HEME TRAFFICKING UNDER LEAD STRESS IN *S. CEREVISIAE*

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HEME TRAFFICKING UNDER LEAD STRESS IN *S. CEREVISIAE*

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SUMMARY

Heavy metals, including lead, are significant for their toxicity to the environment and to organisms' physiology. Lead has been shown to induce oxidative stress in cells through mitochondrial perturbation, as well as affecting heme homeostasis. The initial hypothesis was to study heme as an interorganellar signaling molecule in *S. cerevisiae* cells using a novel genetically encoded fluorescently heme sensor, specifically as a mitochondrial retrograde signaling molecule. In addition to labile and total heme measurements, growth and changes in the protein profile were also assessed to determine how lead affects the cells' viability and stress response. It was found that there was no significant evidence of mitochondrial retrograde signaling with the labile heme pool, but that there was a preservation of the labile heme pool despite a marked decrease in total heme under lead conditions. The results support a model in which high affinity hemoproteins such as catalase are degraded under lead stress, while lower affinity ones such as glyceraldehyde phosphate dehydrogenase (GAPDH) are maintained. These results will help shape the understanding of the presence and purpose of the labile heme pool and how cells respond to oxidative stress in the context of heme.

CHAPTER 1

INTRODUCTION

The mitochondrion is a crucial organelle for cellular metabolism and intermediate metabolites. In addition to energy production, mitochondria are also involved in biosynthesis, metabolite conversion, and signal transduction[3]. The mitochondria and nucleus have a highly conserved endosymbiotic relationship in eukaryotes, with exchange of genetic material between these organelles. Mitochondrial retrograde signaling (MRS) is an intracellular pathway that enables mitochondria to communicate with the nucleus to regulate gene expression and enable the cell to adapt to changes in mitochondrial function. Previous studies have shown the significance of MRS in health and disease in its role in the pathogenesis and progression of diseases from cancer to neurodegenerative disorders[1,2]. Despite its importance in health and disease, the cellular mechanisms of MRS remain poorly understood.

One potential mechanism of MRS could be mediated by heme, a mitochondrial derived metabolite. Heme is an iron containing protein prosthetic group known to be an important cofactor for physiological processes such as electron transfer, gas binding and transport, and catalysis[3]. Heme is synthesized by the insertion of ferrous iron into the tetrapyrrole macrocycle of protoporphyrin IX, which is the product of a conserved eight step biosynthetic pathway that involves four mitochondrial and four cytosolic enzymes. The first committed step is the formation of ALA (d-aminolevulinic acid), which is then catalyzed by ALAD in the cytosol, and the final step involves insertion of the iron into the protoporphyrin ring by ferrochelatase in the mitochondrial matrix[1]. Intracellular heme trafficking is necessary for storage, sequestration, utilization, and incorporation into

hemoproteins[2]. The specific molecules and pathways necessary to facilitate heme transport and delivery have. yet to be elucidated.

Heme's role in MRS is proposed on the basis that there are many heme-dependent nuclear transcription factors, such as HAP1 in *S. cerevisiae*, that control processes such as respiration and energy metabolism and that heme synthesis is sensitive to mitochondrial function[2]. In this manner, mitochondrial dysfunction may be transmitted by changes in heme synthesis and availability, which is in turn sensed by heme-dependent transcription factors that control cell metabolism.

In order to study the role of heme in MRS, we established an experimental paradigm in which lead (Pb) is used to induce mitochondrial dysfunction and heme-mediated MRS. Non-biological heavy metals such as lead (Pb) inhibit enzymes in the biosynthesis of heme (ALAD catalyzed step), and induce mitochondrial oxidative stress through accumulation of reactive oxygen species[2,4]. Mitochondrial stress then results in disruption of heme biosynthesis[3]. As a consequence, there may be changes in the activity of heme dependent nuclear transcription factors that control various processes[3]. Considering these pieces together motivates the question of how heavy metals would perturb heme dependent mitochondrial retrograde signaling. Yeast is a very useful model organism for studying lead toxicity because of its ability to be manipulated easily and its entirely sequenced genome.

Total cellular heme can be conceptualized as being the summation of two pools, kinetically inert and labile heme. The former represents heme buried in the active sites of high affinity hemoproteins such as globins and cytochromes. The latter corresponds to heme that can be readily exchanged between different proteins, relevant for mobilization

in trafficking, utilization, and signaling. To probe the labile heme pool, the Reddi laboratory has developed a class of heme sensors. The first generation heme sensor (HS1) utilizes a tri-domain architecture composed of heme binding protein (cytochrome b562) and two fluorescent proteins, eGFP and mKATE2, which are quenched and unaffected by heme respectively. If heme is bound to the sensor, the ratio of eGFP to mKATE2 fluorescence decreases. HS1 will be used to study the dynamics and availability of heme in a compartment-specific manner in the context of heavy metal induced stress.

The signals and relays that mediate MRS are not well understood and because heme is a mitochondrial derived metabolite and there are numerous heme-dependent nuclear transcription factors, we hypothesize that heme acts as a signaling molecule in MRS. The Reddi lab's novel genetically encoded ratiometric fluorescent probes will be used to measure labile and *in vivo* heme trafficking and signaling throughout the yeast cell, an advantage over the previous lack of tools to examine heme signaling. Herein we establish a yeast model of lead toxicity, and utilize genetically encoded heme sensors and various biochemical approaches to probe the role of heme signaling in response to lead toxicity.

CHAPTER 2

METHODS AND MATERIALS

Sources and Instrumentation

All reagents and chemicals were obtained from Amresco, unless stated otherwise. Optical density measurements were carried out with a Cary 60 spectrophotometer and fluorescence readings were acquired with a Synergy H1 hybrid multi-mode microplate reader (BioTek).

Organism and Culture Conditions

The *S. cerevisiae* strains used in this study were derived from BY4741 from the yeast gene deletion collection (Thermo Fisher Scientific). Wild type (WT) yeast cells expressing empty vector (p415-GPD) and the HS1 sensor (p415-GPD-HS1) driven by the GPD promoter were primarily used, in addition to *hem1*Δ cells with a HEM1 deletion, previously created and tested strains were used[6]. For mitochondrial matrix or nuclear targeted sensors, strains fused with localization sequences to N-terminal Cox4 or C-terminal SV4 were used[6]. For examining HAP1 activity, the CYC1 promoter is positively regulated by HAP1 and was used to drive eGFP expression. Cells were cultured in either yeast extract-peptone-dextrose with 2% glucose (YPD) or synthetic-complete media with 2% glucose and lacking leucine (SC-LEU), with 15 mg/L ergosterol (Alfa Aesar) and 0.5% tween-80 added to the media for growing *hem1*Δ cells. Incubation time was 15-16 hours at 30°C, shaking at 220 rpm.

Transformation and Confirmation of Sensor Addition

To transform cells with the desired sensor plasmid, cells from a single colony with the correct strain were cultured overnight in YPD, then 0.1 M LiOAc was added to the pellet to achieve a 100 OD/mL cell density. The solution (50 µL) was transferred to microcentrifuge tubes for HS1, HS1-M7A, no DNA, and EV, for each clone. Each solution was pelleted, the 0.1 M LiOAc removed, and 240 µL 50% PEG, 30 µL 1 M LiOAc, 10 µL ssDNA, and 50 µL all sterile water or 1 µL DNA and 49 µL sterile water were added onto the cell pellet and vortexed for a minute. The tubes were placed in 30°C water bath for 30 minutes, and 42°C water bath for 20 minutes, then vortexed and re-suspended in 1 mL of sterile water. Each cell solution was added to SC-LEU plates with glass beads, incubated at 30°C for 2 days, and made into freezer stocks with 35% glycerol YPD.

To extract, amplify, and verify the correct DNA sequence the cell solution was incubated overnight at 30°C shaking at 220 rpm, then re-suspend in 1 mL of Solution 1 (from Omega mini-prep box), and transferred to a microcentrifuge tube. After pelleting, an equivalent pellet volume of zirconium oxide (ZrO) beads was added, then subjected to bullet blending (Next Advance, 3 minutes, setting 8). To complete DNA extraction, the Omega mini-prep protocol was followed. PCR was conducted with 100 µL samples and 0.8 µL Taq polymerase. Agarose gel (0.35 g agarose in 50 mL 1X TAE running buffer, 4 µL EtBr) was made and run with 4 µL 100 bp DNA ladder, and samples with 10 µL amplified DNA and 2 µL 6X loading dye for about 80 minutes at 60 mA. The gel was imaged with a UV Transilluminator, then sent for sequencing (barcoding) if there were both 300 and 500 bp bands.

Lead Shock Protocol (Small Scale)

This protocol was developed to grow yeast cells, treat cells with lead, and subsequently perform various assays (Figure 3.1). Cells transformed with sensor were cultured on SCE-LEU+ALA media at 30°C. They were grown overnight for 15-16 hours at 30°C, 220 rpm, then optical density was measured at 600 nm (about 5-9 OD/mL). A volume of cells was transferred for 15 OD to new 50 mL conical tubes, pelleted, and re-suspended in 15 mL MES buffer to 1 OD/mL. MES was chosen based on previous work done by Van der Heggen[5]. Aqueous 100 mM $Pb(NO_3)_2$ (Alfa Aesar) to 500 µM was added and the tubes were left on the benchtop at room temperature (about 23°C) for 30 minutes. The cells' optical density at 600 nm was measured, then cells were transferred for 5 OD to new 50 mL conical tubes, pelleted, and re-suspended in 5 mL SCE-LEU to 1 OD/mL. ALA was added if applicable. The tubes were placed in an incubator at 30°C, 220 rpm for 4 hours. Afterwards, optical density was measured, 1 mL of cells were transferred to new microcentrifuge tubes, pelleted, and re-suspended in a calculated volume of 1x PBS to 7 OD/mL. This solution (100 µL) was added to wells in a black bottomed Fluotrac 96 well plate and fluorescence was measured with a 5 minute kinetic run at the wavelengths for eGFP and mKATE.

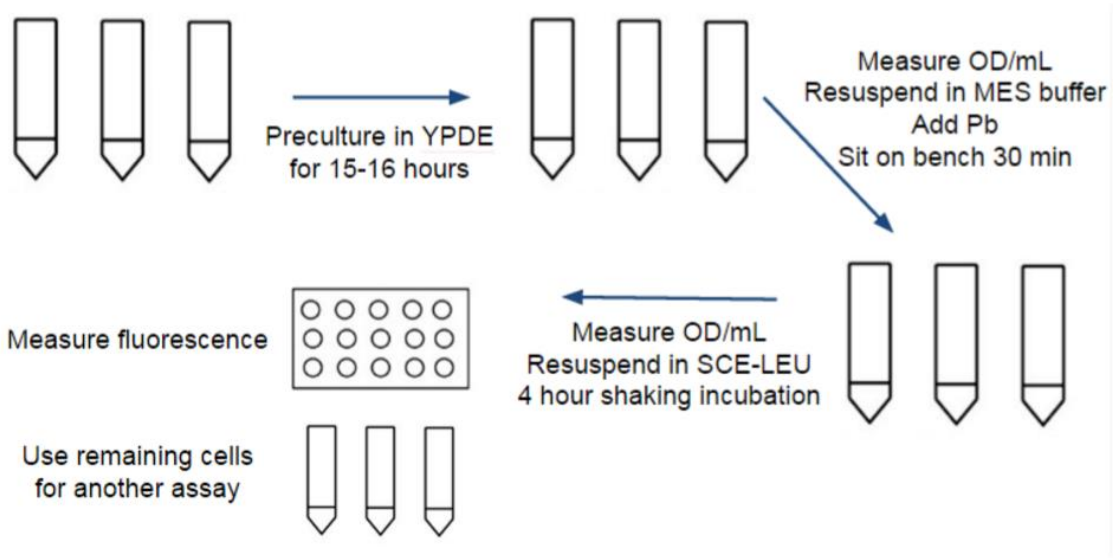

Figure 3.1. Simplified scheme illustrating the lead shock protocol.

Catalase Enzyme Activity

To assess the catalase activity in the cells under each experimental condition, catalase enzyme activity gels were run. Different cell volumes were transferred to locking microcentrifuge tubes to normalize to the lowest OD/mL (10< OD/mL). Cells were lysed with phosphate lysis buffer, ZrO beads, and bullet blending. A Bradford Protein Concentration Assay was performed to determine the lysate protein concentration. Samples were prepared to a total volume of 13 µL (15 µg protein, 2.6 µL 5X SOD loading dye, lysis buffer). A 10% tris-glycine gel (Invitrogen) was run at 13 mA with Tris-glycine pH 7 running buffer. The gel was stained immediately for 2 minutes with a catalase staining solution containing 1 part Dopamine (20mg/mL) in pH 8 0.2 M KPi buffer, 1 part para-phenylenediamine (3.5mg/mL) in pH 8 0.2M KPi, 1 part 15 % H2O2, and 2 parts DMSO mixed in the order listed, followed by imaging.

Coomassie Staining

To determine equal loading and total protein profile, samples were prepared with a total volume of 10 µL (5 µg lysate, 2 µ 5X sodium dodecyl sulfate (SDS) loading dye, 1 µL dithiothreitol (DTT), 1X phosphate buffered saline (PBS)) and ran a 10% polyacrylamide gel (Invitrogen) (30 mA) for about 80 minutes. The gel was stained with Coomassie staining solution (0.1% (w/v) Coomassie blue R350, 20% (v/v) methanol, and 10% (v/v) acetic acid), heated in microwave for 2 minutes, then replaced with Coomassie de-staining solution, and heated in microwave for 2 minutes. Fresh de-staining solution (50% (v/v) methanol in water with 10% (v/v) acetic acid) was added, the gel was left on a rocking platform overnight, and then imaged with transilluminator.

High Performance Liquid Chromatography (HPLC)

HPLC was used to measure and quantify total intracellular heme. The cell pellet was re-suspended from -80°C (output of large scale lead protocol experiment) in 150 µL sterile water. Optical density (using 1/400-1/600 dilution) was measured and acetone-HCl (9.75 mL acetone and 0.25 mL concentrated HCl) was used to lyse cells. The cell solution (50 µL) was combined with 50 µL 0.1% trifluoroacetic acid (TFA) in a 1:1 mixture of water and acetonitrile. A heme standard (hemin chloride (Calbiochem/Merck) in 0.1 M NaOH) with nanomolar concentration was prepared. An Agilent 1260 Infinity HPLC with a diode array detector and C18 column was used. The solvent was switched between 100% acetonitrile, 0.1% TFA to clear the line and 50% acetonitrile in water, 0.1% TFA to run the sample at a 1 mL/min flow rate. Heme was quantified using the

hemin chloride standard using the peak areas at the characteristic retention time of heme of 3.4 minutes.

Total Reflection X-ray Fluorescence (TXRF) Elemental Analysis

Cellular lead uptake was quantified by TXRF spectroscopy. The cell pellet was re-suspended from -80°C (output of large scale lead protocol experiment) in 150 µL sterile water. Optical density (using 1/400-1/600 dilution) was measured, then 9 µL cell solution (100< OD/mL) was transferred to new microcentrifuge tube and 1 µL Ga (10 ppm) was added. The quartz disks were prepared by adding 10 µL silicone, then heating on hot plate (about 5 minutes). Cell solution with Ga (2 µL) was added to center of the silicone, then heated (about 5 minutes). The samples were run on a Bruker S2 Picofox TXRF instrument.

CHAPTER 3

RESULTS

Yeast Model of Lead Toxicity

We first sought to establish a yeast model of lead toxicity to probe heme-based MRS in response to lead stress. Towards this end, we determined the median lethal dose (LD50) of lead required to kill half of the yeast culture was 500 µM $Pb(NO_3)_2$ (Figure 4.1). Viability was measured by determining the ability of the yeast cells to grow and recover after the 3 hour Pb shock.

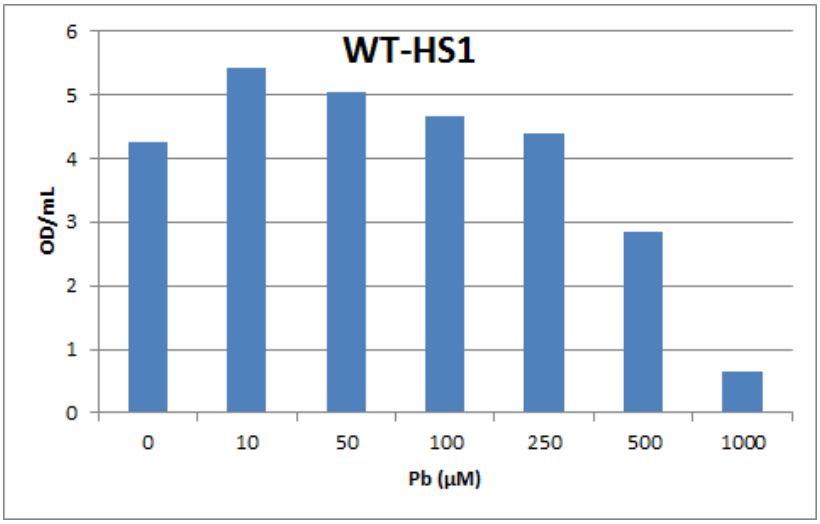

Figure 4.1. Growth density after lead shock (0 to 1000 µM) and 20 hour recovery in fresh SCE-LEU media.

Total Reflection X-Ray Fluorescence Spectrometry (TXRF) verified and measured the amount of lead transported into cells. Figure 4.2 shows that lead significantly accumulated in the cells treated with lead and that there were virtually undetectable levels in cells without lead treatment. Figure 4.2 further shows that the presence of heme does not influence lead uptake.

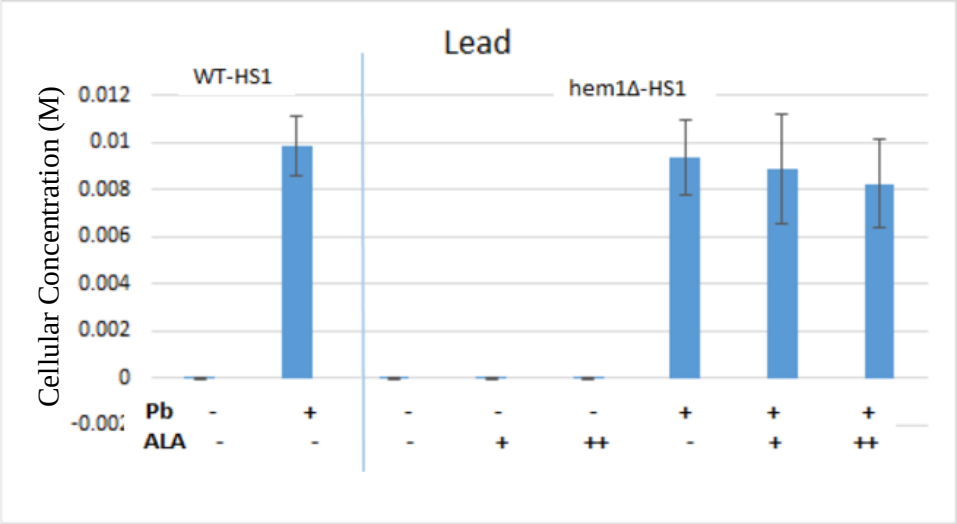

Figure 4.2. Concentration (M) of lead per WT-HS1 or *hem1Δ*-HS1 cell, with either 0 µM (-) or 500 µM (+) lead. ALA concentrations were 0 µM (-), 10 µM (+), and 200 µM (++). The samples with +Pb had very similar amounts of lead uptake (0.007 to 0.011 M/cell), while the -Pb samples had virtually no lead uptake.

Effects of Lead Toxicity on Labile and Total Heme

Having established a yeast model for lead toxicity, we next sought to determine the impact lead has on total and labile heme. As expected, we observed that total heme levels are depleted in lead treated WT cells to undetectable levels (Figure 4.3). This is due to several heme biosynthetic enzymes being inhibited by lead. However, rather surprisingly, labile heme pools are largely preserved as determined by genetically encoded fluorescent sensors.

The amount of total heme in cells was quantified using HPLC and a hemin chloride standard. WT cells exhibit a cellular concentration of heme of 536 nM and lead treatment resulted in undetectable levels of heme similar to the *hem1Δ* strain, which cannot biosynthesize heme (Figure 4.3). As seen in Figure 4.4, there was a decrease in

ratio in the lead treated cells, indicating an increase in the labile heme pool, in contrast to the decrease in total heme concentration.

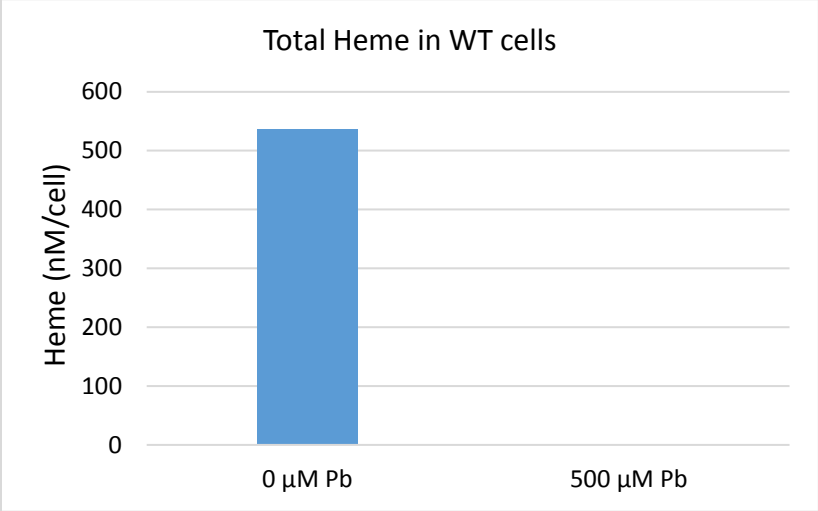

Figure 4.3. The concentration of heme (nM) per cell for WT-HS1 with either 0 or 500 µM Pb. Without lead treatment, there is approximately 5 x 10^{-7} nM heme/cell.

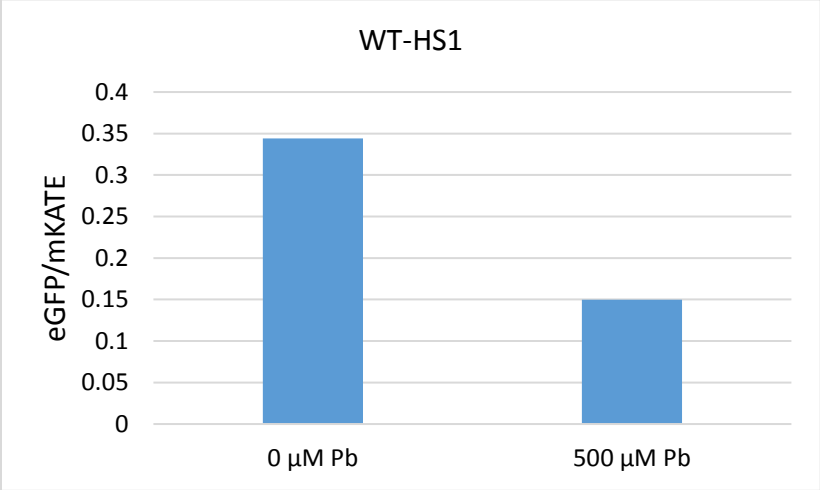

Figure 4.4. The fluorescence ratios for 0 and 500 µM Pb in WT-HS1 cells

Labile Heme in Cellular Compartments

The fluorescence ratios after lead shock and 4 hour recovery were measured in cells with sensor targeted to the cytosol, mitochondria (cox4), and nucleus (NLS). The cytosol and nucleus showed similar relative eGFP/mKATE values between the ±Pb conditions, with an irregular negative ratio value for the lead treated cells with mitochondrial sensor (Figure 4.5). The lack of significant change in cytosolic labile heme pools was especially key in the decision to shift the direction of the project away from mitochondrial retrograde signaling.

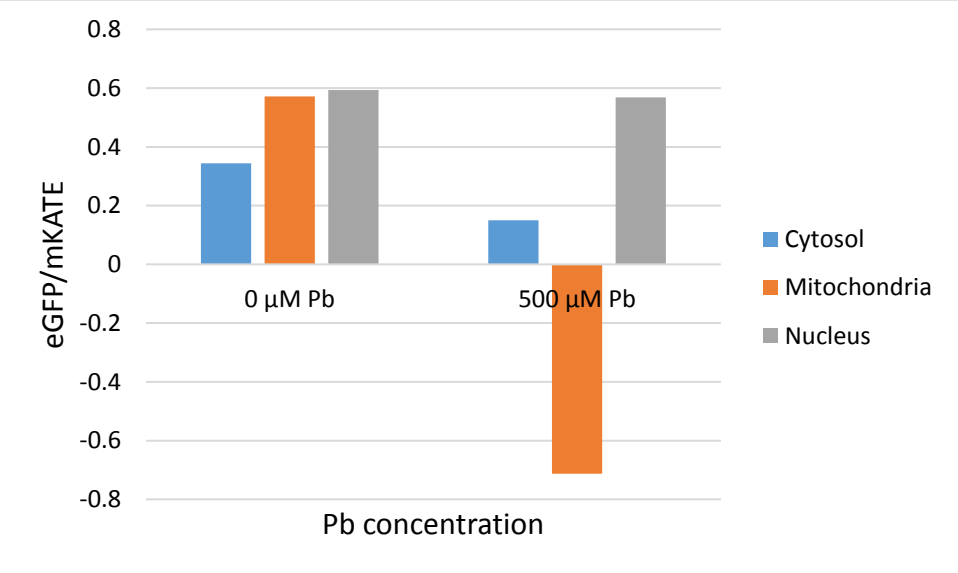

Figure 4.5. Fluorescence ratios of WT cells with HS1 sensor targeted to the cytosol, mitochondria, and nucleus under ±Pb.

Effect of Lead on Heme-Regulated Processes

Having established that the labile heme pool is preserved in response to lead toxicity, we next sought to determine if lead stress effects heme-regulated functions. Towards this end, we sought to assay catalase activity, a heme dependent enzyme, and HAP1 activity, a heme regulated transcription factor.

As shown in Figure 4.6, there is drastically less catalase activity in the WT-HS1 cells that underwent lead shock. Catalase was the only high affinity hemoprotein available to study in yeast.

Presently, the only known heme dependent transcription factor in *S. cerevisiae* is HAP1. Therefore, cells with eGFP driven by cyc1, an indicator of HAP1 activity, were subjected to the lead shock protocol and the fluorescence level results are proportional to HAP1 activity. In the WT-pcyc1-eGFP cells, there was a decrease in HAP1 activity with lead treatment (Figure 4.7).

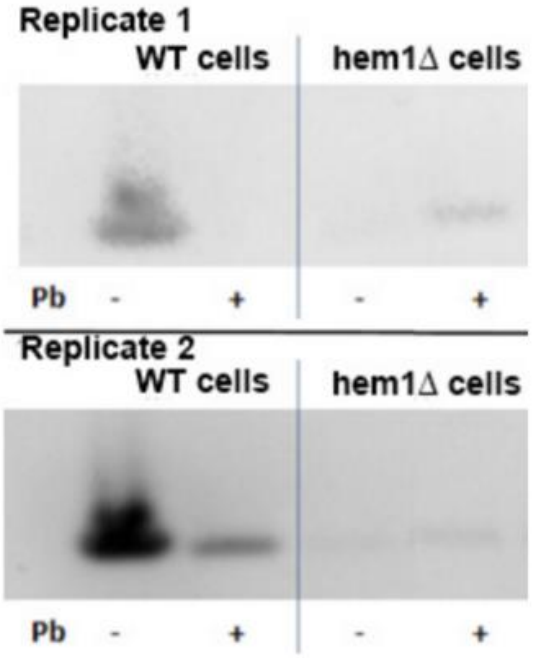

Figure 4.6. The stained catalase activity gel with 15 µg protein loaded. Qualitatively, the WT-HS1 without Pb has significantly more catalase compared to WT-HS1 with Pb.

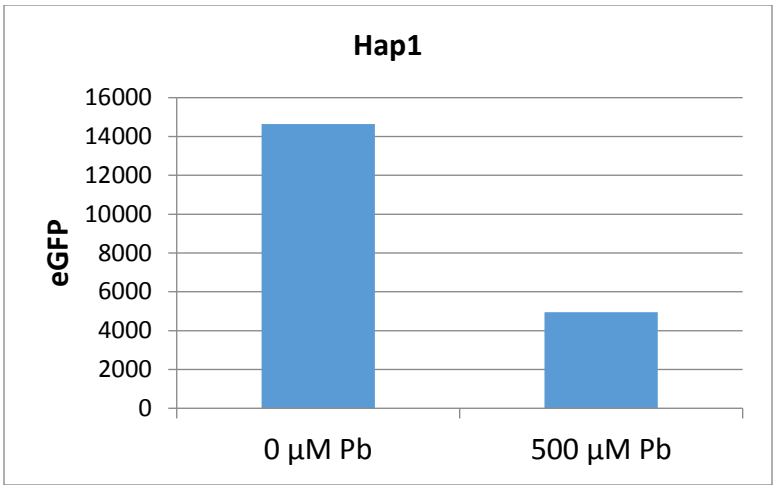

Figure 4.7. The fluorescence results for WT-pcyc1-eGFP after for cells with and without lead shock. There is a decrease in HAP1 activity with addition of lead.

Total Protein Analysis

One mechanism in which to investigate why the total heme pool is depleted while the labile heme pool is preserved during lead stress is to probe protein abundance using SDS-PAGE analysis and tandem mass spectrometry. If the high affinity hemoproteins, accounting for a large fraction of heme in cells, are selectively degraded, this would help explain the mechanism behind the heme pool discrepancy. Qualitatively examining the Coomassie gel, it appears that there is significantly less protein in WT-HS1 cells with Pb, except for two bands (Figure 4.8). The higher molecular weight (MW) band has consistent intensity across all the samples, but the lower molecular weight one appears to be stronger in WT with Pb. Protein ID done in collaboration with the Torres lab at Georgia Tech identified the higher MW band as enolase, and the lower as GAPDH.

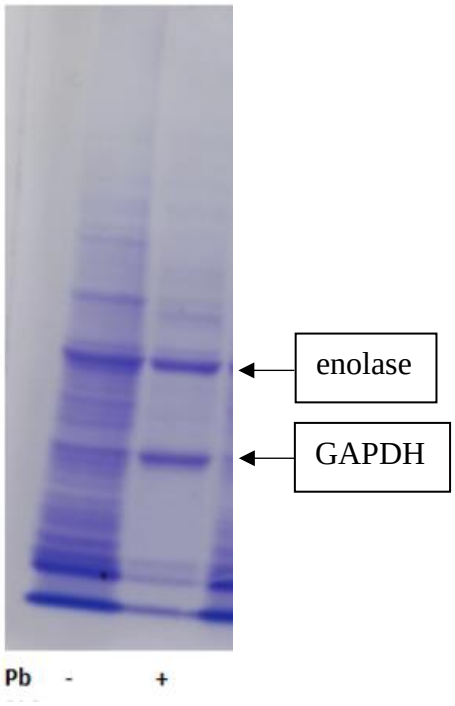

Figure 4.8. The stained Coomassie total protein gel. Qualitatively there is significantly less protein in WT-HS1 +Pb, with the exception of two bands, which were determined to be enolase and GAPDH.

CHAPTER 4

DISCUSSION AND CONCLUSION

The original hypothesis was that heme acts as a MRS molecule after lead induced stress. However, the results do not indicate a clear connection between lead shock and changes in labile heme between the cytosol, mitochondria, and nucleus. The cytosol did experience a decrease in labile heme, however, this was not matched with a change in the nucleus or mitochondrial labile heme pools. Unknown factors caused the negative eGFP/mKATE ratio in the 500 µM $Pb(NO_3)_2$ with mitochondrial sensor cells, and it was generally difficult to probe these compartments in a dependable and quantitative manner. Future experiments should be done to continue exploring MRS with other techniques in combination with the HS1 sensor.

The results instead suggest a previously undiscovered discrepancy in total and labile heme tracking together under lead stress. A few key experiments were performed to provide a basis of support for the results. It was found that 500 µM $Pb(NO_3)_2$ results in a LD50 dose that can still induce changes in heme and retain viability of some cells. TXRF elemental analysis verified that the lead uptake was working as expected, without influence from the presence or lack of heme.

When examining the relative amounts of total and labile heme between WT-HS1 with and without $Pb(NO_3)_2$, the total heme is completely knocked down with the addition of lead, while labile heme remains preserved to similar (slightly lower) levels as WT without $Pb(NO_3)_2$. It does not appear that this preserved labile heme is being used for HAP1 transcription factor activity because HAP1 activity decreased in lead treated cells.

Catalase is a high affinity hemoprotein that also showed significant diminishment in activity in the lead treated cells.

Interestingly, it was found that enolase and GAPDH proteins were maintained in lead-treated cells, when all other proteins were greatly reduced. GAPDH is a lower affinity hemoprotein found to buffer intracellular heme and regulate the heme-dependent transcription factor heme activator protein (Hap1p) activity[6]. Enolase is mainly a cytosolic glycolytic enzyme, but has also been found to be involved in vacuole fusion; a hypothesis for future experiments is the role of the vacuole and vacuole transporters in the maintenance of the labile heme pool[7].

CHAPTER 5

FUTURE WORK

The established results provide a firm foundation for many promising and exciting studies to further investigate the mechanisms and significance of heme trafficking under lead toxicity. Another approach is to remove the heme pools with biosynthetic inhibitors such as succinyl acetone and very high affinity but non-active proteins like cytb562, to assess how lead's effect on cell growth and protein activity changes.

The total heme experiments using HPLC need further replication and reduced error. In conjugation with HPLC, Coomassie, catalase, and GAPDH activity should be assessed by their respective gels to gain a clearer picture of the mechanism or pathway by which labile heme is being preserved and what function it is being preserved for. Some of the genes controlling proteins for heme transport and degradation are known and can be knocked out to find the impact on cells under lead toxicity. *Tdh3Δ* and *pug1Δ* strains would remove the ability of cells to synthesize glyceraldehyde-3-phosphate dehydrogenase and enable protoporphyrin uptake, fixing part of the labile heme pool flux[8,9]. *Hmx1Δ* would inactivate heme oxygenase, the enzyme that degrades heme, thus removing another source of the labile pool losing heme[10]. Expanding the scope of the study to mammalian cells would also be a worthwhile future study.

Preliminary work is being done using heme agarose beads with affinity chromatography to quantitate GAPDH changes in conjunction with mass spectrometry (Stable isotope labeling with amino acids in cell culture (SILAC)) to determine proteome

changes caused by lead toxicity. Additional studies are needed to completely elucidate how and why labile heme is being preserved for low affinity hemoproteins like GAPDH).

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