response did not evaluate the potential importance of HRI. We have compared the requirement for PERK, GCN2, PKR, and HRI in the arsenite translational response. Our studies support the
idea that arsenite treatment activates HRI to phosphorylate eIF2α and that eIF2α phosphorylation is essential to inhibit protein synthesis. In addition, our studies support that PERK, GCN2, and PKR do not significantly mediate phosphorylation of eIF2α in response to arsenite. It was recently established that HRI is activated by arsenite in erythroid cells [30]. However, it was unknown whether eIF2α phosphorylation is required and whether HRI is expressed at physiologically significant levels to mediate eIF2α phosphorylation and inhibition of translation upon arsenite exposure to non-erythroid cells.

Our findings that support that HRI is the kinase that mediates eIF2α phosphorylation upon arsenite treatment include: 1) arsenite treatment did not induce eIF2α phosphorylation or significantly inhibit protein synthesis in HRI+/+ MEFs, whereas protein synthesis was inhibited and eIF2α was phosphorylated in wild-type, GCN2−/−, PKR−/−, and PERK−/− MEFs; 2) arsenite treatment disaggregated polysomes to a lesser degree in eIF2α A/A mutant or HRI−/− MEFs, whereas polysomes were dissociated in wild-type and PKR−/− MEFs; 3) eIF2α A/A mutant and HRI−/− MEFs did not form stress granules upon arsenite exposure compared to control cells that did form stress granules; 4) HRI−/− MEFs were more sensitive to arsenite treatment compared to control MEFs; and 5) HRI−/− mice were more sensitive to arsenite toxicity compared to wild-type or PKR−/− mice. In addition, we have demonstrated that HRI mRNA is expressed in MEFs at a low level. Therefore, this low level of expression appears physiologically sufficient to mediate the translational inhibition in response to arsenite. Analysis of the PKR−/−, PERK−/−, GCN2−/− and HRI−/− mutant MEFs strongly support the hypothesis that HRI is the only eIF2α kinase that signals the arsenite stress response under these conditions.

These results also establish an essential requirement for phosphorylation of eIF2α in SG assembly. Homozygous A/A eIF2α mutant MEFs did not assemble SGs in response to arsenite, heat shock or FCCP. In addition, transfection of a Ser51Asp eIF2α mutant, to mimic the phosphoserine, was sufficient to induce SGs in these cells in the absence of exogenous stress. These findings indicate that phosphorylation of eIF2α is essential and sufficient for SG assembly. Previous studies demonstrated that acute energy starvation mediated by uncoupling oxidative phosphorylation through FCCP treatment induces the assembly of SGs without detectably increasing the phosphorylation of eIF2α [35]. These findings lead to propose that reduced levels of eIF2-GTP-tRNA^Met ternary complex is the key trigger for SG assembly, rather than phosphorylation of eIF2α per se [33, 35]. It is likely that basal levels of constitutively phosphorylated eIF2α are sufficient to induce SGs in wild-type cells treated with FCCP. Regardless, the essential requirement for phosphorylated eIF2α demonstrated in this study requires a reconsideration of current models for SG assembly [36], that currently predict that SG assembly is a result of ternary complex insufficiency rather than due to eIF2α phosphorylation per se. The data presented here suggest a more direct role for phospho-eIF2α in SG assembly.

Phosphorylation of eIF2α is implicated in both apoptotic and anti-apoptotic responses. It is proposed that eIF2α phosphorylation promotes apoptosis by preventing translation of inhibitors of apoptosis [41, 52]. Alternatively, eIF2α phosphorylation is implicated in survival by inhibiting translation when protein-folding conditions are suboptimal and by mediating the preferential translation of protective factors, such as the transcription factor ATF4 [27, 38, 53]. Therefore, it is possible that eIF2α phosphorylation may contribute to both survival and death responses upon arsenite exposure. However, our findings in MEFs and in mice support that HRI-mediated phosphorylation of eIF2α is a protective response to arsenite exposure. In contrast, the utility of arsenite as an anti-cancer agent may be attributed to its effects on another signaling pathway, such as activation of JNK or inhibition of NFκB activation [5, 6]. Since HRI-mediated phosphorylation of eIF2α protects cells from arsenite exposure, there should be consideration to developing agents to inhibit HRI that may be used in conjunction with arsenite for therapeutic intervention in cancer.

Chronic arsenite exposure is a major public health concern affecting a large percentage of the world population. We have shown that in
vivo survival upon acute arsenite exposure requires HRI activity. eIF2α phosphorylation in liver tissue was increased in an HRI-dependent manner, consistent with reports of liver toxicity and arsenite accumulation in the liver [48, 49]. The identification of HRI as a protective factor for arsenite toxicity may also provide an avenue toward rational development of HRI agonists for the treatment and/or prevention of arsenite-induced disease and death.

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References

Figure Legends

Figure 1. Arsenite inhibits protein synthesis through eIF2α phosphorylation.
Wild-type eIF2α (S/S) and homozygous Ser51Ala mutant eIF2α (A/A) MEFs were incubated in 0-200 μM arsenite (As) for 30 min and then pulse-labeled with [35S]-methionine and cysteine.
A. SDS-PAGE analysis of metabolically labeled proteins in cell extracts.
B. Quantitation of protein synthesis after treatment with sodium arsenite. Analysis was performed in triplicate as described in Experimental Procedures. SEM error bars were small and not apparent on the graph.
C. Protein synthesis after sodium arsenite treatment. MEFs were treated with 100 μM sodium arsenite for 30 min and then pulse-labeled with [35S]-methionine and cysteine. Cells were harvested and incorporation measured as described in Experimental Procedures. Results are presented as mean ± SEM from three independent experiments.
D. Phosphorylation of eIF2α and p38 upon sodium arsenite treatment. Western blot analysis was performed after exposure of MEFs to 100 μM arsenite for 30 min. eIF2α and p38 phosphorylation were quantified and normalized to total eIF2α. The ratio of the normalized phosphorylation signal of arsenite treated versus untreated is indicated.

Figure 2. Arsenite-mediated eIF2α phosphorylation and inhibition of protein synthesis requires HRI.
Wild-type or the indicated eIF2α kinase-null MEFs were incubated in 100 μM arsenite or 1μM thapsigargin (Tg) for 30 min and then pulse-labeled with [35S]-methionine and cysteine.
A. Western blot analysis of MEFs treated with 0 and 100 μM arsenite using anti-phospho-eIF2α, anti-phospho-p38, and anti-total eIF2α antibody. eIF2α and p38 phosphorylation were quantified and normalized to total eIF2α. The ratio of the normalized phosphorylation signal of arsenite treated versus untreated is indicated.
B. MEFs were treated with increasing concentrations of arsenite (0, 10, 50, 200 mM) for 30 min, then pulse-labeled with [35S]-methionine and cysteine, and then harvested as described in Experimental Procedures. Equal amounts of protein extract were analyzed by SDS-PAGE and autoradiography. Quantitation of protein synthesis after treatment with sodium arsenite was performed in triplicate as described in Experimental Procedures. Data are mean ± SEM.

Figure 3. Arsenite-mediated eIF2α phosphorylation and inhibition of protein synthesis requires HRI.
Wild-type or the indicated eIF2α kinase-null MEFs were incubated in 100 μM arsenite for 30 min and then pulse-labeled with [35S]-methionine and cysteine.
A. MEFs were treated with 0 and 100 μM for 30 min and then analyzed by Western blot using anti-phospho-eIF2α and anti-total eIF2α antibody. eIF2α phosphorylation was quantified and normalized to total eIF2α and the ratio of arsenite treated versus untreated is indicated for each MEF.
B. MEFs were treated with 100 μM sodium arsenite for 30 and then pulse-labeled with [35S]-methionine and cysteine. Cells were harvested and incorporation measured as described in Experimental Procedures. Results are presented from three determinations.
**Figure 4.** HRI kinase and phosphorylation of eIF2α inhibit translation initiation. Polyosome profiles were obtained from cell extracts prepared after incubation of wild-type S/S MEFs, homozygous mutant eIF2α A/A MEFs, PKR−/− mutant MEFs, PKR+/− (C57Bl/6J MEFs for control of PKR−/− genetic background), HRI+/+ wild-type MEFs, and HRI−/− mutant MEFs in 0 [-] or 250 μM [+] arsenite for 30 min. The values presented are the ratio (P:M) of polysomes(P)/80S(monosomes(M)) species for arsenite-treated relative to the untreated cells. Samples treated with arsenite are shown in bold.

**Figure 5.** HRI kinase mRNA is expressed in wild-type MEFs. A. RNA was extracted from wild-type and HRI−/− mutant MEF cultures and RT-PCR was performed. The amplification primers produce a 1549 bp product from the wild-type HRI mRNA, amplifying from bp 311 of the coding sequence to the stop codon of the mRNA. In the mRNA of the HRI+/+ MEFs, the primers amplify a truncated 1145 bp product. B. Northern blot analysis was performed as described in Experimental Procedures.

**Figure 6.** HRI-mediated phosphorylation of eIF2α is required for stress granule formation. Wild-type eIF2α S/S, homozygous eIF2α mutant A/A, HRI wild-type and HRI+/− mutant MEFs were treated with increasing concentrations of sodium arsenite for 45 min, then processed for immunofluorescence and visually scored for SGs, using TIA-1 and eIF3 as SG markers. More than one hundred cells were scored per treatment. Upper panels, graphic data; lower panels, representative images of cells treated with 1000 μM arsenite and stained for TIA-1 or eIF3 to reveal the presence or absence of SGs. No granules were detected in the mutant eIF2α A/A MEFs at any concentration of arsenite, thus there are no visible data on bar graph (A). A. Wild-type eIF2α S/S and homozygous eIF2α A/A MEFs. B. Wild-type HRI+/+ and homozygous HRI−/− MEFs. C. Morphology of wild-type eIF2α S/S, homozygous eIF2α A/A, wild-type HRI+/+, and HRI−/− mutant MEFs treated with 1000 μM arsenite and stained for either TIA-1 or eIF3 as indicated. D. Homozygous mutant eIF2α A/A MEFs were co-transfected with a β-galactosidase expression vector in the presence of a vector Ser51Asp mutant eIF2α expression. At 48 hr post-transfection, cells were analyzed for SGs as described in Experimental Procedures.

**Figure 7.** HRI is required for survival to arsenite. A. MEF viability. Wild-type and HRI−/− MEFs were exposed to (100 μM) arsenite for 0-3 h and cell viability was measured after 48 h. B. Dose-response for survival to arsenite injection into wild-type mice. Arsenite was injected subcutaneously at dosages of 5-20 mg/kg and survival was determined after 24 h. n=2-12 mice per dosage. C. Arsenite survival in wild-type, HRI−/−, PKR−/−, and PKR−/− HRI−/− double- mutant mice. Mice were injected with 15 mg/kg arsenite and survival was determined after 24 h. N = number of mice. D. Red blood cell counts in phenylhydrazine- and arsenite- treated mice. Mice were treated with phenylhydrazine or arsenite (15 mg/kg) and red blood cell counts were determined as described in Experimental Procedures. E. eIF2α phosphorylation in liver extracts. Wild-type and HRI−/− mice (3 each) were treated with sodium arsenite (15 mg/kg) or vehicle and liver extracts were prepared after 4 h for Western blot analysis using antibodies specific to phosphorylated eIF2α as
described in Experimental Procedures. Blots were stripped and reprobed with antibodies specific for total eIF2α. Each lane represents a liver extract sample from different mice. The ratio of phosphorylated to total eIF2α was determined by averaging the densities for each set of three individual mice.
Figure 1

A. 
As, μM  1  10  50  100  200  1  10  50  100  200

1  2  3  4  5  6  7  8  9  10  11  12

B. 

S/S  A/A

500  400  300  200  100  0

0.1  1  10  100  1000

As, μM

C. 

Mock
As (50μM)

S/S  A/A

D. 

A/A  S/S

-  +  -  +  As

eIF2α-P  0.0  3.3

eIF2α  5.6  5.0

p38-P  1  2  3  4

Downloaded from http://www.jbc.org/ by guest on October 5, 2017
Figure 2

[Image of a gel with bands labeled elF2α-P, elF2α, and p38-P, showing results for HRI+/+, HRI−/−, PKR−/−, and HRI+/− treated with As, 100 µM.]

[Bar graph showing 32S-cpm/µg for HRI+/+, PKR−/−, and HRI+/− treated with different concentrations of As (0, 10, 50, 200 µM).]
Figure 3

A. 

<table>
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<th>S/S</th>
<th>A/A</th>
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B. 

![Graph showing 3S-cpm/μg for different genotypes](http://www.jbc.org/)

- **Mock**
- **As**
- **Tg**
Figure 4

80S Polys
S/S
P/M (+As)
P/M (-As)
0.13

80S Polys
A/A
P/M (+As)
P/M (-As)
0.24

PKR\(^{+/+}\)
0.17

PKR\(^{-/-}\)
0.15

HRI\(^{+/+}\)
0.33

HRI\(^{-/-}\)
0.60

Top  Bottom  Top  Bottom
Figure 5

A. RT-PCR

B. Northern

2036 1636 1018 506

1kb ladder HRI +/+ HRI −/− HRI cDNA

1549 1145

HRI +/+ HRI −/−

28S 18S

ethidium bromide 18S
Figure 6

A. S/S vs A/A

- Bar graph showing % cells with SGs at different As, μM concentrations.

B. HRI+/+ vs HRI−/−

- Bar graph showing % cells with SGs at different As, μM concentrations.

C. Images comparing:

- S/S
- HRI+/+
- HRI−/−
- A/A
- HRI−/−
- HRI−/−

Legend:
- TIA-1
- eIF3

Scale bar: 10 μm
Figure 6D

A/A + p51D + β GAL

β gal / eIF3

β gal / TIA-1
Figure 7

A. Viability

B. Survival %

C. Survival % (15 mg/kg)

D. Strain/mouse Treatment | RBC x 10^9/ml
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E. Control | As

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Heme-regulated inhibitor (HRI) kinase-mediated phosphorylation of eukaryotic translation initiation factor 2 (eIF2) inhibits translation, induces stress granule formation, and mediates survival upon arsenite exposure
Edward McEwen, Nancy Kedersha, Benbo Song, Donalyn Scheuner, Natalie Gilks, Anping Han, Jane-Jane Chen, Paul Anderson and Randal J. Kaufman

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