Analyzing the Cellular Stress Response through Exposure to Polycyclic Aromatic Hydrocarbons (PAHs)

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Abstract

The growing concern over exposure risks of polycyclic aromatic hydrocarbons (PAHs) has called for more comprehensive research into the toxicity and carcinogenic character of common PAHs. Exposure to benzo(a)pyrene (BAP) and benzo(e)pyrene (BEP) has been related to the phosphorylation of translation initiation factor eIF2α, an event which often leads to stress granule (SG) formation. Using drug exposure assays and western blotting, we examined the relationship between various PAHs and the formation of SGs, and the associated degree of phosphorylation of eIF2α in multiple human cell lines following treatments with BAP and BEP. We find that PAHs do not cause SG formation or increased phosphorylation of eIF2α relative to a known control in either cell line. This research calls for more investigation into the toxicity of PAHs that examines the effects of exposure on other stress-related pathways.

Acknowledgements

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Table of Contents

Introduction

Cellular Stress Response

Stress is commonly understood as a homeostatic disruption. The cellular stress response (CSR) is the cell's defense mechanism against stressors from the environment. The CSR can be triggered by shifts in temperature, DNA damage, changes in pH, reactive oxygen species, etc. (Locke, 1997). The CSR consists of many different pathways that can be specific or non-specific, based on the type of stressor involved and the potential damage caused (Kültz, 2005). These pathways can range from the promotion of survival to programmed cell death and depend upon the cell's ability to cope with the stressor. Some cellular reactions to stress include cell cycle and metabolic interventions, DNA repair, and degradation of damaged proteins (Fulda et al., 2010). There are many stress response pathways, and proteins associated with the main functions of the CSR are widely conserved among organisms (Kültz, 2005). It has been shown that deletion of bacterial genes that encode stress proteins causes cell death when the cells are exposed to environmental conditions that trigger the stress response. Similarly, when stress related proteins are compromised in mammalian cells there is cell death after exposure to a known stressor (Welch, 1992).

Stress Granules and Processing Bodies

In response to certain types of stress, cells produce membraneless assemblies in distinct structures referred to as stress granules (SGs) and processing bodies (p-bodies). Stress granules form as accumulations of mRNA bound by RNA-binding proteins to aggregate into large cytoplasmic bodies in response to translation inhibition (van Leeuwen and Rabouille, 2019). Specifically, translation initiation is halted, which leads to a buildup of non-translating messenger ribonucleoprotein particles (mRNPs) that comprise the stress granule (Ivanov, Kedersha, & Anderson, 2019). P-bodies are composed of mRNAs that are translationally inactive and proteins that either repress translation or degrade mRNA. Both stress granules and p-bodies are known to sequester important molecules in the formation of assemblies during stress-specific conditions and inhibit downstream signaling pathways that would otherwise reduce cellular survival outcomes (van Leeuwen and Rabouille, 2019). However, SGs and p-bodies differ from one another according to formation, duration, localization within the cell, and specific function.

Stress granules in mammalian cells form when a cell is exposed to oxidative stress, endoplasmic reticulum stress, heat shock, nutrient deprivation, or other toxic conditions (Nostramo and Herman, 2017). The granules typically contain clusters of translation initiation factors including eIF2α, eIF3, eIF4A/B, eIF4E, and eIF4G, polyadenylated mRNAs of 40S ribosomes, and RNA-binding proteins PAB1, Caprin, FMR1, TDP-43, TIA1, and G3BP1/2. A precipitating event in the formation of stress granules is the phosphorylation of eIF2α by kinases that are activated by stress. (van Leeuwen and Rabouille, 201). The pathway for eIF2α kinase

activation is depicted in Figure 1. Phosphorylated eIF2α inhibits recycling of the initiator eIF2α-GTP-tRNA^{Met} ternary complex, which halts translation initiation and leads to mRNP buildup as previously described (Tsai et al., 2016). Some studies have shown that genetic loss of eIF2α kinases result in increased susceptibility to oxidative stress (Rajesh, K. et al., 2015).

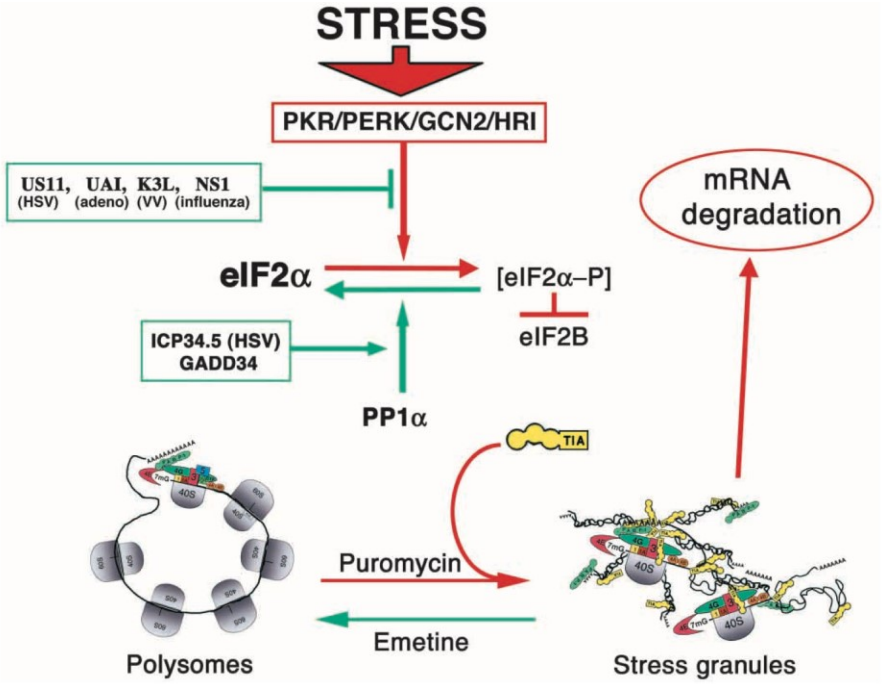

Fig. 1 - eIF2α Kinase Activation Pathway and Availability of eIF2α-GTP-tRNA^{Met}. Taken from Anderson and Kedersha (2002).

This eIF2α phosphorylation event is followed by dephosphorylation of G3BP1 (GTPase-activating protein [SH3 domain] binding protein 1), which further leads to G3BP1 multimerization and later SG formation (Scholte et. al, 2015). For this reason, G3BP1 is commonly used as a marker for SGs. However, while G3BP1 is a consistent marker of SGs, their composition is heavily influenced by the type of stress to which they are exposed (Chen, L., & Liu, B., 2017).

Unlike stress granules, p-bodies may be present in a cell even in the absence of stress, though they are enlarged when mRNA has been deadenylated, translationally silenced, or targeted for degradation (Ivanov, Kedersha, & Anderson). P-bodies are known to act as sites of mRNA turnover, yet they also house translationally repressed mRNAs that are not marked for degradation. Furthermore, p-bodies are postulated to serve as protectors of these mRNAs, inhibiting them from destruction, and isolating them from the rest of cellular functions for later release into translation after stress has been relieved (van Leeuwen and Rabouille, 2019). Stress granules and p-bodies, though structurally and functionally different, interact with one another upon stressful conditions. These assemblies often “dock” with each other in mammalian cells and overlap in yeast, yet the specific functions and constituents they exchange are not as well

understood. Stress granules in mammalian cells may accumulate non-translating mRNAs during heat shock, and then relocate to p-bodies afterwards (Decker and Parker, 2012). As such, mRNAs marked for degradation may first be sorted by SGs and later shuttled to p-bodies to complete the process (see Figure 2; Kedersha et. al, 2008). Specifically, the transfer of mRNA only occurs in one direction, from SGs to p-bodies (Wilbertz et al., 2019). While general function of SGs and p-bodies have been described, the exact roles that SGs and p-bodies assume during cellular stress, especially between one another, have yet to be thoroughly defined.

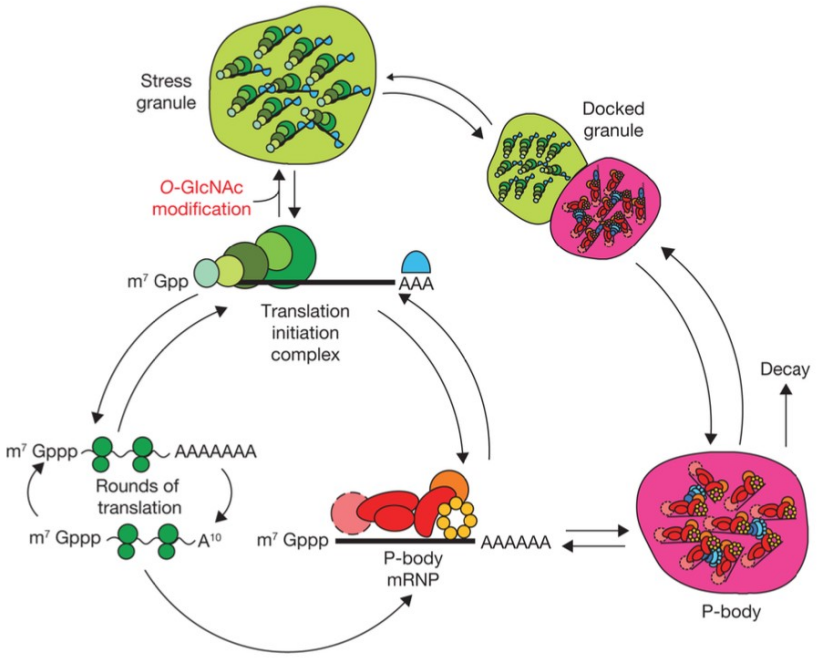

Fig. 2 - Potential Pathway from SGs to P-bodies. Taken from Hilliker and Parker (2008).

Polycyclic Aromatic Hydrocarbons (PAHs)

Polycyclic aromatic hydrocarbons (PAHs) belong to a large group of organic compounds composed of two or more fixed benzene rings arranged in numerous configurations, as shown in Figure 3 (Kim et. al, 2013).

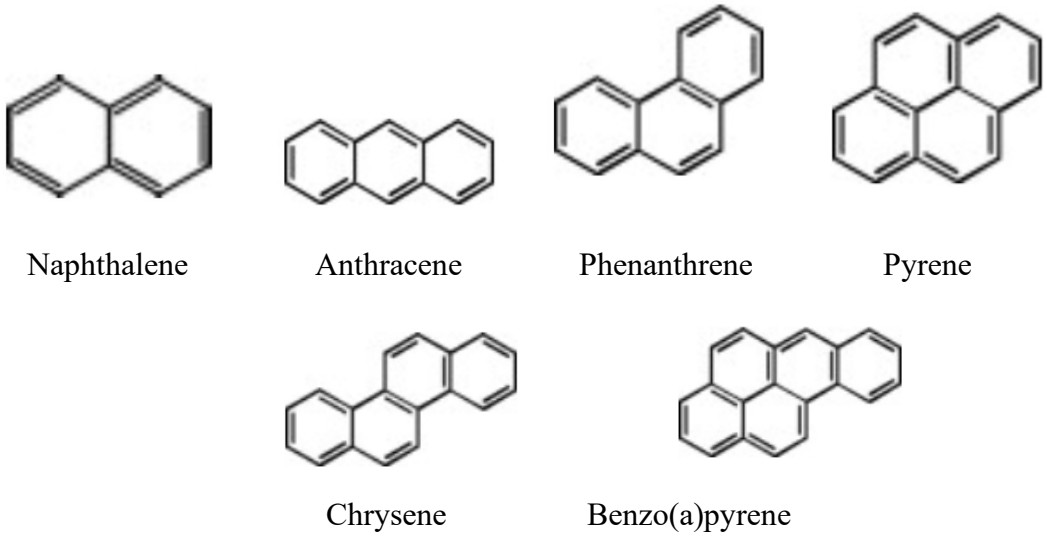

Fig. 3 - Structures of Common Benzopyrenes. Taken from Kim et al (2013).

Benzopyrenes are a type of PAH and are commonly encountered as a result of incomplete combustion of organic materials such as fossil fuels, car exhaust, smoke from wood fires, tobacco, oil and gas products, and charred foods (NCI Dictionary of Cancer Terms, 2020). The increase of PAHs in the atmosphere is becoming more concerning because of their suspected carcinogenicity and mutagenicity. In the early 2000s, the toxicity of PAHs was not understood, and all assessed risks posed by mixtures of PAHs assumed that all carcinogenic PAHs are as potent as benzo(a)pyrene (BAP), which is one of the most dangerous PAHs (C.T. Nisbet, et. al, 2004). Since then, most information on the toxicity of PAHs suggests that most are not as potent as benzo(a)pyrene; however, the Environmental Protection Agency (EPA) continues to overestimate the risks for safety purposes (C.T. Nisbet et. al, 2004). Benzo(a)pyrene is very dangerous and contact can cause skin rashes, skin color changes, warts, bronchitis and in severe cases cancer (NCI Dictionary of Cancer Terms, 2020). Some *in vitro* studies on mammalian cells showed that exposure to BAPs induced sister chromatid exchange, chromosomal aberrations, and sperm abnormality (Phale et al., 2019).

Relationship Between PAHs and Stress

It has been previously understood that PAH exposure induces a general stress response that can include DNA damage and genomic instability, stress kinase activation, endoplasmic reticulum (ER) stress, and an increase in eIF2α phosphorylation, among other responses (Fu et al., 2012). DNA damage is triggered by the creation of reactive oxidative species (ROS); when exposed to UV irradiation, PAHs can absorb the photon energy to induce photo-excited states (Fu et al., 2012). These compounds can then react with oxygen and other naturally existing molecules in the body to produce ROS and ROS intermediates at the cellular level such as

oxygenated PAHs and free radicals, which causes DNA damage through strand breakage or formation of adducts (Fu et al., 2012). Oxidative stress can also generate ROS through oxidative protein folding and mitochondrial respiration and detoxification which induces several intracellular pathways including activating eIF2α and/or inactivating phosphatases to facilitate cellular death or survival (Rajesh, K. et al., 2015).

As previously established, phosphorylation of eIF2α occurs under stressful conditions like ROS-mediated ER stress or heme deficiency, and recently this phosphorylation event has been linked to a benzo(a)pyrene (BAP)-induced stress granule formation (Cnop et al., 2017; Kim et al., 2017). This finding motivated this study to examine the link between phosphorylation of eIF2α and subsequent formation of stress granules from various types of PAHs. One study that informed this relationship was conducted by Kim et al. (2017), which demonstrated that BAP exposure to JEG-3 and BeWo human placenta choriocarcinoma cell lines induces phosphorylation of eIF2α. (see Figure 4). This evidence suggests that PAHs, especially BAP, might play a role in some SG related stress responses, but the relationship between most PAHs and stress granule formation is not well defined. We focus our research on this relationship, with the hypothesis that acute exposure to BAP will induce the formation of stress granules in human epithelial osteosarcoma cells of the U2OS-DS cell line and human placenta choriocarcinoma cells of the JEG3 cell line. Our research will also examine the relationship between stress granule formation and various PAHs. We additionally hypothesize that U2OS-DS exposure to sodium arsenite at a low concentration combined with a high concentration of BAP/BEP will increase the formation of stress granules in U2OS cells compared to low-dose sodium arsenite alone.

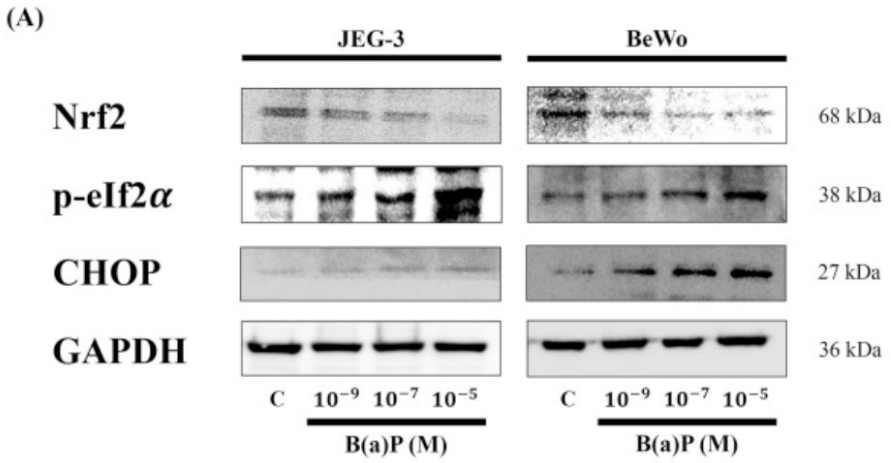

Fig 4 - Western Blot Showing Phosphorylation of eIF2α. This image was taken from Kim et al., 2017. This research informed our hypothesis that exposure to BAP induces phosphorylation of eIF2α, leading to the formation of stress granules in various human cell lines.

Materials and Methods

Cell Culture

The U2OS double-stable human osteosarcoma cell line (ATCC HTB-96) containing green fluorescent protein (GFP)-G3BP1 and red fluorescent protein (RFP)-DCP1 were created as described (Kedersha et al, 2008). These cells were cultured in Dulbecco's Modified Eagle Medium (DMEM)-high glucose medium, 10% Fetal Bovine Serum (FBS), and 1% Penicillin-Streptomycin (PS). Additionally, the JEG3 human placenta choriocarcinoma cell line (ATCC HTB-36) was cultured in Eagle's Minimum Essential Medium (EMEM)-high glucose medium, 10% (FBS), and 1% PS. All cells were kept in a humidified incubator containing 5% CO_2 at 37°C and split 1:3 every other day.

Cell Plating

For every experiment, the cells were plated in 12-well plates at a concentration of $8x10^4$ cells in 1mL of medium per well. The plates were then placed in the humidified incubator at 37℃ for approximately 48 hours.

Representative Sodium Arsenite Assay

To validate sodium arsenite as a positive control, an initial acute stress assay was conducted on U2OS cells. First, the cells were plated and 100µM and 500µM concentrations of sodium arsenite, as well as a growth medium negative control were prepared in 2mL of pre-conditioned medium taken from the wells. The remaining medium was aspirated from all wells, and each prepared treatment condition was added to its own row on a 12-well plate. The plate was incubated for one hour at 37℃ and then prepared for fixation and staining.

Acute Cellular Stress Assay

The following PAHs were tested on U2OS-DS cells in a series of acute cell stress exposure assays for a one hour treatment period: benzo(a)pyrene (BAP) (Sigma B1760-100MG), benzo(e)pyrene (BEP) (Sigma B10102-25MG), naphthalene (Sigma 184500-5MG), anthracene (Sigma 10580-25MG), phenanthrene (Sigma P11409-25MG), and pyrene (Sigma 571245-1G). The cells were plated and 100µM, 250µM, and 500µM concentrations of each drug, as well as 500µM sodium arsenite (positive control) and 500µM dimethyl sulfoxide (DMSO, negative control) were prepared in 1.5mL of medium from the wells. The remaining medium was aspirated and 0.5mL of each variable was added to a corresponding column on the plate, as shown in Figure 5. The plates were incubated for one hour at 37℃ and then prepared for fixation and staining. There was also a four hour assay with all concentrations of BAP and BEP, and the positive and negative control.

The same protocol was followed for treatment of the JEG3 cells with BAP and BEP. A one hour treatment and a four hour treatment with 100µM, 250µM, and 500µM concentrations of BAP and BEP, a DMSO negative control, and 500µM sodium arsenite positive control were carried out separately.

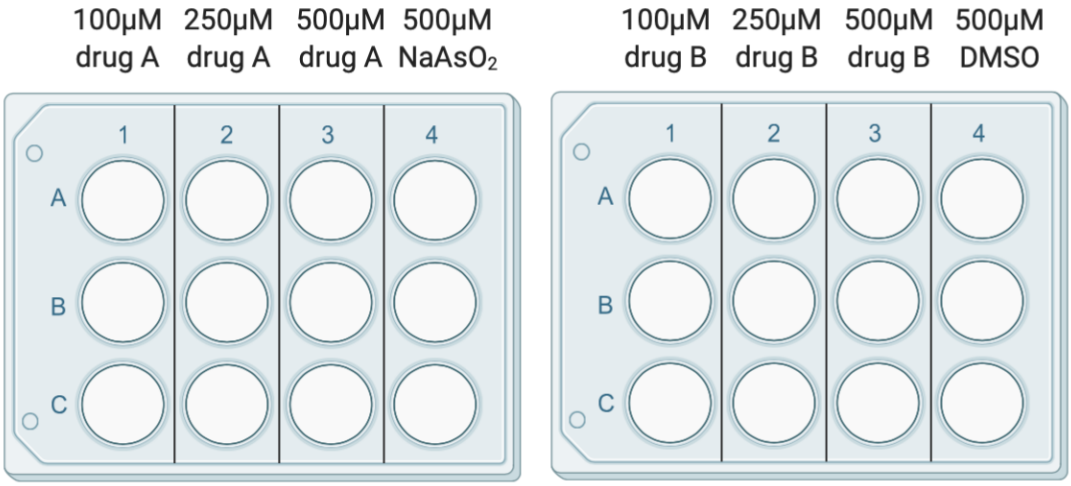

Fig. 5 - Plate Setup. The general layout of the 12-well plates used for each weekly cell stress assay on U2OS-DS and JEG3 cells. Amount of plates and columns of wells utilized varied depending on the quantity of drugs tested each week.

Combined Low-Dose Sodium Arsenite and BAP/BEP Exposure Assay

U2OS-DS cells were plated and exposed to a low dose of the positive control, 75µM sodium arsenite in combination with BAP and BEP, 75µM sodium arsenite alone, and the DMSO negative control for one hour. Three combination assays were conducted and the cells were fixed, stained, mounted on microscope slides, and counted as done for previous assays.

Fixation and Staining

The cells were fixed and stained immediately after drug treatment. First, the medium and PAH mixture was removed from each plate and cells were rinsed with PBS. The PBS was removed, 0.5mL of 4% paraformaldehyde (PFA) was added to each well, and the plates incubated on a rotator at 1,500rpm at room temperature for 10 minutes. PFA was then removed and 0.5mL of 100% methanol was added to each of the wells and placed back on the rotating table for 5 minutes. The solution was then removed and the well plates were rinsed with PBS. The stain was created using a 1:5000 dilution of Hoechst 33342 dye in PBS, using 0.5mL of Hoechst/PBS for each well (Product information for all staining and antibody reagents is available in Table 1). The plates were then incubated on the rotating table for 10 minutes. The solution was removed and the plates were rinsed with PBS twice; the third time the PBS was left in the wells to prevent the cells from drying

Once fixed and stained, the cells were mounted onto microscope slides to be viewed and counted. Poly(vinyl alcohol) mounting medium (Sigma 363073025G) was warmed in a water bath at 42°C and used to adhere the glass coverslips containing the fixed and stained cells to slides. Two coverslips corresponding to each variable were mounted per slide. The slides were then labeled and covered with tape to blind for cell counting.

This same protocol was used for the JEG3 cell line, however 0.5mL of 5% bovine serum albumin (BSA) in PBS was added to the cells as a blocking solution after rinsing off the methanol. The plate was then placed on a rotator at 1,500rpm for at least one hour. Then, a 1:1000 dilution of primary rabbit anti-G3BP1 antibody in 5% BSA/PBS was added to the cells at 0.5mL of solution per well (Appendix A). After an hour on the rotator, the cells were washed three times with PBS. 0.5mL of secondary antibody (Anti-Rabbit IgG) diluted 1:2000 in 5% BSA/PBS as well as Hoechst stain diluted 1:5000 in PBS were added for another hour. An additional three washes with PBS were performed and mounting was carried out as described previously.

Counting

The fixed and stained cells were counted under a fluorescence microscope. The number of cells with stress granules, appearing as small green clusters within the cell cytoplasm, were counted along with the number of cells without stress granules (Figure 6). Cells were counted from at least three fields of view at 400X magnification until at least 250 cells were counted in total. A percentage of cells with stress granules for each experimental condition was calculated from these numbers and collated to form a complete data set.

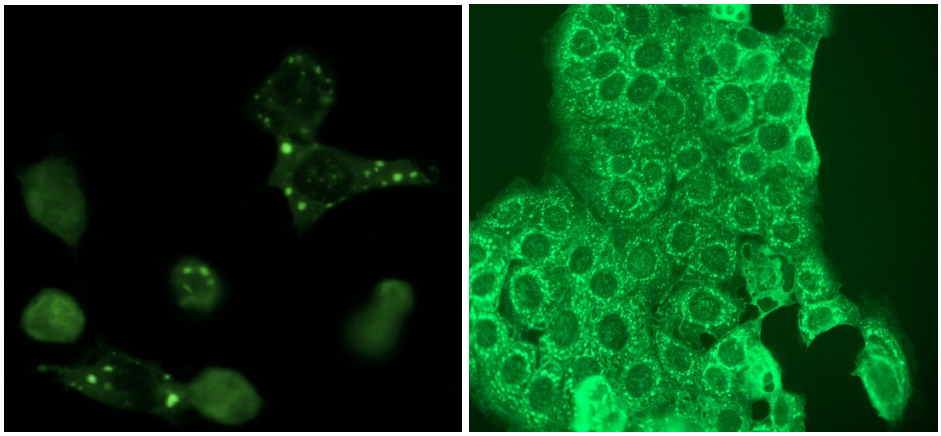

Fig. 6 - Cells Expressing Stress Granules. Above shows U2OS-DS cells (left) and JEG3 cells (right) expressing stress granules visualized as glowing dots within the cell cytoplasm. Cells expressing these bright masses were scored as positive for the presence of stress granules. Images shown are from respective cell lines exposed to 500µM sodium arsenite positive control for one hour.

Western Blot Assay

Both U2OS-DS (two replicates) and JEG3 (one replicate) cell lines were exposed to 500µM BAP, 500µM BEP, 500µM sodium arsenite, and DMSO for one hour following the same setup as was done for the acute exposure assays. A four hour stress component was included in these assays for only 500µM BAP and 500µM BEP in the U2OS-DS replicates. The JEG3 replicate additionally included a four hour exposure to the positive and negative control, which was deemed unnecessary for further blots due to excessive cell death (see Figure 11). Upon completion of stress exposure, the medium was aspirated from all wells and the cells were washed once with PBS. A lysis buffer solution was prepared with 1.3mL 2X SDS sample buffer and 13µL 1M DTT to solubilize the cell membranes and reduce disulfide bonds in preparation for gel electrophoresis. 100µL of the mixture was added to each well after removing the PBS. The wells were scraped with a rubber paddle to collect the lysate and reduce viscosity of the buffer for easier pipetting. The samples were then placed into separate tubes using a micropipette and heated in an 80℃-water bath for 20 minutes prior to freezing for storage until moving onto gel electrophoresis.

Gel Electrophoresis

The samples were heated at 37°C prior to starting gel electrophoresis and spun down at 1,000 rpm for 2 minutes. The gel used was BioRad Mini-Protean TGX Stain-Free Gel, 4-15%, denaturing and reducing gel with a 12-well comb which can hold up to 20µL per well. The gel ran at 100V for one hour in 1X tris-glycine buffer. The New England BioLabs Color Protein Broad Range P7719S standard was loaded at a volume of 5µL and all other samples were loaded at 20µL.

Western Blot - Wet Transfer

A wet western blot transfer was conducted to transfer the separated proteins from the gel to a membrane for later imaging. The transfer buffer was made of 20% methanol, 75% H_2O, and 5% 10X tris/glycine buffer (0.25M tris, 1.5M glycine, 0.1% SDS). The fiber pads and filter paper were soaked in the transfer buffer prior to building and setup. The transfer membrane was first rinsed in methanol and then soaked in the transfer buffer. The cassette was placed with the black side down and the order of the build was placed consecutively as follows: fiber pad, filter paper, protein gel, PVDF transfer membrane, filter paper, fiber pad. The cassette was placed in the buffer tank, which was then placed inside the wet tank along with a stir bar. A container holding ice was inserted next to the buffer tank which helps prevent the gel from overheating and melting during the transfer. The entire apparatus was placed on a stir plate and the wet tank transfer ran at 100V for 90 minutes.

Image Development

After the transfer, the membrane was rinsed with deionized water and then Ponceau stained to check that the protein bands had transferred onto the membrane. The Ponceau dye was rinsed off using a washing buffer (PBS 0.5% Tween). The membrane was then immersed in 5% Bovine Serum Albumin in PBS (5% BSA) overnight; BSA was used to occupy unbound sites on the membrane to prevent antibodies from sticking nonspecifically. The blocking solution was poured off and primary antibody (P-eIF2α Rabbit mAb) was added to the membrane at 1:1,000 in 5% BSA and left overnight. The primary was removed the following day, and the membrane was washed with the washing buffer three times for 5 minutes on a rotator at 1,500rpm. The secondary antibody (Anti-Rabbit IgG) at 1:10,000 in 10mL of 5% BSA was added to the membrane and left on for 45 minutes on the rotator. The membrane was then washed with the buffer wash three more times for 5 minutes each and placed in a PBS bath for imaging. During the washes, the developing solution was made by adding 1mL of SuperSignal West Pico PLUS Luminol/Enhancer Solution (Prod #1863092) to 1mL of SuperSignal West Pico PLUS Stable Peroxide Solution (Prod#1863093). The membrane was placed in the Bio Rad ChemiDoc XRS and the developer solution was added right before the imaging protocol ran. A 10-minute exposure time was used, and images were taken every 20 seconds.

Once the p-eIF2α image was taken, the membrane was placed back in the container and the total antibody (eIF2α Rabbit mAb) was added to the membrane at 1:1,000 in 5% BSA. The membrane was placed on the rotator at 1,500rpm for 1 hour. Then the total antibody was rinsed off with 3 washes at 5 minutes each with the washing buffer. After the washes, a 10mL solution containing 1:10,000 secondary antibody (Anti-Rabbit IgG) in 5% BSA was poured onto the membrane. The membrane was placed on the rotator again for 45 minutes. The membrane was then washed with the buffer wash three more times for 5 minutes each and placed in a PBS bath for imaging. The developing solution was made, and the imaging process was conducted as was done for the phospho-specific image.

Western Blot Data Analysis

All blots were analyzed using ImageJ software. The images of the membrane were first inverted to represent increasing signal intensity with phosphorylation. One selection box was created to analyze each band with the same surface area. An additional area on the membrane was selected to serve as background. All mean intensity values were normalized by subtracting the background and then calculating ratio of peIF2α to total eIF2α. From these values, the percent of phosphorylation relative to the sodium arsenite positive control was calculated by setting the positive control ratio to 100% and scaling the rest accordingly.

Statistical Analysis

Standard error was calculated for both the representative sodium arsenite control experiment and the acute-low dose sodium arsenite combined with BAP/BEP experiment to be shown as error bars. This was done by first calculating the average and standard deviation amongst each respective sample category. The average value was reported as shown, while the standard deviation was divided by the square root of the number of samples collected in order to calculate standard error, which was then reported as an error bar.

Results

A single acute exposure assay was performed to measure stress granule formation within U2OS-DS cells after exposure to varying concentrations of sodium arsenite.

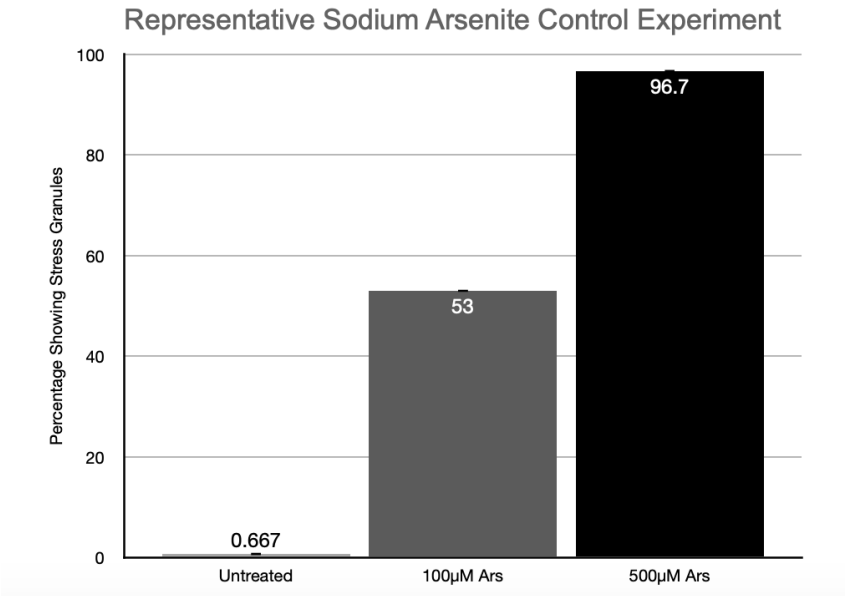

Fig. 7 - U2OS-DS Acute Cell Stress Response to $NaAsO_2$. The error bars represent the standard error between three individual counts using the same slides.

Figure 7 shows a 53% stress response at 100µM sodium arsenite and a complete stress response, approximately 97%, at a 500µM sodium arsenite. These results confirmed the ability to move forward with a concentration of 500µM sodium arsenite as a positive control for following experiments, such as in Figure 8. Additionally, the coverslips were counted by three different researchers, with standard error between each count represented as error bars. This was done to show that all researchers followed the same criteria to score slides and there was little margin of error between individual counts. Figure 8 shows the percentage of stress granule formation in U2OS-DS cells after a single acute exposure assay using varying concentrations of BAP and BEP. Two negative controls were included, a treatment of DMSO only and an untreated condition with only growth medium.

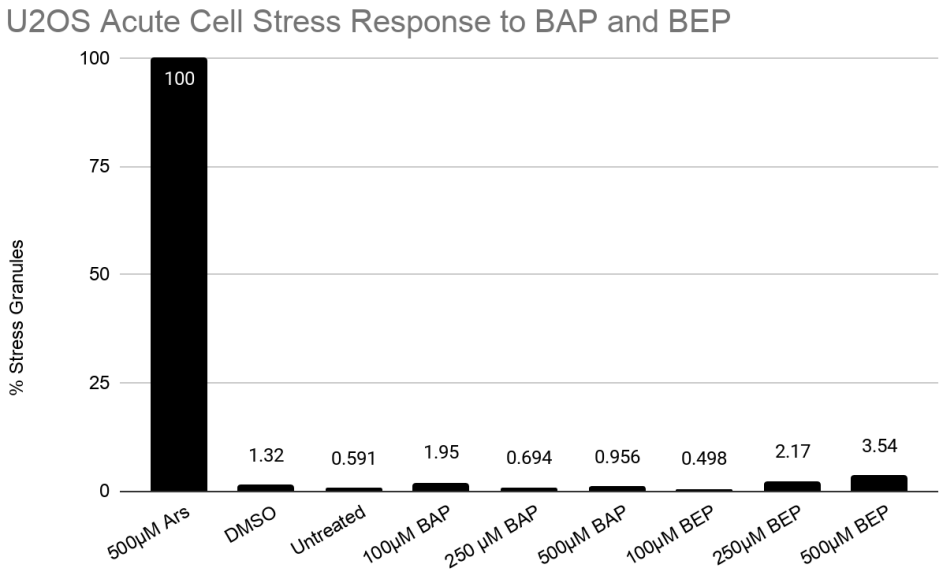

Fig. 8 - U2OS-DS Acute Cell Stress Response to Various Concentrations of BAP and BEP.

A low level of stress granules was observed under all treatment conditions other than the positive control. The maximum percentage observed was 3.54% for the 500μM BEP condition. All other BAP and BEP conditions exhibited a percentage between around 0.5% and 2%. The negative control, DMSO, yielded a 1.32% stress response, indicating most of the stress granule formation for all other conditions was at or near background.

The investigation continued with the analysis of four more PAHs. U2OS-DS cells were exposed to three different concentrations of anthracene, phenanthrene, pyrene, and naphthalene to measure the acute stress response from one assay each. The results are shown in Figure 9.

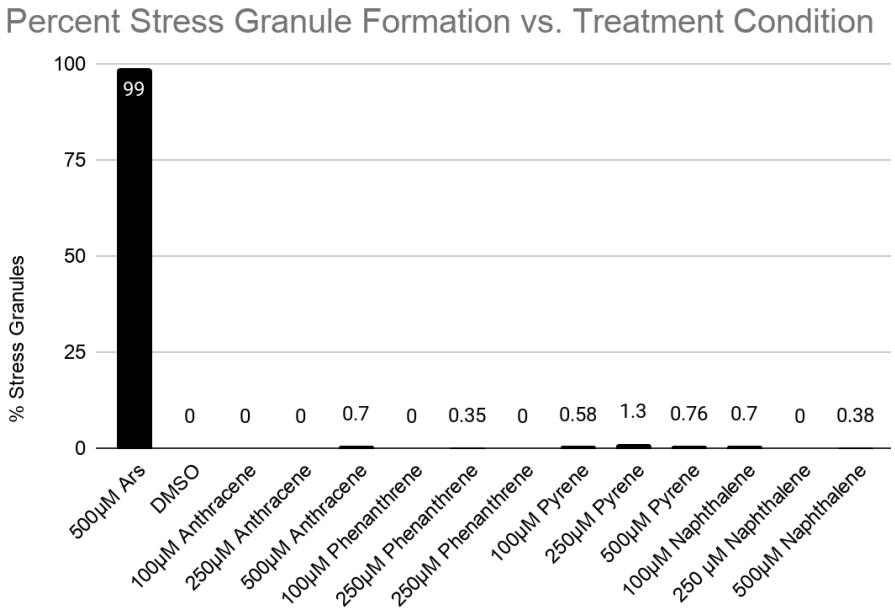

Fig. 9 - U2OS-DS Acute Cell Stress Response to Anthracene, Phenanthrene, Pyrene, and Naphthalene.

Similar to the previous experiment, there was no above-background stress response observed under any condition apart from the positive control. The maximum response observed was 1% stress granule formation with acute exposure to 500µM anthracene, 100µM pyrene, 250µM pyrene, 500µM pyrene, and 100µM naphthalene. Due to the minimal stress response exhibited, these PAHs were not investigated further.

Following the experiments performed on the U2OS-DS cell line, the JEG3 cell line was chosen for use under similar experimental conditions to observe the stress response. This was done to examine stress granule formation in the same cell line that Kim et. al, (2013) reported phosphorylation of eIF2α following exposure to BAP. Figure 10 demonstrates an acute exposure assay of three concentrations of BAP and BEP on the JEG3 cells.

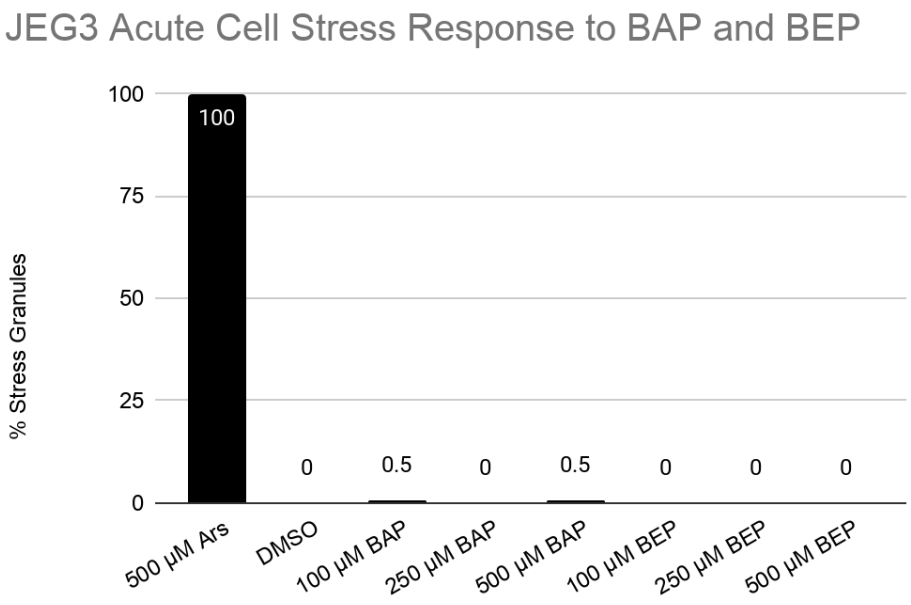

Fig. 10 - JEG3 Acute Cell Stress Response to Various Concentrations of BAP and BEP.

A minimal stress response was observed under all treatment conditions apart from the positive control. The maximum response was 1% stress granule formation for 100µM BAP and 500µM BAP. All other conditions showed a 0% response.

To further analyze the effect of BAP and BEP on both U2OS-DS and JEG3, a four hour exposure was performed to compare to the one hour exposure of all previous experiments. The same positive control, 500µM sodium arsenite, was used for this experiment.

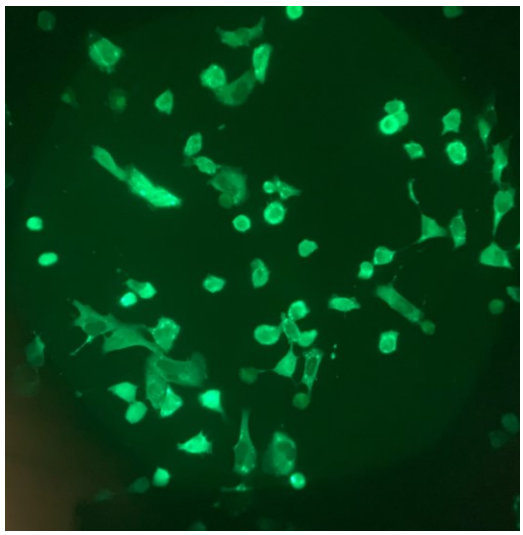

Fig 11 - U2OS-DS Cells at 400X Following Exposure to 500µM Sodium Arsenite for 4 Hours.

In both the U2OS-DS and JEG3 cell lines, extensive cell death was observed in the samples treated with the positive control, which was seen as a decrease in cell number and

abundance of rounded cells (see Figure 11). No formal count was conducted, as the four hour exposure was deemed too long to quantify stress granule formation due to the frequency of cell death and lack of stress granule formation by either BAP or BEP.

Motivated by the study in which Kim et al. (2017) reported phosphorylation of eIF2α in response to BAP (see Figure 4), a western blot was conducted to measure the levels of phosphorylated eIF2α and total eIF2α under one hour and four hour exposure times to BAP, BEP, a negative control, and positive control. JEG3 and U2OS-DS cell lines were analyzed under these conditions and the blot results are shown in Figure 12. Phosphorylation of eIF2α was then quantified for each blot by calculating the ratio of phosphorylated eIF2α to total eIF2α and percentage of phosphorylation relative to positive control for all treatment conditions. This quantification is shown in Figure 13 for JEG3 and Figure 14 for U2OS-DS.

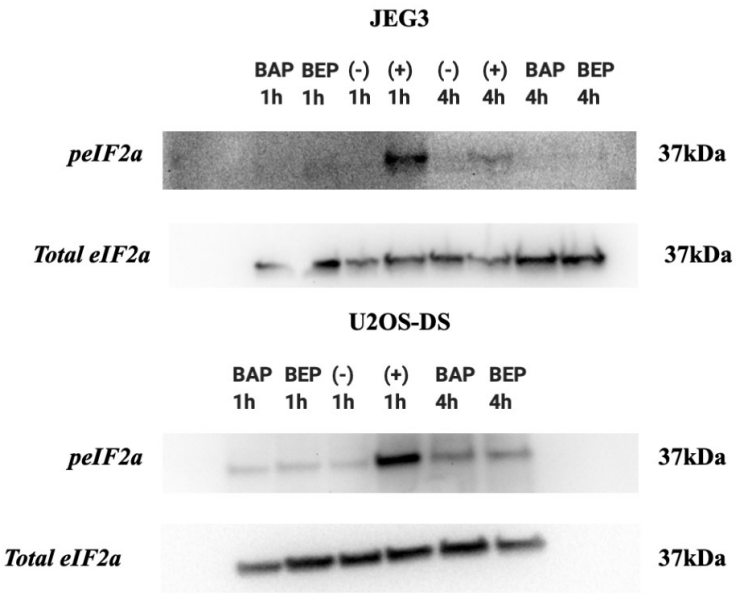

Fig. 12 - JEG3 and U2OS-DS Western Blots Probed for peIF2α and Total eIF2α.

The blots specific to total eIF2α were conducted to show background expression of eIF2α (approx. 37kDa) in all samples in comparison to the amount of phosphorylated protein. Both blots show high levels of peIF2α in the one hour positive control (500μM sodium arsenite) exposure lane. The four hour positive control lane in the JEG3 blot also shows a slightly greater signal for peIF2α than the other non-control lanes, but not as much as that of the one hour exposure. Therefore, the four hour control was eliminated from the U2OS-DS western blot due to increased cell death. All other BAP and BEP treatments for both JEG3 and U2OS do not show a significant amount of phosphorylation of eIF2α, if any at all.

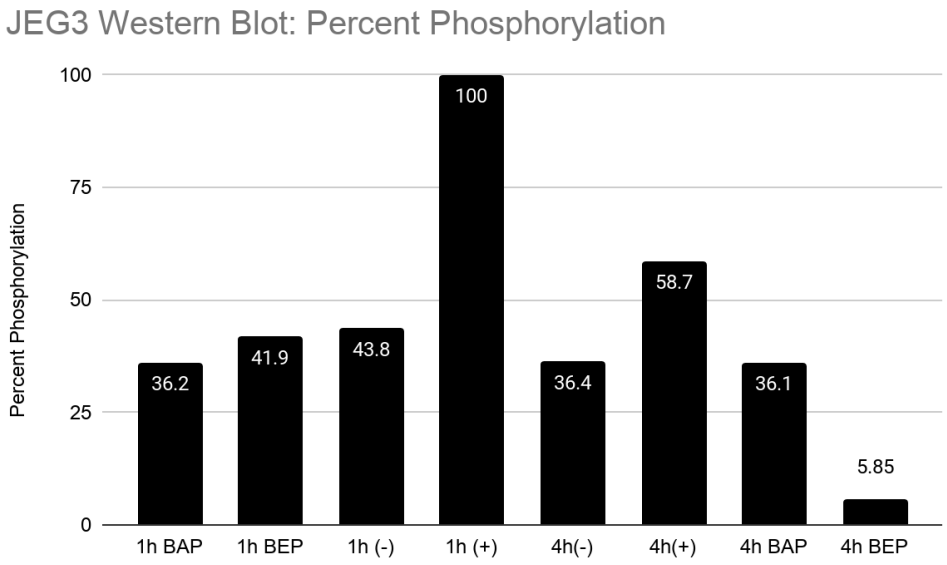

Fig. 13 - JEG3 Western Blot Quantified for Percent of Phosphorylation.

Above shows the percent of phosphorylation for a single western blot experiment on JEG3 cells. All values were related to the sodium arsenite positive control set as 100% phosphorylation. Both the one hour and four hour exposures to BAP and BEP show some phosphorylation relative to the negative control, with the exception of 4h BEP. The four hour positive and negative controls were eliminated from future blots with U2OS-DS due to cell death and greater phosphorylation value in the one hour positive control.

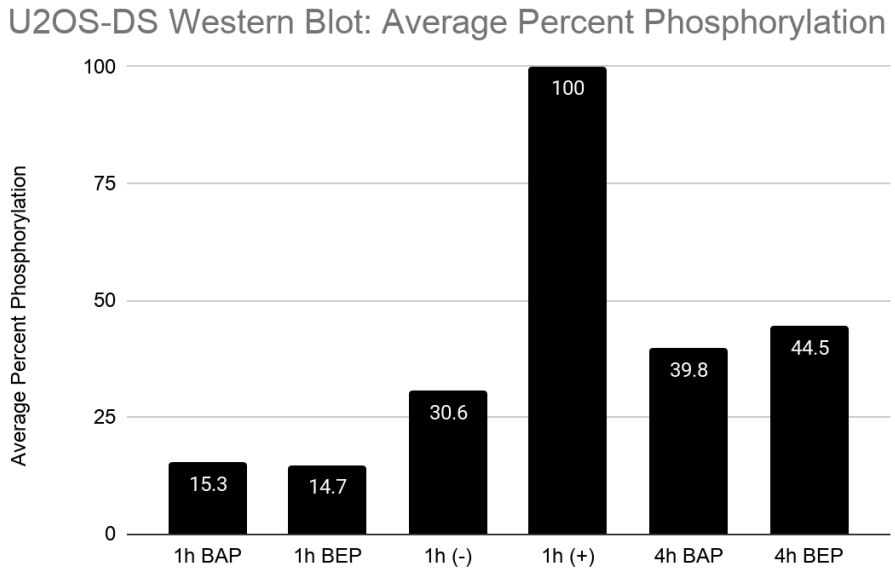

Fig. 14 - U2OS-DS Western Blot Quantified for Average Percent of Phosphorylation.

Above shows the average percent of phosphorylation across two western blots, with the sodium arsenite positive control serving as 100%, and all other values scaled accordingly. The data show that the four hour exposure to BAP and BEP produced more phosphorylation than that of the one hour exposure, but not as significant as that of the positive control . Overall, these data show a maximum phosphorylation response from the one hour positive control treatment, evidenced clearly in Figure 12. The other treatments show a varying response.

A final set of experiments was conducted to examine the effects of a combined treatment of 500µM BAP or BEP with 75µM arsenite on U2OS-DS cells. This was to determine whether the limited amount of stress granule formation from BAP or BEP alone could be additive when combined with a low dose of sodium arsenite to lower the cellular stress threshold. The controls in these experiments were 500µM BAP and BEP, and 75µM arsenite. DMSO was used as a negative control. The results for these experiments are shown in Figures 14 and 15.

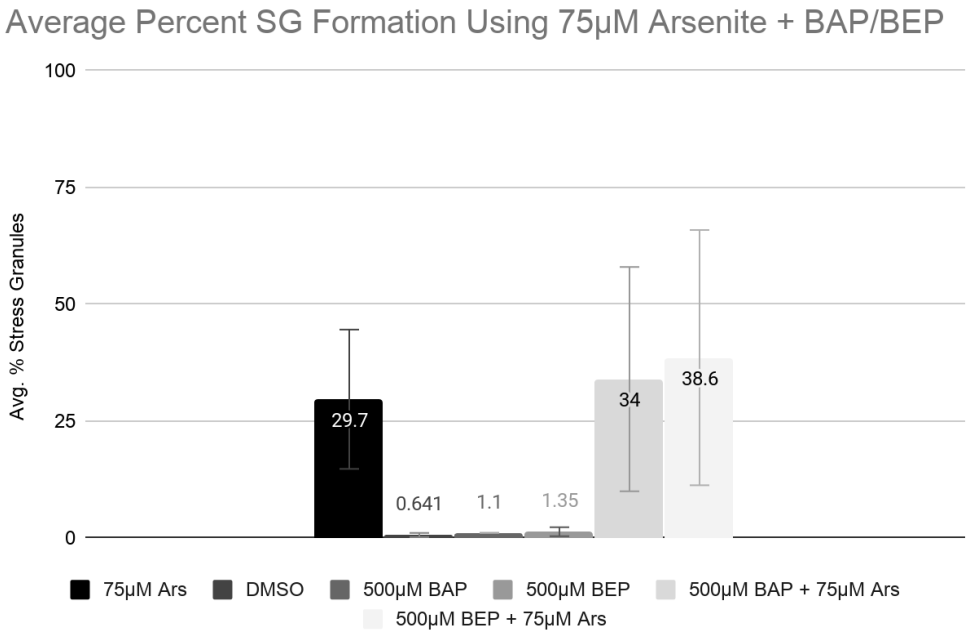

Fig. 15 - U2OS-DS Cell Stress Response to Acute Low-Dose Sodium Arsenite Combined with BAP and BEP. This figure shows the results of two separate one hour exposure assays as a representation of average expression of stress granules with error bars indicating standard deviation.

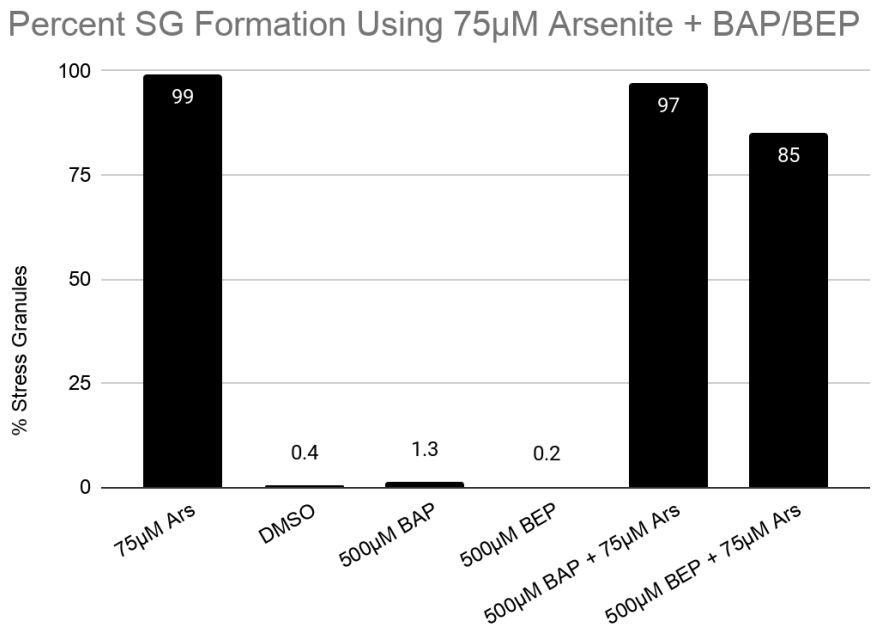

Fig. 16 - U2OS-DS Cell Stress Response to Acute Low-Dose Sodium Arsenite Combined with BAP and BEP, Plated the Day Before Stress. The cells used for this stress assay were plated one day prior to the experiment, rather than two days as usual. The cells were plated at a concentration of 160,000 cells per well rather than the typical 80,000 cells per well. For this reason, the data are analyzed separately from the average stress granule formation of the previous two replicates of the acute low-dose exposure assay.

As depicted in Figure 15, combination of both BAP and BEP with 75µM sodium arsenite produced a slightly higher stress response in U2OS cells than just arsenite alone. BAP and BEP alone did not show stress granule formation. Comparatively, the data in Figure 16 show that there is a strong stress response from both 75µM sodium arsenite alone, as well as when combined with either 500µM BAP or BEP. Neither 500µM BAP nor BEP alone produced a significant stress response in the form of stress granules.

Discussion

U2OS-DS and JEG3 Acute Exposure Assays

Research for this project began with one hour assays on U2OS-DS cells exposed to 100µM, 250µM, and 500µM concentrations of several PAHs (BAP, BEP, anthracene, phenanthrene, pyrene, and naphthalene) to analyze stress granule formation via fluorescence microscopy in comparison to a known positive control: sodium arsenite at 500µM. Overall, none of these experiments showed evidence of stress granule formation by any of the PAHs in U2OS-DS cells, as evidenced by the low percentages in all conditions except for the positive control seen in Figures 6 and 7. This observation motivated the next step to examine stress granule formation in the JEG3 cell line. The same concentrations of BAP and BEP were tested on this cell line, as Kim et al. (2017) had found evidence of phosphorylation of eIF2α in JEG3 cells

exposed to BAP (Figure 4). Because phosphorylation of eIF2α is known to play a key role in the beginning stages of SG formation, it was hypothesized that BAP or BEP exposure to the same cell line might show evidence of stress granules. Clearly seen in Figure 10, this assay also yielded no SG formation relative to the sodium arsenite positive control. Finally, a four hour exposure experiment was conducted on each of the cell lines with the same concentrations of BAP and BEP. This experiment was conducted to determine if a longer exposure time would increase the formation of SGs, which might indicate that the previous acute exposure was not long enough to produce a positive stress response. This experiment did not show SG formation in U2OS-DS or JEG3 by either BAP or BEP, and the cells treated with arsenite appeared to experience a high prevalence of cell death. Depicted in Figure 11, both cell lines showed many cells with rounded edges, spaced far apart. This is very different from the untreated cell morphology that appears as wide projections with cells linked tightly to each other. Therefore, the four hour exposure time was deemed too long to quantify SG formation. This data does not support a relationship between phosphorylation of eIF2α and subsequent kinase activation as a key mechanism of action by PAHs. However, it is plausible that PAHs activate the cellular stress response in an approach that is not related to this pathway or stress granule formation. To test this further, research was inspired to explore the cellular stress response in these cell lines at a molecular level.

U2OS-DS and JEG3 Western Blots

The next phase of this study was to conduct western blots on both cell lines using one hour and four hour exposure assays using 500μM BAP and 500μM BEP. Since none of the benzopyrenes had induced the formation of stress granules, disproving the original hypothesis, it was hypothesized that there would also be no evidence of phosphorylation of eIF2α from either one hour or four hour exposure to BAP/BEP. From the images in Figure 12, it appeared that neither BAP or BEP increased the levels of peIF2α in either cell line compared to the strong band seen in the arsenite positive control. Once again, the first blot was conducted on JEG3 cells that included a four hour exposure to controls, but this variable was eliminated for further blots because the band for peIF2α in the one hour treatment was much stronger. To further investigate peIF2α content, all blots were quantified by determining peIF2α relative to total eIF2α. These results revealed that both JEG3 and U2OS-DS cells showed apparent phosphorylation at four hour time points, but less than half of the amount seen with 500μM sodium arsenite. U2OS-DS cells exclusively showed slightly more peIF2α from a four hour exposure to BAP and BEP compared to the one hour exposure, but neither of these were more than 45% phosphorylated compared to 100% by arsenite. Overall, while there was some evidence of phosphorylation of eIF2α after four hour exposure, it was minimal compared to sodium arsenite, a known stressor. Additionally, the amount of phosphorylation by BAP or BEP was not enough to trigger stress granule formation from the methods tested. We conclude from this information that future research should examine other potential pathways that may contribute to the toxicity and carcinogenicity of PAHs, as the formation of SGs does not appear to be strongly affected by

exposure to some of the most common PAHs. However, as phosphorylation of eIF2α prevents translation initiation, further investigation into whether PAHs decrease translation after four hours of exposure to BAP or BEP could be useful.

Acute Low-Dose Sodium Arsenite Combined with BAP/BEP Experiments

The final set of experiments in this study were conducted to measure the effects of combined exposure to a high dose of BAP and BEP with a low dose of the arsenite positive control on U2OS-DS cells over a one hour period. It was hypothesized that the small amount of SG formation from 500μM BAP and 500μM BEP alone acted as a pre-stressor to sensitize the cells to respond more readily to the addition of 75μM sodium arsenite. This would result in greater stress granule formation from the combination of BAP, BEP, and arsenite in comparison to arsenite alone. We found that U2OS-DS cells showed SG formation at about 30% in the 75μM positive control, less than 2% in BAP and BEP alone, and just over 30% in the combination treatments. Since both results showed a slight increase in SG formation from the combined exposure over that of the positive control, we inferred that using a lower dose of arsenite in combination with a high dose of BAP or BEP would lower the stress threshold enough to increase SG formation from the PAHs. However, the error bars in Figure 15 represent that there is a considerable margin of variability among quantified results, potentially due to having only two replicates of this experiment. More replicates with these conditions would be required to accurately determine whether a high concentration of BAP or BEP can serve as a pre-stressor to increase SG formation upon combination with a low dose of arsenite. We propose that the next step for this combination experiment would be to conduct the same assay with a four-hour exposure to BAP and BEP while adding arsenite upon the last hour. While minimal, our results showed that four hours of exposure to BAP or BEP mounts a slight stress response, seen in the western blot data. This next phase of the combination experiment would show whether a longer exposure time would further prime the cells to intensify the stress response beyond that of the one-hour treatment.

When this experiment was conducted an additional time (see Figure 16), the cells were plated a day before treatment, rather than the usual two days prior as was described in the methods for cell plating. The cells were plated at a concentration of 160,000 cells instead of 80,000 cells per well and then treated 24 hours later, rather than 48 hours later. This experiment showed a very strong stress response in the formation of SGs by 75μM arsenite that closely matched that of what had previously been seen from 500μM arsenite. Again, there was no evidence of SGs in either BAP or BEP alone, but the combined treatment with 75μM arsenite showed strong SG formation. These results are remarkably different from what was seen from the same experiment conducted on cells that were plated two days prior to treatment, shown in Figure 15. We postulated that because cells are very sensitive to environmental changes, it is likely that passaging cells a day before experimenting increased their susceptibility to become stressed, thereby intensifying the cellular response to any noxious agents like sodium arsenite.

Conclusion

Recently, research surrounding the prevalence of PAHs in the environment has indicated several ways in which humans can be exposed in everyday life. PAHs may be found in petroleum products, diesel fuels, charred foods, cigarettes, firewood, and even in roasted coffees.

All of the PAHs tested in this study are considered “priority”, and most of them have been classified as possible carcinogens by the National Toxicology Program and the EPA (All about PAHs, 2020). For this reason, it is critical that ongoing research examine the effects of common PAHs in human cells. This study has shown just one of the many stress related pathways that cells utilize to protect themselves from harm, but additional research is required to fully understand how PAHs may evade responses such as SG formation. The combination experiment of low-dose sodium arsenite with BAP/BEP is the first step in the direction of understanding how PAHs may enhance the cellular stress response when combined with another carcinogen. PAHs are certainly not the only potentially toxic chemical which humans encounter in day to day life; since it is likely that human cells will come into contact with several PAHs and other noxious chemicals all at once. This study hopes to participate in fortifying and adding to the limited knowledge about how PAHs may potentially be harmful to human cells, as well as emphasizing the need for more exploration of the topic.

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Appendices

Appendix A: Products and Dilutions Used in this Study

Product	Application	Dilution	Manufacturer	Product Number
Rabbit-anti-G3BP 594	Immunofluorescence primary stain	1µL/mL in 5% BSA/PBS	Santa Cruz Biotechnology	8889S
Anti-rabbit IgG Alexa Fluor 488 (green) stain	Immunofluorescence secondary stain	1µL/2mL in 5% BSA/PBS	Cell Signaling Technology	4412S
Hoechst 33342 (blue) nuclear stain	Immunofluorescence nuclear stain	1µL/5mL in PBS	Life Technologies	1642791
Rabbit-anti-peIF2α	western blot primary stain	1µL/mL in 5% BSA/PBS	Cell Signaling Technology	3398T
Rabbit-anti-eIF2α	western blot primary stain	1µL/mL in 5% BSA/PBS	Cell Signaling Technology	5324T
HRP-linked-anti-rabbit IgG	western blot secondary stain	1µL/10mL in 5% BSA/PBS	Cell Signaling Technology	7074P2

Appendix B - Raw Data for Representative Sodium Arsenite Assay

	Percent Containing Stress Granules			
	Untreated	**100μM Ars**	**500μM Ars**	
	0	48	99	
	1	54	98	
	1	57	93	
Average	0.667	53.0	96.7	
STDEV	0.577350269189626	4.58257569495584	3.21455025366432	
N	3	3	3	
SQRT	1.73205080756888	1.73205080756888	1.73205080756888	
STDEV/SQRT	0.333333333333333	2.64575131106459	1.85592145427667	
Mean untreated	0.667 +/- 0.003			
MEAN 500μM Sodium Arsenate	96.7 +/- 0.019			
Mean 100μM Sodium Arsenite	53.0 +/- 0.027			
Treatment Condition	Percent (-) Stress Granules	Percent (+) Stress Granules		
100μM BAP	**98.0**	**1.95**		
250 μM BAP	**99.3**	**0.694**		
500μM BAP	**99.0**	**0.956**		
100μM BEP	**99.5**	**0.498**		
250μM BEP	**97.8**	**2.17**		
500μM BEP	**96.5**	**3.54**		
500μM Arsenite	0	100		
DMSO	**98.7**	**1.32**		
Untreated - Growth Medium	**99.4**	**0.591**		

Appendix C - Raw Data for Combined Low-Dose Sodium Arsenite and BAP/BEP Exposure Assay

1st experiment			2nd experiment		
Sample Identity	Percent SG		Sample Identity	Percent. SG	
75µM Arsenite	44.5		75µM Arsenite	14.8	
DMSO	0.77		DMSO	0.511	
500µM BAP	1.21		500µM BAP	0.997	
500µM BEP	1.53		500µM BEP	1.17	
500µM BAP + 75µM Arsenite	54.6		500µM BAP + 75µM Arsenite	13.3	
500µM BEP + 75µM Arsenite	54.9		500µM BEP + 75µM Arsenite	22.3	
Sample Identity	Avg. % Stress Granules	ST DEV	n	sqrt	stdev/sqrt
75µM Ars	29.7	21.0010714	2	1.414213562	14.85
DMSO	0.641	0.1831406563	2	1.414213562	0.4532554467
500µM BAP	1.1	0.1506137444	2	1.414213562	0.7778174593
500µM BEP	1.35	0.2545584412	2	1.414213562	0.9545941546
500µM BAP + 75µM Ars	34	29.20351006	2	1.414213562	24.04163056
500µM BEP + 75µM Ars	38.6	23.05168107	2	1.414213562	27.29432175

Appendix D - Raw Data for U2OS-DS and JEG3 Western Blots

Blot 1 U2OS (ours)

phospho-specific band	area	mean	min	max	avg - background
1h BAP	0.054	39.625	19	64	30.546
1h BEP	0.054	48.643	27	75	39.564
1h (-)	0.054	60.145	30	99	51.066
1h (+)	0.054	181.765	76	255	172.686
4h BAP	0.054	120.64	73	255	111.561
4h BEP	0.054	94.261	46	159	85.182
Background	0.054	9.079	2	18	0

ours	phospho/total	%phospho
1h BAP	0.4485264966	28.00414036
1h BEP	0.3631690548	22.67477453
1h (-)	0.4783476184	49.57045008
1h (+)	1.60164351	100
4h BAP	0.964985425	60.24970093
4h BEP	1.135426941	70.89136466
Background	0	0

natalie	phospho/total	%phospho
1h BAP	0.03247598589	2.694926946
1h BEP	0.0808983757	6.713120682
1h (-)	0.1393400677	11.5627376
1h (+)	1.205078525	100
4h BAP	0.2323410189	19.28015595
4h BEP	0.2194167172	18.20766968
Background	0	0

total

band	area	mean	min	max	avg - background
1h BAP	0.089	72.636	10	201	68.103
1h BEP	0.089	113.474	21	255	108.941
1h (-)	0.089	111.288	23	255	106.755
1h (+)	0.089	112.351	18	255	107.818
4h BAP	0.089	120.142	22	255	115.609
4h BEP	0.089	79.555	10	219	75.022
Background	0.089	4.533	1	12	0

Treatment Condition	average percent phosphorylation
1h BAP	15.3
1h BEP	14.7
1h (-)	30.6
1h (+)	100
4h BAP	39.8
4h BEP	44.5

Blot 2 U2OS (Natalie)

phospho-specific band	area	mean	min	max	avg - background
1h BAP	0.001	4019.6	2164	7215	840.147
1h BEP	0.001	5575.781	2804	10924	2396.328
1h (-)	0.001	7179.354	3251	13262	3999.901
1h (+)	0.001	36296.181	5790	65535	33116.728
4h BAP	0.001	10435.983	5754	20377	7256.53
4h BEP	0.001	9635.4	6156	14230	6455.947
Background	0.001	3179.453	2164	4119	0

total

band	area	mean	min	max	avg - background
1h BAP	0.001	27468.567	3376	63206	25869.792
1h BEP	0.001	31220.235	4924	65534	29621.46
1h (-)	0.001	30304.811	4754	65535	28706.036
1h (+)	0.001	29079.746	4786	65532	27480.971
4h BAP	0.001	32831.013	5577	65534	31232.238
4h BEP	0.001	31021.998	3967	65531	29423.223
Background	0.001	1598.775	1367	1875	0

JEG3

Treatment Condition	phospho/total	Percent Phosphorylation
1h BAP	0.2753536412	36.2
1h BEP	0.3192202872	41.9
1h (-)	0.3337901509	43.8
1h (+)	0.7612957776	100
4h(-)	0.2772090309	36.4
4h(+)	0.4470939873	58.7
4h BAP	0.2749465013	36.1
4h BEP	0.04455156296	5.85

phospho-specific band	area	mean	min	max	avg-background
1h BAP	0.066	130.447	77	181	14.716
1h BEP	0.066	148.762	76	226	33.031
1h (-)	0.066	145.202	90	231	29.471
1h (+)	0.066	202.167	105	255	86.436
4h(-)	0.066	111.422	50	186	32.304
4h(+)	0.066	120.104	54	196	40.986
4hBAP	0.066	91.813	15	158	12.695
4hBEP	0.066	85.828	27	143	6.71
background(left)	0.066	115.731	59	210	0
background(right)	0.066	79.118	27	144	0

total band	area	mean	min	max	avg-background
1h BAP	0.059	58.918	15	203	53.444
1h BEP	0.059	108.948	16	255	103.474
1h (-)	0.059	93.766	36	178	88.292
1h (+)	0.059	119.012	39	243	113.538
4h(-)	0.059	122.007	41	226	116.533
4h(+)	0.059	97.146	34	205	91.672
4hBAP	0.059	154.543	48	255	149.069
4hBEP	0.059	156.086	42	255	150.612
background	0.059	5.474	3	8	0