Understanding the Cellular Stress Response of Redox Metals

A Major Qualifying Project

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Abstract

When eukaryotic cells are stressed by acute levels of arsenite, they cause stress granule formation through the phosphorylation of eIF2alpha through the HRI kinase pathway, to conserve cellular resources and restrict mRNA translation. However, it is unclear if other metals create a similar cellular stress response. Using fluorescent microscopy, we measured the presence stress granules in U20S-DS cells and HAP1 cell lines exposed to acute doses of arsenic, cadmium, and lead. We find that cadmium (Cd) causes stress granule formation likely via activation of the HRI kinase pathway, but lead (Pb) does not initiate stress granules.

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Table of Contents

Background

Cellular Stress Response

As eukaryotic cells develop, they are subject to many different environmental stressors. Some of these stressors include oxidative stress, viral infection, heavy metal stress and others (Kültz 2003). To conserve resources, eukaryotic cells elicit a cellular stress response (CSR) that encompass the cellular processes needed to assess and prevent damage to the cell. In doing so, the CSR works to temporarily increase the cell's ability to tolerate stress but if damage is too severe to repair, the cell CSR will induce cell death or apoptosis (Kültz 2003; Kültz 2005; Arimoto et al. 2008).

The CSR encompasses different times frames and stages. Shorter responses act to manage damaged macromolecules, and long-term responses focus on restoring cellular homeostasis. (Kültz 2003; Kültz 2005). During the initial stage of the CSR in the shorter response, cell growth is halted, nucleic acids and proteins are repaired, and any macromolecular debris is cleared (Kültz 2003). The initial response is stress-specific and contributes to the formation of stress granules (Arimoto et al. 2008).

Stress Granules

In the cellular stress response, mRNA translation is halted. Untranslated mRNAs transcripts under conditions of transient acute stress consolidate into mRNA-containing protein aggregates known as stress granules (SGs) (Anderson and Kedersha 2008). In addition to mRNA, stress granules also contain proteins associated with the stress response like G3BP, TIA-1, and other proteins that are dependent on the mechanism of stress. The untranslated mRNAs are released from the polysomes and packed into cytoplasmic foci to form SGs. The related proteins get sorted and some are packed into SGs (Aulas et al. 2017).

SGs are commonly formed by the phosphorylation of eukaryotic translation initiation factor 2α (eIF2α). eIF2α is responsible for the delivery of initiator tRNAs pre-initiation complexes required for the translation initiation of mRNA transcripts. Under a stress response, specific kinases are activated and phosphorylate eIF2α at serine 51 (S51) position, which regulates the global rates or protein synthesis in eukaryotic cells (McEwen et al. 2005, Aulas et al. 2017). eIF2α is a GTP binding protein that binds the tRNA initiator methionine to form a ternary complex required for 40S ribosomal submit recognition of AUG initiation complex. In addition to its role in translation regulation, eIF2α phosphorylation has cell-cycle progression and memory consolidation in the nervous system and therefore has been linked to many diseases such as cancer and neurodegenerative diseases (Boye, E., & Grallert, B. 2019). Therefore, it is important that we understand the molecular pathways to potentially provide insight into the pathology to SG related diseases.

There are four known kinases that can perform this phosphorylation event of eIF2α in mammalian cells, including protein kinase R (PKR), general control non-repressed 2 kinase

(GCN2), heme-regulated inhibitor kinase (HRI), and PKR-like ER kinase (PERK) (Basu et al. 2017, McEwen et al. 2005). These four known kinases are stress specific. PKR is known to be induced by viral stress caused by class 1 interferons (Basu et al. 2017, McEwen et al. 2005). GCN2 is induced by amino acid starvation and mediates translational adaption to starvation (Basu et al. 2017, McEwen et al. 2005). PERK is induced by the accumulation of unfolded proteins in the endoplasmic reticulum (Basu et al. 2017, Liu, Jin, et al.2019). HRI is important in erythroid cells where it activated by deficiency in heme, through heme binding domains (Basu et al. 2017, McEwen et al. 2005, Wang, X., Wang, L., & Liu, S. 2015). When the environmental stress subsides, the stress granules will dissolve, and the mRNAs can once again be translated or can be targeted for decay.

Arsenite

High doses of arsenite have been known to cause damage to different tissues by induction of apoptosis or necrosis (McEwen et al., 2005). The most immediate cellular response to arsenic exposure is the inhibition of protein synthesis that correlates with the phosphorylation of the eukaryotic translation initiation factor 2 alpha at Ser51. Out of the four protein kinases known to phosphorylate elF2α, the expression of HRI is essential to mediate the phosphorylation of eIF2α in an arsenite induced response (McEwen et al., 2005). It was once thought that arsenite could be reliant on the PERK or PKR kinase in response to oxidative stress. In a viral stress study, researcher looked at flavivirus infected cells that induced oxidative stress but developed resistance to arsenite induced SG response after infection (Basu, M., Courtney, S. C., & Brinton, M. A. 2017). Previous studies also did not look into the significance or HRI compared to the other kinases. Measuring the phosphorylation of eIF2α and in the presence of SGs in knockout lines of the four kinases showed that PERK, GCN2, and PKR do not significantly mediate phosphorylation of eIF2α in response to arsenite (McEwen et al., 2005).

Toxic Redox Metals: Cadmium and Lead

Toxic metals like arsenic, cadmium, and lead are widely found in our environment in contaminated air, water, soil, and food. These metals act as catalysts in oxidative reactions of biological macromolecules, and their toxicities are associated with oxidative stress in cells (Ecral et al., 2001). These metals deplete cells major antioxidants like thiol-containing antioxidants and enzymes and cause an increase in reactive oxygen species (ROS). An increase of these species is what result in oxidative damage caused to lipids, proteins, and DNA. Tracing heavy metal toxicity to diverse cells has been reported to cause a wide array of detrimental health problems including neurological disorders, cancers, anemia, and more. However apart from arsenite, little is understood about whether other toxic metals like cadmium and lead that induce oxidative stress, can also illicit the CSR to provide protective adaptive mechanisms like stress granules to alleviate stress in cells (Chen L, Liu B 2017). Further, understanding of the metal-induced oxidative stress in cells in other heavy metals can help to better understand the toxic effects of heavy metals.

Previous studies have demonstrated that cadmium has shown evidence of some stress granules and that it may be through eIF2α phosphorylation. A study showed that sub-lethal levels of cadmium inactivate several DNA repair enzymes and in turn show a presence of cytoplasmic

"stress granules" appearing structures (Bravard, A. et al., 2010). It was previously demonstrated through confocal microscopy analyses that cells exposed to Cd recruited hOGG1 (a major DNA glycosylase responsible for the initiation of the base excision repair (BER) of 8-oxoguanine)-GFP into discrete structures in the cytoplasm (Bravard, A. et al., 2010). While the description of this recruitment is consistent with mechanism of stress granules, the confocal microscopy or western blots revealed no indicators of proteins, or examination of eIF2α to confirm that the structures are indeed SGs. A study examining the effects of cadmium induced neuronal injury by inhibiting the endoplasmic reticulum (ER) stress eIF2α, western blots revealed levels of eIF2α phosphorylation (p-eIF2α) and ER proteins (Yuan, Y. et al., 2019; Liu, J. et al., 2019). Studies have also found there to be indication of the PERK and HRI kinase playing a role in cadmium induced stress. Studies have shown phosphorylation of PERK in cadmium stressed ovarian cell tissue (Liu, J. et al., 2019; Shi, X-T. et al., 2020). HRI has been noted to play a protective role in mitigating the damage caused by cadmium induced liver and kidney tissue (Wang, L. et al., 2017). However, despite these studies, it remains unclear which eIF2α kinase is primarily responsible for Cd-induced SGs. Given that lead and cadmium are both redox metals and share potential linkages eIF2α kinases, it may be that they could have similar cellular stress responses, though this has not been directly studied.

The cellular level effects of cadmium and lead, particularly related to the activation of cellular stress response pathways, are not well understood. While there seems to be reference to the different individual aspects of the eIF2α pathway, there is not yet sufficient information to indicate whether this works to create a protective adaptive response to cellular stress. This project aims to determine stress granule formation and eIF2α kinase pathway activation in response to cadmium and lead. We used fluorescence microscopy to analyze stress granule formation and stress pathway activation in cells. We find that Cd, but not Pb, causes stress granules in two different cell lines. Further, we map the pathway activation of Cd to the HRI pathway. The work provides further information about the cellular effects of cadmium and lead exposure, and has implications for heavy metal exposure in cells.

Materials and Methods

Cell Line Maintenance

Double-stable osteosarcoma (U2OS-DS) cells containing GFP-G3BP with RFP-DCP1 (Kedersha et al., 2008) were maintained for the first exposure assay to assess stress granule formation. For the rest of the exposure assays, the near-haploid human HAP1 cells (Aulas et al., 2017) derived from chronic myelogenous leukemia cells were maintained as a tool to study the translational control further.

Complete DMEM media (DMEM with 10% FBS, 1% Penicillin/Streptomycin and 1% glutamine) was used for the U2OS-DS and HAP1 lines. HAP1 cells either were the wild-type condition, had a S51A mutation, or had a PERK or HRI kinase knockout lines. All cell lines were incubated at 37°C with 5% CO2. These cells were sub-cultured approximately every other day at a ratio of 1:4 or 1:6 U2OS-DS cell line, and 1:8, 1:10, or 1:12 for HAP1 cell line.

Acute Exposure Assays

U2OS-DS or HAP1 cell lines were plated in a 12-well plate with coverslips at about 8 x 104 - 1.2 x 105 per well. Each well contained 1 mL media. The plate was incubated for ~48 hours at 37˚C. Then 0.5 mL media was taken from each of the wells and combined with media from other wells that received the same treatment contained the same cell line. Media was either mixed with the drug treatment as indicated on the well plates in Figure 1 or left untreated. The remaining media was aspirated from each well, 0.50 mL of the treated media was immediately returned to the appropriate wells, and the plate was incubated at 37˚C for 60 minutes

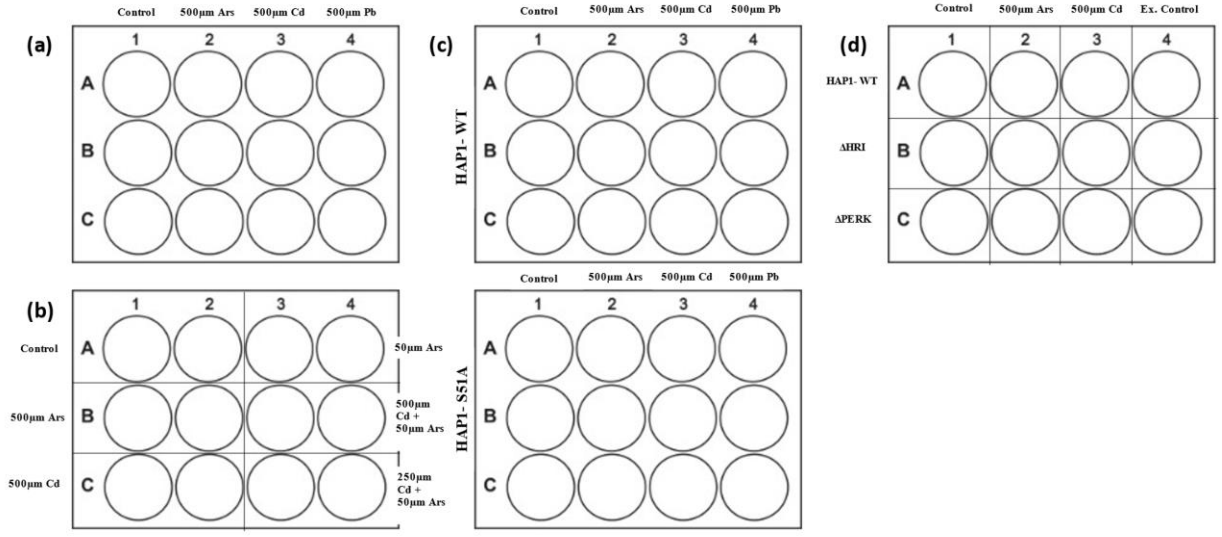


Figure 1: Acute exposure assay plate arrangement used for **(a)** U2OS-DS cells, **(b)** HAP1 cells **(c)** HAP1 cell lines **[top]** HAP1-S51A cell lines **[bottom] (d)** HAP1-WT cell lines, HAP1-ΔHRI cell line, HAP1-ΔPERK cell line. Unlike the other plates, the cell lines were separated **horizontally [A, B,C],** and the treatments were indicated **vertically [1,2,3,4].** An extra untreated control was maintained in the last well column **[4]**

After incubation, the media was aspirated, and the wells were rinsed with sterile 1X PBS. About 0.5 mL 4% paraformaldehyde was added to each well, and the plate rotated on an orbital shaker at room temperature for 10 minutes. The 4% paraformaldehyde was removed from each well and discarded in the labeled waste container. About 0.5 to 1 ml of cold 100% methanol was added to each well and the plate rotated on an orbital shaker for 10 minutes. The 100% methanol was discarded from each well and put into the labeled waste container. Followed by four rinses with 1X PBS. Following the rinses, U2OS-DS cells were directly mounted onto glass slides. For HAP1 cell lines experiments the cells were first stained using the staining protocol below. Following staining, the coverslips were mounted onto glass slides. One cover slip was mounted per slide with ~1mL of vinyl mounting media (Fukui et al. 1987). The percentage of cells positive for stress granules was calculated based on manual counts using fluorescence microscopy. The cells were viewed at 40X magnification, and a minimum of 250 cells over 4 fields for the U20S DS cell line were counted on each slide. A minimum of 125 cells were viewed over 4 fields for the HAP-1 cell lines. The labels on the slides were blinded, and the results from three independent counts were averaged to reduce bias.

Staining Cells for Fluorescence Microscopy

Following the acute exposure assay and fixing of the cells with paraformaldehyde, each well was treated with 0.5 mL of a 5% BSA (bovine serum albumin) in PBS blocking solution, and the plate was incubated on a rotator for 1 hour. The blocking solution was removed from the wells, 0.5mL of the primary antibody solution (Table 1) was added to each well, and the plate was incubated on a rotator for 1 hour. The antibody was then removed and saved, and the wells were washed three times by addition of 1X PBS and incubation on a rotator for five minutes. The PBS was removed between washes and after the final wash. 0.5mL of the secondary antibody was then added to each well (Table 1). The secondary stain was applied for 1 hour while the plate was incubated on an orbital shaker. The secondary stain was then removed, and wells were washed three times with 1X PBS as before. The coverslips were then mounted as described above.

Table 1: Antibodies and Dilutions Used in This Study

Antibody	Application	Dilution	Manufacturer	Product Number
Hoschst 33342 nuclear stain	Immunofluorescence secondary stain	1:5000	Life Technologies	1642791
rabbit -anti-G3BP	Immunofluorescence primary stain	1:1000	AbCam	ab181150
Anti-rabbit IgG Alexa Fluor RT 488 (green) stain	Immunofluorescence secondary stain	1:500	Cell Signaling Technology	4412S

Results

Ars, Cd, Pb acute exposure comparison assay

To determine whether cadmium and lead formed stress granules, an acute exposure assay was performed on U2OS-DS cells. As shown in Figure 3, stress granules appear as many small white dots around the cytoplasm of the cell. Cells that did not have stress granules has a cytoplasm that appeared grey throughout. The results from the assay can be found in Figure 4.

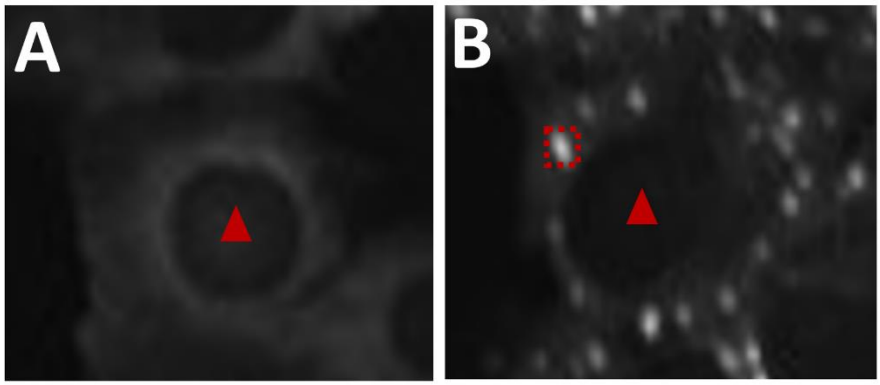
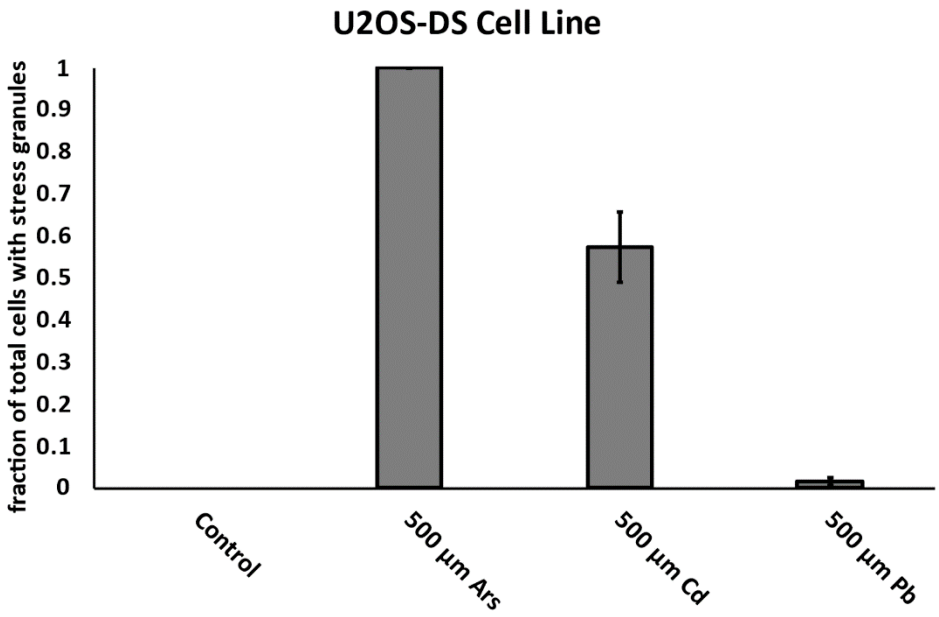


Figure 2: A U2OS-DS cell viewed at 40X. (A) U2OS-DS cell that contain no stress granules (B) and that contain stress granules that appear as white dots. The red dotted square circles a singular stress granule. The triangle (A&B) indicts where the nucleus is. The lighter area is the cytoplasm which is where most the stress granules appear on the slides

A



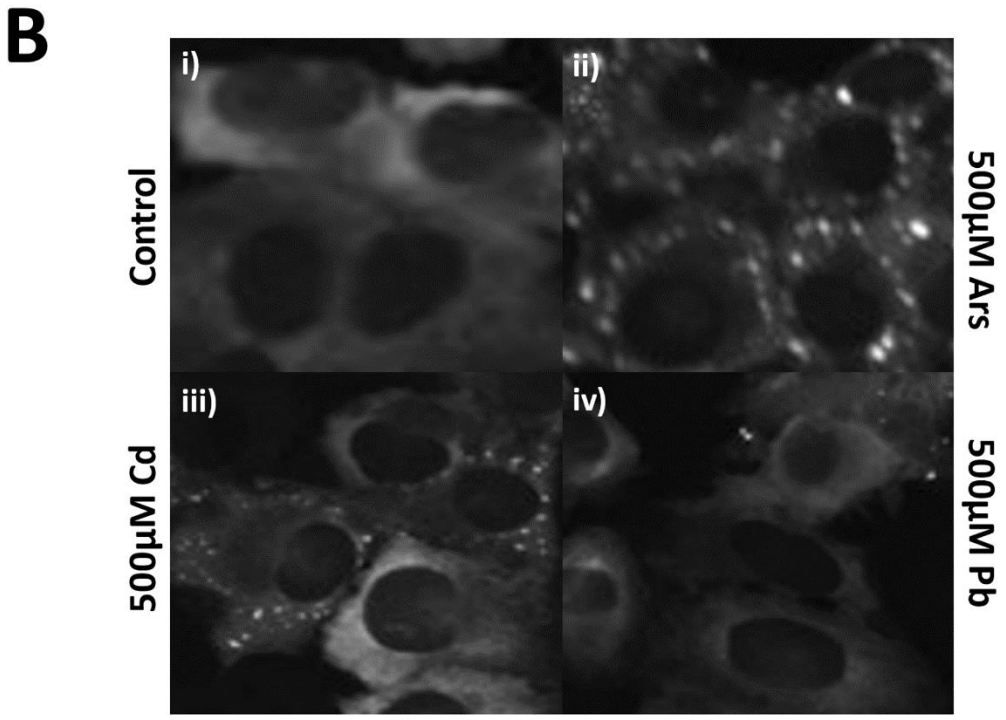


Figure 3: (A) U2OS-DS cells were treated for 60 minutes with 500µM solutions of the treatments indicated on the chart, and the percentage of cells containing stress granules was determined. n=3, error bars +/- S.E.M **(B)** The cells were viewed at magnification 40X, and a minimum of 250 cells over 4 fields were counted on each slide. To reduce bias, the labels on the slides were blinded, and the results from three independent counts were averaged.

It was found that 100% of cells formed stress granules under 500µM of arsenite. Arsenite was the positive control of this experiment and was consistent with previous experiments and the following experiments where arsenite is used as a positive control (Hoppe, M., Friend, C., & Wu, J. 2018). 0.572 or 57.2% percent of cells formed SGs under 500µm of cadmium. Lead resulted in the lowest fraction of stress granule formation accounting for 0.0142 or 1.42% cells with stress granules. There was little to no stress granules in lead but around more than half of the cells in Cd expressed stress granules.

Ars and Cd acute exposure comparison assay

An arsenite and cadmium combined exposure assay was performed to observe whether arsenite and cadmium were potentially working in the same pathway. Using the same controls from the Ars, Cd, Pb acute exposure comparison assay, this experiment included drug treatments of 50µM of Ars(ii), 500µM Cd + 50µM of Ars(iv), 250µM Cd + 50µM of Ars(vi). This experiment abd the following experiment used HAP1 cell lines. Figure 5A shows the fraction of stress granules in these treatments. Our negative (untreated) control showed no stress granules. 500µM Ars showed 100%. 500µm Cd had a fraction of 0.551 or 55.1%. 50µM Ars reported 0.00830 or

0.830%. 500µM Cd + 50µM Ars reported a fraction of 0.823 or 82.3% and 250µM Cd + 50µM Ars reported a fraction of 0.633 or 63.3% of cells.

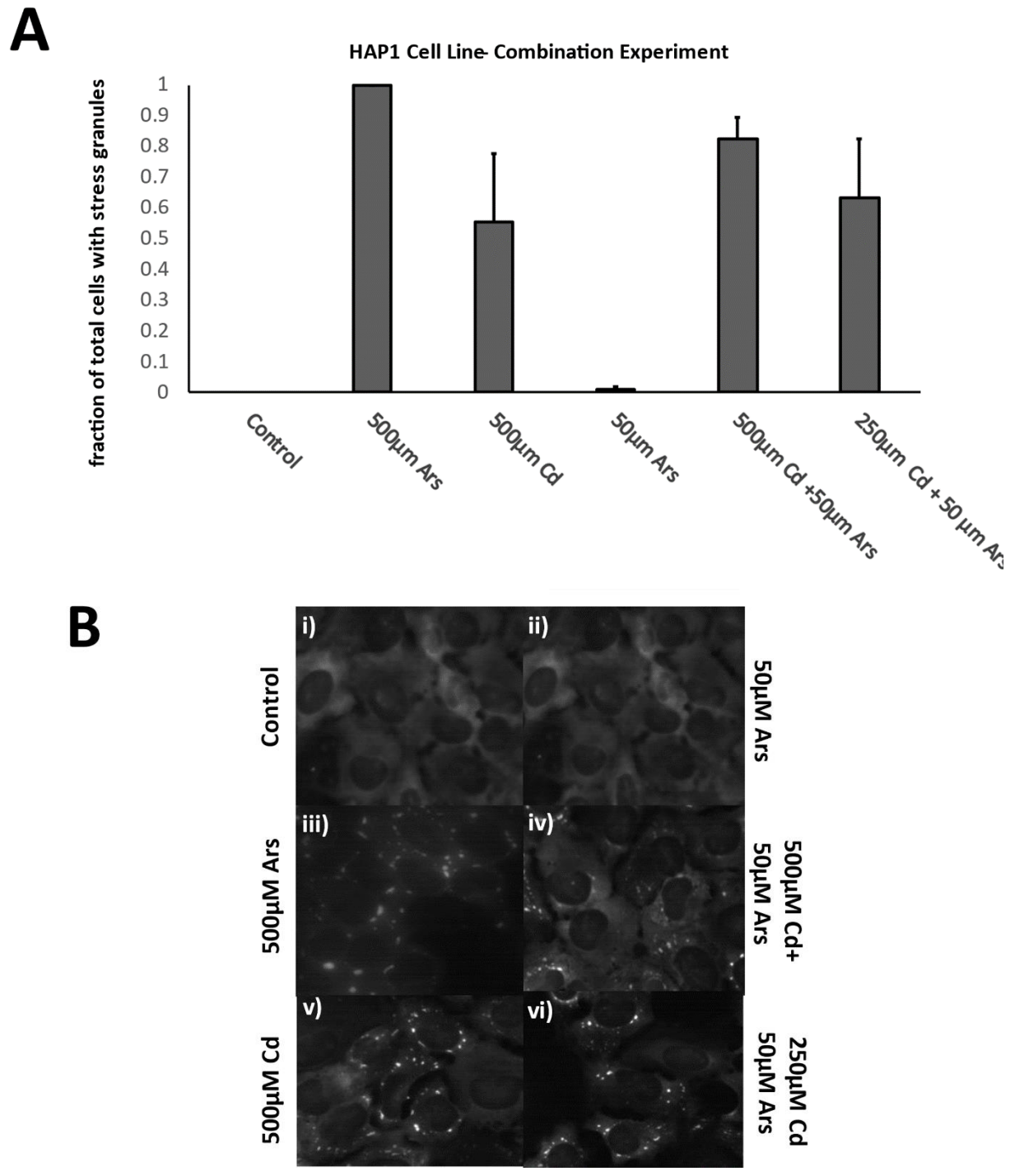


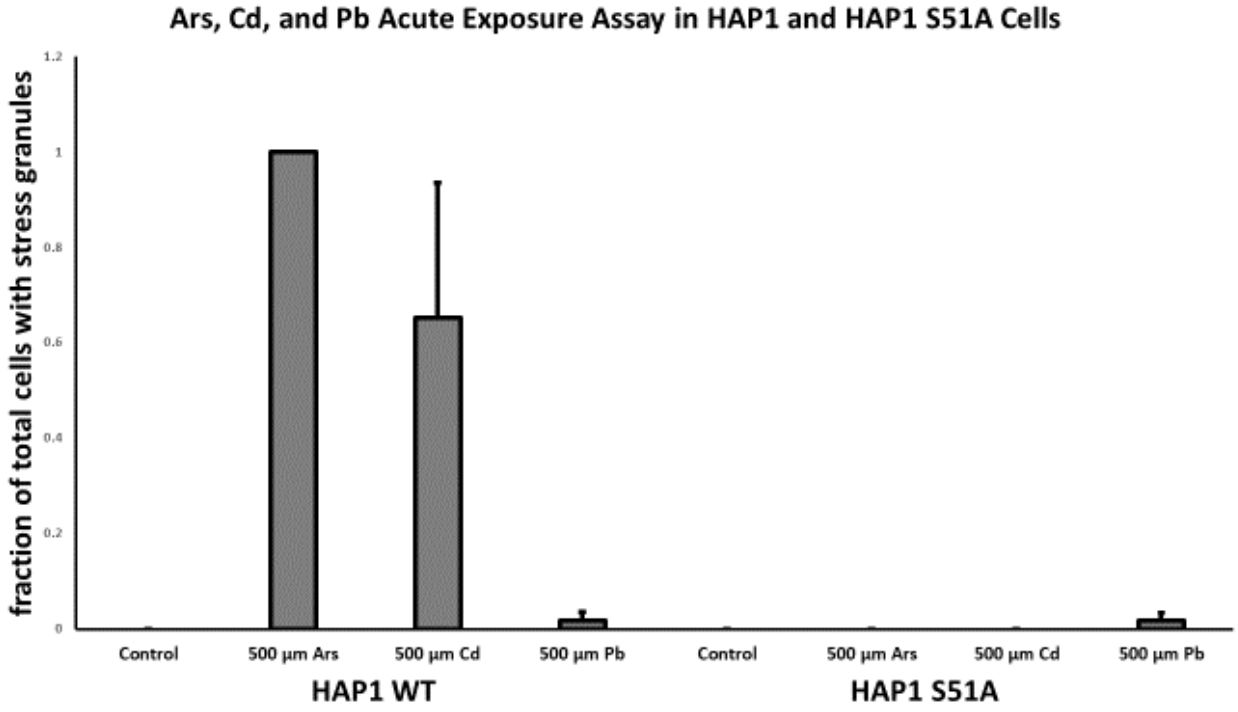
Figure 4: (A) HAP-1 cells were treated for 60 minutes with indicated µM solutions of the treatments indicated on the chart, and the percentage of cells containing stress granules was determined. n=3, error bars +/- S.E.M. Experiment 2 was voided due to inconsistent results in +/- controls**. (B)** The cells were viewed at magnification 20, and a minimum of 125 cells over 4 fields were counted on each slide. To reduce bias, the labels on the slides were blinded, and the results from three independent counts were averaged.

Compared to 500µM Cd, the addition of 50µM Ars caused at least a 10% increase in 250µM of Cadmium and 30% increase in 500µM. Indicating that these two metals could be acting in through a similar cellular stress pathway

Ars, Cd, and Pb acute exposure comparison assay in HAP1 WT and S51A cell line

To confirm if arsenite and cadmium were acting in a similar cellular stress pathway a cellular stress assay similar to the first experiement was done using the HAP1 WT cells and HAP1 cells with a serine 51 mutation. Phosphorylation on serine 51 of eIF2α by stress-responsive kinases leads to the formation of stress granules. With the mutation it was predicted that cells reliant on the eIF2α would not show a presence of stress granules. The HAP1-WT cells did not contain this mutation. Three trials of this experiment were performed but only 2 trial results are present for 500µM Cd due to the third trial's coverslip was misplaced.

A



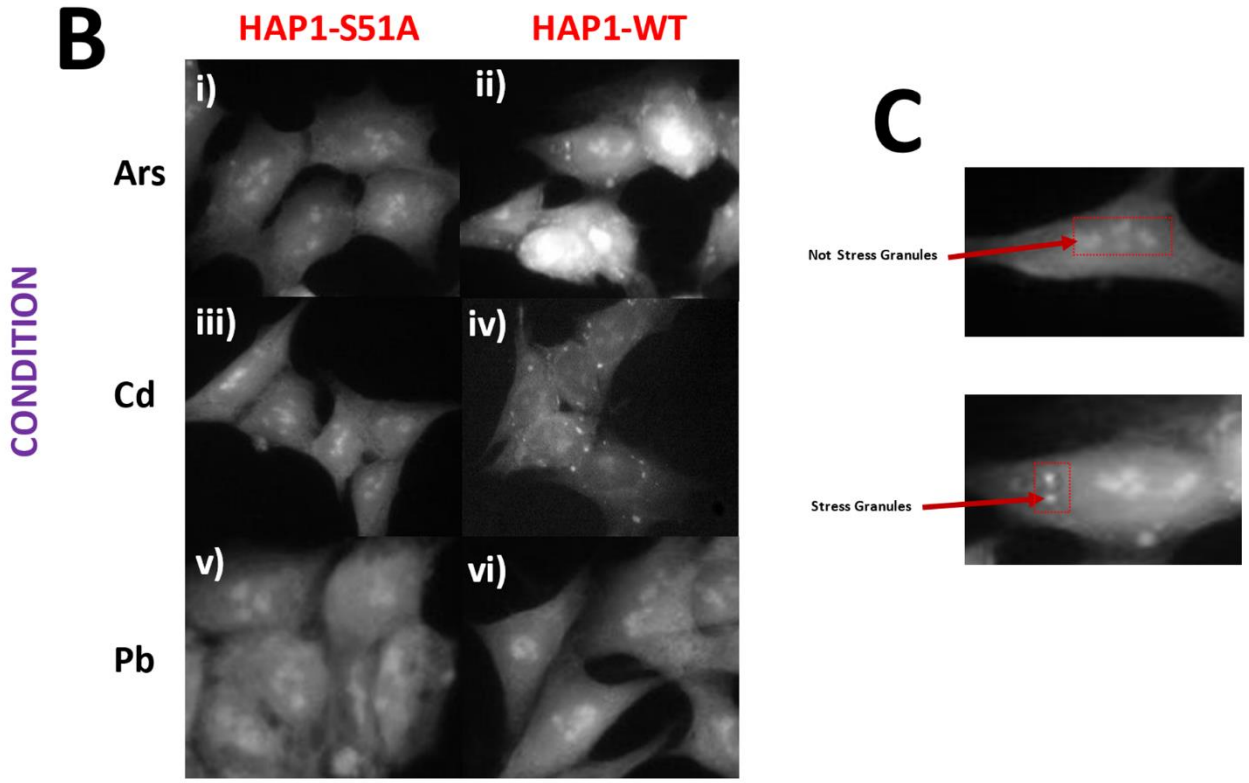


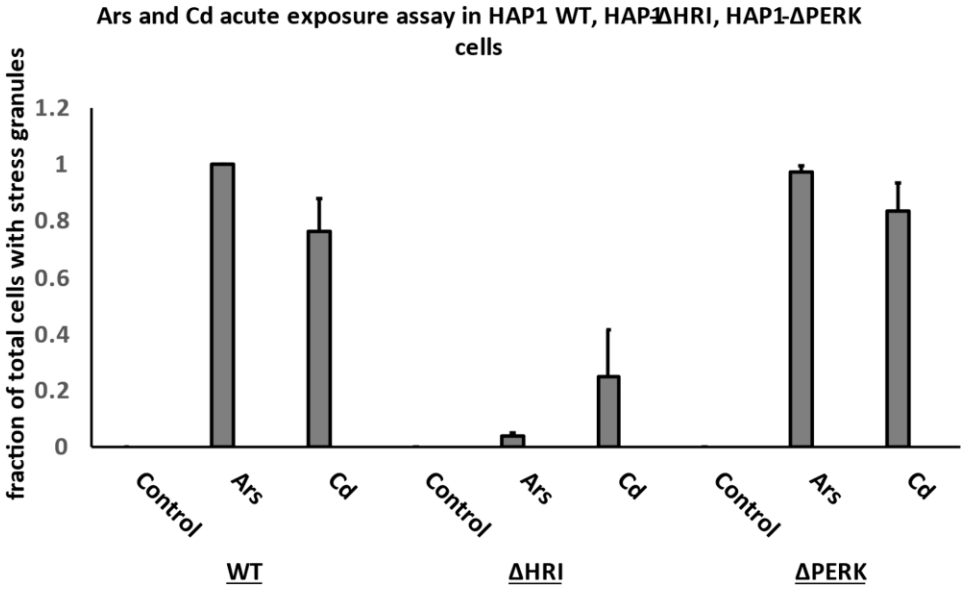
Figure 5: (A) HAP-1 cells were treated for 60 minutes with 500µM solutions of the treatments indicated on the chart, and the percentage of cells containing stress granules was determined. n=3, error bars +/- S.E.M. n=2 for Cd cells because third trial results were misplaced. **(B)** The cells were viewed at magnification 40X, and a minimum of 125 cells over 4 fields were counted on each slide. To reduce bias, the labels on the slides were blinded, and the results from three independent counts were averaged. **(C)** These cells were viewed under fluorescent microscopy with an old stain. Therefore, there are filaments that in the nucleus that indicate that. Stress granules continue to appear to be round white dots located in the periphery of the cell in the cytoplasm.

Like previous experiments, 100% of the cells exposed to 500µM of arsenite presented stress granules but 0.00% presence in HAP1 S51A cells. 500µM Cd had a fraction of 0.652 or 65.2% of SG presence in HAP1-WT cells but 0.00 present in HAP1 S51A cells. However Pb has of 1% in both HAP1-WT and HAP1 S51A cells. This led us to conclude that it's likely that Pb doesn't work through the eIF-2α pathway, but Cd does.

Ars and Cd acute exposure assay in HAP1 WT, HAP1-ΔHRI, HAP1-ΔPERK cells

The final experiment was performed to determine the kinase pathway induced by cadmium to form stress granules. For this experiment three cell lines were obtained—the same HAP1WT and cell lines of knockouts with HRI and PERK. Previous showed that correlation between cadmium induced stress and PERK phosphorylation and ER stress proteins. Connections between HRI and cadmium were also referenced in liver stress. It was hypothesized that cadmium induced phosphorylation be dependent on both HRI and PERK but reliant in PERK and that higher fraction of cells with stress granules would be present HRI knockout cells than in PERK knockout cell lines. In Figure 6A we see that 500µM of Ars had a fraction of a 1.00 in WT cells

A



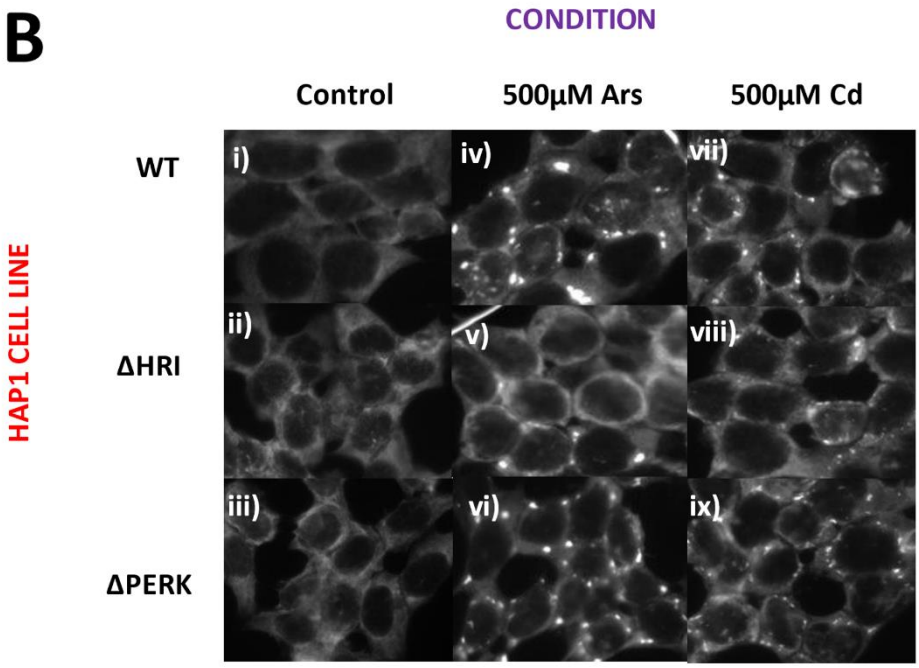


Figure 6: (A) HAP-1 cell lines were treated for 60 minutes with 500µM solutions of the treatments indicated on the chart, and the percentage of cells containing stress granules was determined. n=2, error bars +/- S.E.M**. (B)** The cells were viewed at magnification 40X, and a minimum of 125 cells over 4 fields were counted on each slide. To reduce bias, the labels on the slides were blinded, and the results from three independent counts were averaged.

0.03 or 3% in ΔHRI cells and 0.972 or 97.2% in ΔPERK cells. 500µM of Cd showed a fraction of 0.763 or 76.3% in WT cells, 0.250 or 25.0% in ΔHRI cells, and 0.836 or 83.6% in ΔPERK cells. As indicated in Figure 7B vii) there is significant less presence of stress granules cadmium treated cells in ΔHRI cell than in ΔPERK cells. ΔPERK cells had a higher level of stress granules than the fraction presented in WT cells treated with cadmium.

Discussion

Cd in acute exposure assay with HAP1 WT, HAP1-ΔHRI, HAP1-ΔPERK cells

The data suggests that Cd induced stress creates a cellular stress response that is dependent on the eIF2α response pathway, via the kinase HRI. There were higher levels of stress granules in 500µM Cd exposure HAP1-WT cells than in U2OS-DS cells. We suggest that this could be due to the increased sensitivity of the near-haploid cells. Experiments continued with HAP1 cell line instead of keeping the U2OS lines since the HAP1 cells had the genetically modified eIF2α kinase deletions and mutations needed for the purpose of this study. On average Cd induced stress appeared to cause more than 50% of stress granule formation in U2OS-DS cells.

Previous experiments that looked at p-PERK phosphorylation concluded that Cd could work though PERK but did not investigate the possibility that cadmium could also work through other kinases to produce stress granules. Due to time constraints, we narrowed our kinase investigation to HRI and PERK. Cd presented involvement of HRI and PERK kinases in the literature and since it was a redox inactive metal and metal that caused oxidative stress. It could be likely that like arsenite in the flavivirus experiment, Cd induced oxidative stress could affect the amino acid deprivation. Future experiments measuring 500µM of Cd stress granule formation ΔGCN2 and ΔPKR lines could aid in exploring and mapping Cd's cellular stress pathway further. The knockout experiment revealed that cadmium works primarily through HRI kinase for stress granule formation.

Pb in acute exposure comparison assay with U2OS-DS cells, HAP1 WT and HAP1 S51A Cells

Unlike the stated hypothesis, Lead did not show significant stress granule formation and maintained a consistent expression of about 1% in all acute exposure assays. Lead results also appeared to be the same in the HAP1WT and HAP1-S51A mutation. Serine 51A is a mutant form of serine 51. Serine 51 is responsible for phosphorylating eIF2α meaning a mutation on this would inhibit stress granule formation. Not only does lead not form significant stress granules but it is more likely that lead does not work through the eIF2α pathway since its low stress granule response seems to be independent of whether or not eIF2α is phosphorylated.

Lead was examined under acute conditions and at 500µM of stress. It could be that lead forms stress granules at higher concentrations and that the 500µM Pb to 1mM of Pb is like what 50µM Ars is to 500µM of Ars where 50µM is not enough to produce significant formation of stress granules but 500µM is. One thing that would have strengthened our experiment would have been to test the combination of Pb and Ars in an acute exposure assay to see if Pb could also be inhibiting SG assembly or not.

Recommendations

Stress granule research is ever growing and is critical for understanding the extent of the role of trace heavy metals in associated diseases. Future studies could investigate the role redox metals to better understand the connections between oxidative stress levels on stress granule formation.

Acute exposure combination experiments between known metal stress granule inducers and unknowns metals can provide insight of which metals inhibit suppress stress granule response.

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References

Arimoto, K., Fukuda, H., Imajoh-Ohmi, S., Saito, H., & Takekawa, M. (2008). Formation of stress granules inhibits apoptosis by suppressing stress-responsive MAPK pathways. *Nature cell biology*, *10*(11), 1324-1332.

Aulas, A., Fay, M. M., Lyons, S. M., Achorn, C. A., Kedersha, N., Anderson, P., & Ivanov, P. (2017). Stress-specific differences in assembly and composition of stress granules and related foci. *Journal of cell science*, *130*(5), 927-937.

Basu, M., Courtney, S. C., & Brinton, M. A. (2017). Arsenite-induced stress granule formation is inhibited by elevated levels of reduced glutathione in West Nile virus-infected cells. *PLoS pathogens*, *13*(2), e1006240.

Boye, E., & Grallert, B. (2019). eIF2α phosphorylation and the regulation of translation. *Current genetics*, 1-5.

Bravard, A., Campalans, A., Vacher, M., Gouget, B., Levalois, C., Chevillard, S., & Radicella, J. P. (2010). Inactivation by oxidation and recruitment into stress granules of hOGG1 but not APE1 in human cells exposed to sub-lethal concentrations of cadmium. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis*, *685*(1-2), 61-69.

Chen, L., & Liu, B. (2017). Relationships between stress granules, oxidative stress, and neurodegenerative diseases. *Oxidative medicine and cellular longevity*, *2017*.

Ercal, N., Gurer-Orhan, H., & Aykin-Burns, N. (2001). Toxic metals and oxidative stress part I: mechanisms involved in metal-induced oxidative damage. *Current topics in medicinal chemistry*, *1*(6), 529-539

Fukui, Y., Yumura, S., & Yumura, T. K. (1987). Agar-overlay immunofluorescence: high-resolution studies of cytoskeletal components and their changes during chemotaxis. *Methods in cell biology*, *28*, 347-356.

Hoppe, M., Friend, C., & Wu, J. (2018). Stressed Out! Effects of Bisphenols on the Cellular Stress Response. : Worcester Polytechnic Institute.

Kültz, D. (2003). Evolution of the cellular stress proteome: from monophyletic origin to ubiquitous function. *Journal of Experimental Biology*, *206*(18), 3119-3124.

Liu, J., Luo, L. F., Wang, D. L., Wang, W. X., Zhu, J. L., Li, Y. C., ... & Zhang, W. C. (2019). Cadmium induces ovarian granulosa cell damage by activating PERK-eIF2α-ATF4 through endoplasmic reticulum stress. *Biology of reproduction*, *100*(1), 292-299

Luo, B., Lin, Y., Jiang, S., Huang, L., Yao, H., Zhuang, Q., ... & Lin, Z. (2016). Endoplasmic reticulum stress eIF2 α–ATF4 pathway-mediated cyclooxygenase-2 induction regulates cadmium-induced autophagy in kidney. *Cell death & disease*, *7*(6), e2251-e2251.

McEwen, E., Kedersha, N., Song, B., Scheuner, D., Gilks, N., Han, A., ... & Kaufman, R. J. (2005). Heme-regulated inhibitor kinase-mediated phosphorylation of eukaryotic translation

initiation factor 2 inhibits translation, induces stress granule formation, and mediates survival upon arsenite exposure. Journal of Biological Chemistry, 280(17), 16925-16933.

Shi, X. T., Zhu, H. L., Xiong, Y. W., Liu, W. B., Zhou, G. X., Cao, X. L., ... & Wang, H. (2020). Cadmium down-regulates 11β-HSD2 expression and elevates active glucocorticoid level via PERK/p-eIF2α pathway in placental trophoblasts. *Chemosphere*, *254*, 126785.

Wang, L., Zhang, S., Wang, Z., Xu, M., Yuan, L., Cui, J., & Liu, S. (2017). A protective role of Heme-regulated eIF2α kinase in cadmium-induced liver and kidney injuries. *Chemosphere*, *185*, 284-289.

Wang, X., Wang, L., & Liu, S. (2015). Heme-Regulated eIF2α kinase plays a crucial role in protecting erythroid cells against Pb-induced hemolytic stress. *Chemical research in toxicology*, *28*(3), 460-469.

Wang, T., Yuan, Y., Zou, H., Yang, J., Zhao, S., Ma, Y., ... & Zhu, J. (2016). The ER stress regulator Bip mediates cadmium-induced autophagy and neuronal senescence. *Scientific reports*, *6*(1), 1-14.

Yuan, Y., Yang, J., Chen, J., Zhao, S., Wang, T., Zou, H., ... & Liu, Z. (2019). Alpha-lipoic acid protects against cadmium-induced neuronal injury by inhibiting the endoplasmic reticulum stress eIF2α-ATF4 pathway in rat cortical neurons in vitro and in vivo. *Toxicology*, *414*, 1-13.

Appendix A: Ars, Cd, Pb acute exposure comparison assay

	Control				Ars.				Cd				Pb.		
Presence of Stress Granules	Yes	No	Total		Yes	No	Total		Yes	No	Total		Yes	No	Total
Experiment 1	0	265	265		285	0	285		205	74	279		8	244	252
Experiment 2	0	272	272		295	0	283		126	150	276		2	290	292
Experiment 3	0	259	259		283	0	283		92	175	267		1	249	250
	Control				Ars.				Cd				Pb.		
Presence of Stress Granules: Total	Amount of Stressed Cells	Amount of Total Ce	Ratio		Amount o	Amount o	Ratio		Amount of Stress	Amount of Tot	Ratio		Amount o	Amount o	Ratio
Experiment 1	0	265			285	285	1		205	279	0.734767		8	252	0.031746
Experiment 2	0	272			295	295	1		126	276	0.456522		2	292	0.006849
Experiment 3	0	259			283	283	1		92	175	0.525714		1	250	0.004
		Average	0			Average	1			Average	0.572334			Average	0.014198
		Standard Deviation	0			Standard I	0			Standard Devia	0.144863			Standard I	0.015263
		Error	0			Error	0			Error	0.083636			Error	0.008812
				Control	500 µm Ar	500 µm Cd	500 µm Pb								
			Average		1	0.572334	0.014198								
			Standard I	0	0	0.144863	0.015263								
			Error	0	0	0.083636	0.008812								

Appendix B: Raw Data for Ars and Cd acute exposure comparison assay

	Control			
Presence of Stress G	Yes	No	total	%+
Experiment 1	0	265	0	
Experiment 2	0	269	0	
Experiment 3	0	250	0	
Experiment 4	0	311	0	
Average	0			
Standard I	0			
Error	0			

	500 Cd			
Presence of Stress G	Yes	No	total	%+
Experiment 1	31	224	255	0.121569
Experiment 2	0	287	287	
Experiment 3	134	18	152	0.881579
Experiment 4	200	107	307	0.651466
Average	0.551538			
Standard I	0.389735			
Error	0.275584			

	50 Ars			
Presence of Stress G	Yes	No	total	%+
Experiment 1	7	274	281	0.024911
Experiment 2	151	112	263	
Experiment 3	0	255	255	0
Experiment 4	0	286	286	0
Average	0.008304			
Standard I	0.014382			
Error	0.008304			

	500 Ars			
Presence of Stress G	Yes	No	total	%+
Experiment 1	259	259	259	1
Experiment 2	47	237	283	
Experiment 3	280	280	280	1
Experiment 4	290	290	290	1
Average	1			
Standard I	0			
Error	0			

	Control	500µm Ar	500µm Cd	50µm Ars	500µm Cd	250µm Cd + 50 µm Ars
Average	0	1	0.551538	0.008304	0.823089	0.63355
Standard I	0	0	0.389735	0.014382	0.118642	0.330074
Error	0	0	0.225013	0.008304	0.068498	0.190568

	500 Cd + 50 Ars			
Presence of Stress G	Yes	No	total	%+
Experiment 1	202	82	284	0.711268
Experiment 2	281	0	281	
Experiment 3	289	16	305	0.947541
Experiment 4	248	58	306	0.810458
Average	0.823089			
Standard I	0.118642			
Error	0.068498			

	250 Cd + 50 Ars			
Presence of Stress G	Yes	No	total	%+
Experiment 1	91	202	293	0.31058
Experiment 2	0	260	260	
Experiment 3	294	9	303	0.970297
Experiment 4	163	100	263	0.619772
Average	0.63355			
Standard I	0.330074			
Error	0.190568			

Appendix C: Raw Data for Ars, Cd, and Pb acute exposure comparison assay in HAP1 WT and S51A cell line

	HAP 1- WT Control				HAP-1 WT Cd				HAP 1- WT Ars				HAP 1- WT Pb				HAP 1- S51A Ars				HAP 1- S51A Cd				HAP 1- S51A Pb				HAP 1- S51A Control			
Presence of Stress Granules	Yes	No	Total	% +	Yes	No	Total	%+	Yes	No	Total	%+	Yes	No	Total	%+	Yes	No	Total	%+	Yes	No	Total	%+	Yes	No	Total	%+	Yes	No	Total	%+
Experiment 1	0	154	154	0	42	96	138	0.304348	125	0	125	1	0	125	125	0	0	131	131	0	0	131	131	0	7	127	134	0.052239	0	115	115	0
Experiment 2	0	131	131	0	103	0	103	1	138	0	138	1	6	103	109	0.055046	0	125	125	0	0	128	128	0	0	125	125	0	0	126	126	0
Experiment 3	0	152	152	0	missing results				139	0	139	1	0	135	135	0	0	119	119	0	0	147	147	0	0	119	119	0	0	117	117	0
	Average	0			Average	0.652174			Average	1			Average	0.018349			Average	0			Average	0			Average	0.017413			Average	0		
	Standard I	0			Standard I	0.4919			Standard I	0			Standard I	0.031781			Standard I	0			Standard I	0			Standard I	0.03016			Standard I	0		
	Error	0			Error	0.283999			Error	0			Error	0.018349			Error	0			Error	0			Error	0.017413			Error	0		

	HAP 1 WT				HAP-1 S51A			
	Control	500 µm Ars	500 µm Cd	500 µm Pb	Control	500 µm Ars	500 µm Cd	500 µm Pb
Average	0	1	0.652174	0.018349	0	0	0	0.017413
Standard I	0	0	0.4919	0.031781	0	0	0	0.03016
Error	0	0	0.283999	0.018349	0	0	0	0.017413

Appendix D: Raw Data for Ars and Cd acute exposure assay in HAP1 WT, HAP1-ΔHRI, HAP1-ΔPERK cells

	ΔHRI Ctrl				ΔHRI Ars				ΔHRI Cd			
Presence of Stress Granules	Yes	No	Total	% +	Yes	No	Total	% +	Yes	No	Total	% +
Experiment 1	0	146	146	0	7	124	131	0.053435	82	100	182	0.450549
Experiment 2	0	151	151	0	3	123	126	0.02381	8	151	159	0.050314
			Average	0	Average	0.038622					Average	0.250432
			Standard I	0	Standard I	0.020948					Standard I	0.283009
			Error	0	Error	0.014813					Error	0.200117

	ΔPERK Ctrl				ΔPERK Ars				ΔPERK Cd			
Presence of Stress Granules	Yes	No	Total	% +	Yes	No	Total	% +	Yes	No	Total	% +
Experiment 1	0	158	158	0	170	10	180	0.944444	144	6	150	0.96
Experiment 2	0	128	128	0	138	0	138	1	141	61	198	0.712121
Average	0				Average	0.972222			Average	0.836061		
Standard I	0				Standard I	0.039284			Standard I	0.175277		
Error	0				Error	0.027778			Error	0.123939		

	WT- Control				WT- Ars				WT- Cd			
Presence of Stress Granules	Yes	No	Total	% +	Yes	No	Total	% +	Yes	No	Total	% +
Experiment 1	0	129	129	0	133	0	133	1	102	62	164	0.621951
Experiment 2	0	132	132	0	154	0	154	1	113	12	125	0.904
Average	0				Average	1			Average	0.762976		
Standard I	0				Standard I	0			Standard I	0.199439		
Error	0				Error	0			Error	0.141024		

	WT			ΔHRI			ΔPERK		
	Control	Ars	Cd	Control	Ars	Cd	Control	Ars	Cd
Average	0	1	0.762976	0	0.038622	0.250432	0	0.972222	0.836061
Standard Deviation	0	0	0.199439	0	0.020948	0.283009	0	0.039284	0.175277
Error	0	0	0.141024	0	0.014813	0.200117	0	0.027778	0.123939