ARSENIC TOXICITY AND ALTERED MITOCHONDRIAL BIOENERGETICS IN RESPONSE TO OXIDATIVE STRESS

BY

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Submitted to the graduate degree program in Toxicology and the Graduate Faculty of the University of Kansas in partial fulfilment of the requirements for the degree of Master of Science.

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Date Defended: April 16, 2015

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ABSTRACT

Environmental exposure to arsenic is a worldwide health concern which is linked to a number of diseases. Areas with arsenic levels above the current safe concentration have higher levels of skin, lung, liver, and bladder cancers and non-carcinogenic pathologies, including neuropathy, cardiovascular diseases and diabetes mellitus. Understanding the mechanism by which arsenic exerts its toxic effects is crucial to developing preventative strategies and more effective treatments for individuals with long term exposure to arsenic compounds.

This thesis outlines the epidemiological evidence for arsenic-linked diseases as well as the current understanding of arsenic metabolism and the mechanism(s) of how arsenic exerts its cytotoxicity.

The liver is a major site of arsenic uptake and biotransformation and thus is affected by high arsenic levels. Generation of reactive oxygen species (ROS) has been shown to play a major role in the toxic effects of arsenic. Using primary and immortalized hepatocyte cell lines these studies investigate how arsenic generates the production of ROS. The localization of superoxide was shown to overlap with mitochondria and antioxidant enzymes were shown to be induced after arsenic treatment. Measurement of mitochondria function through oxygen consumption rate (OCR) shows that arsenic affects oxidative phosphorylation and the ability for mitochondria to efficiently couple electron transport with ATP production. Understanding the source of ROS from arsenic exposure and how cells cope with

altered mitochondrial bioenergetics is an important building block for future studies on arsenic toxicity and for developing treatments for arsenic exposure.

Dedicated to my grandmothers:

The late Marilyn Mickey-Clay and Tokuko Nishiyama Reaster

ACKNOWLEDGEMENTS

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LIST OF ABBREVIATIONS

Abbreviation	Full name
ARE	Antioxidant response element
ATPsyn	Adenosine triphosphate synthase
DMA	Dimethylarsenous acid
ETC	Electron transport chain
EPA	Environmental Protection Agency
GCK	Glucokinase
GCLC	Glutamate-cysteine ligase, catalytic subunit
GCLM	Glutamate-cyteine ligase, regulatory subunit
GSS	Glutathione sythetase
GSTP1	Glutathione-S-transferase pi 1
GSTT1	Glutathione-S-transferase theta 1
HK-2	Hexokinase-2
HO	Heme oxygenase
HPH	Human primary hepatocytes
MMA	Monomethylarsenous acid
MPH	Mouse primary hepatocytes
mtGAT	mitochondrial glycerol-3-phosphate acyltransferase
NAC	N-acetylcysteine
NRF1	Nuclear respiratory factor 1
Nrf2	Nuclear factor-erythroid 2-related factor 2
OCR	Oxygen consumption rate
PDH	Pyruvate dehydrogenase

PGC1α	Peroxisome proliferator-activated receptor (PPAR) gamma coactivator 1α
ROS	Reactive oxygen species
SOD2	Superoxide dismutase 2
SMR	Standardized mortality ratio
TCA	Tricarboxylic acid
TFAM	Transcription factor A, mitochondrial
WHO	World Health Organization

LIST OF FIGURES AND TABLES

Chapter 1: **BACKGROUND AND INTRODUCTION**

1.1 Arsenic as a human health hazard

ENVIRONMENTAL EXPOSURE TO ARSENIC

Arsenic is a metalloid naturally present in the Earth's crust. Its presence in the soil results in the contamination of drinking water from ground sources, the primary route of exposure to humans (1). Consumption of food grown in soil with high arsenic levels is another major source to humans (2). Seafood may also be another route of exposure, however the typical concentration and form of arsenic in seawater presents a lesser problem. Industrial exposure remains of importance, although efforts to remove it from industrial processes in the last thirty years have improved. Arsenic has been used in the making of pigments, pesticides and chemical warfare agents. Its use in industrial processes has presented a source of exposure by inhalation routes through arsine gas production. Arsenic concentrations vary between regions, with some areas having little to no detection. However many countries, such as Bangladesh, India, China, Argentina and the United States have areas with arsenic levels that are considered hazardous (3). The World Health Organization (WHO) initially recommended arsenic levels in water not exceed 0.05 mg/L, or 50 ppb, but lowered the limit to 10 ppb in 1992 as evidence accrued demonstrating the carcinogenic effects of arsenic at low concentrations (4,5). The US limit set by the Environmental Protection Agency (EPA) was adjusted to 10 ppb in 2006 (6). In the world, over 100 million people are exposed to unhealthy levels of arsenic (7). Many community water sources exceed this limit, and a search for alternative water sources or methods to remove arsenic from water is of high importance.

EPIDEMIOLOGICAL EVIDENCE LINKING ARSENIC EXPOSURE TO HUMAN HEALTH

Arsenic exposure has been implicated in a variety of health hazards. Arsenic-associated diseases have been linked to chronic exposure of arsenic levels even below the WHO's recommendation of 10 ppb (8). Epidemiological studies show higher cases of skin, lung, liver, and bladder tumors in areas with high levels of arsenic in the water supply compared with the general population or areas with low arsenic exposure. Skin and bladder tumors are particularly common. Because of this, inorganic arsenic has been classified as a carcinogen by the WHO and EPA. Reviews of epidemiological studies from Argentina, Taiwan and the United States concluded that bladder cancer diagnoses and deaths were higher in these areas with arsenic-contaminated well water (9). More convincingly, when tap water systems which removed arsenic were implemented, there was an eventual gradual decrease in bladder cancer-related deaths. Meta-analysis has recently shown that the standardized mortality ratio (SMR) is significantly increased with respect to liver cancer mortalities reported in high-level arsenic areas (10).

In addition to carcinogenic effects, arsenic is implicated in numerous nonmalignant diseases. Peripheral vascular disease, cardiovascular disease, skin lesions, and diabetes mellitus are several important results of sustained arsenic exposure. In areas with high-chronic exposure (>0.05 mg/L), cardiovascular disease, coronary heart disease, stroke, and peripheral arterial disease were 32, 89, 8, and 17% more likely to occur (8). A meta-analysis of epidemiological studies in China on the occurrence of

type 2 diabetes mellitus suggested that risk for type 2 diabetes increased by 13% for every 100 µg/L increment of inorganic arsenic in drinking water (10).

1.2 Mechanisms of arsenic toxicity

METABOLISM OF ARSENIC

Arsenic is found within soil and water primarily as pure, oxide, sulfur, or compounded with metals. Oxides are the most common arsenic species in drinking water and are most correlated with toxicity. The trioxide and pentoxide forms of arsenic are well absorbed through oral and inhalation routes, and transdermal exposure is another possible entry of arsenic. All these routes pass eventually through the liver, where hepatocytes take up arsenite (trioxide form) and arsenate (pentoxide form) (1). Aquaporin 9 has been strongly linked to intracellular accumulation of arsenic in primary mouse hepatocytes (11,12). It has been shown that inhibition of aquaporin 9 reduces cellular concentration of arsenic, while overexpression increases arsenic toxicity. A series of biotransformation and detoxification reactions take place in the liver to enhance excretion (13,14). Arsenic pentoxide is reduced to arsenic trioxide by an arsenate reductase, purine nucleoside phosphorylase. With S-adenosylmethionine as a methyl donor, a series of methylations and reductions occur by arsenite/MMA^{III} (monomethylarsenous acid) methyltransferase and MMA^{V} reductase, respectively (15) (Figure 1). Glutathione is used for the reduction reactions in between methylations. A mixture of mono-, di-, and tri-methylated forms of arsenic are formed and readily excreted in the urine. The average proportions of these forms in human urine are 10-30% inorganic arsenic, 10-20% MMA and 60-80% dimethylarsenous acid (DMA) (15). These methylated species may exert some toxicity on hepatocytes, but the (III) and (V) oxides have shown the most potential for toxic effects (1). Multidrug resistance-

associated protein 1 (MRP-1) has been implicated in arsenic resistance and as a transporter responsible for efflux of arsenite-glutathione conjugates (16,17). The sites of absorption that receive the highest exposure to toxic arsenic concentrations are skin, lung, liver, and bladder. The higher incidences of cancer in these organs suggest a strong correlation.

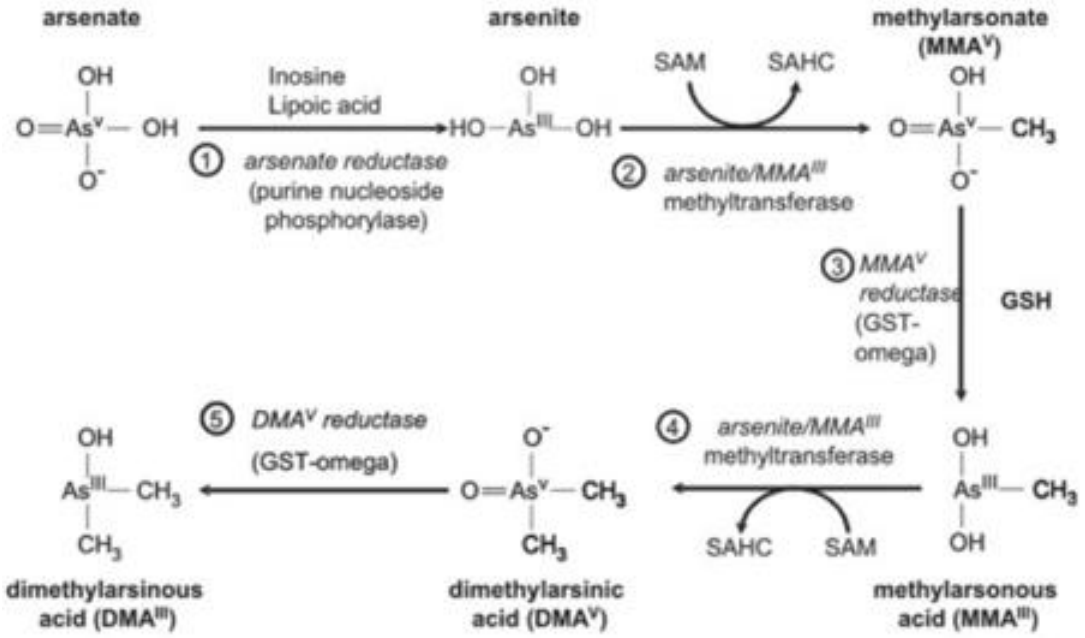


Figure 1. Known pathways of arsenic metabolism in vertebrates (18).

EVIDENCE FOR OXIDATIVE STRESS IN ARSENIC-INDUCED TOXICITY AND CARCINOGENICITY

Myriad toxic effects are seen intracellularly with arsenic poisoning. Stable As-S bonds can occur with sulfur groups within proteins to inhibit enzymes (19). Pyruvate dehydrogenase, succinic dehydrogenase, glutathione reductase, and thioredoxin reductase inhibition by arsenite have been reported (20-22). Unstable arsenoesters can form from interactions of arsenate with the phosphate groups within ATP, disrupting the bioenergetic profile of the cell (13). Inhibition of key antioxidant and metabolic enzymes provide strong evidence for the production of reactive oxygen species (ROS) seen from arsenic poisoning.

Production of ROS is suggested to be a major source of the toxic effects seen from arsenic. Oxidative stress is known to be involved in the degradation of cellular macromolecules, such as lipid peroxidation and DNA damage. Disturbing enzyme activity and production of various ROS (H_2O_2, OH^-, ROO·, O_2^-) is hypothesized to be a source of genotoxicity that produces hyperplasia and cancer phenotypes (23,24). Inhibition of DNA ligase, chromosomal aberrations, altered DNA methylation, and activation of the mitogen activated protein kinase pathway are avenues that have been explored to provide causes of increased cell proliferation frequently seen with chronic arsenic exposure (13,25).

If the antioxidant response of the cell is sufficient to detoxify arsenic species and its ROS byproducts, cellular adaptation will allow survival of affected cells and prevent organ damage. A primary mechanism of antioxidant response is via the Nrf2-Keap1

pathway. The transcription factor nuclear factor-erythroid 2-related factor 2 (Nrf2) is normally sequestered and ubiquitinated in the cytoplasm. Accumulated ROS disrupts Nrf2-Keap1 and as a result Nrf2 accumulates and translocates to the nucleus (26). Nrf2 forms a heterodimer with a small Maf protein to bind the Antioxidant Response Element (ARE) in the promoter region of target genes. Genes regulated by this pathway include enzymes that reduce oxidative stress, phase I and II metabolism enzymes, and transporters. Heme oxygenase-1 (HO-1) is regulated by Nrf2 and is highly inducible in response to arsenic. Previous studies show HO-1 to exert a protective effect from oxidative stress by positive feedback with Nrf2 (27). As such, HO-1 provides an established positive control to indicate ROS and arsenic poisoning (28).

Despite consistent observation of arsenic-induced ROS, little is known about the components and origins of arsenic induced mammalian oxidative stress. Understanding the origins and mechanism by which arsenic induces oxidative stress could open up potentially new therapeutic avenues for prevention and intervention strategies against the toxic effects of arsenic.

1.3 Mitochondrial dysfunction in normal and pathophysiology

MITOCHONDRIA AND ARSENIC

Mitochondria are the largest source of ATP production and are very sensitive to the oxidative state of the cell. Under normal conditions a proton gradient is built from the transfer of electrons from various electron donors, with molecular oxygen being the final electron acceptor. The established proton gradient is coupled to the production of ATP from ADP. While this provides the cell with a large portion of its ATP pool, there are many points in this process where reactive by-products can be produced (29). Electron transfer to oxygen must be tightly controlled or electron "leaking" can result in superoxide production, a highly reactive oxygen species (30). Malfunction of electron transfer not only reduces the cell's available ATP, the ROS produced from this reacts with many cellular macromolecules. Excessive cellular damage will initiate apoptosis or give rise to a variety of pathological conditions (31,32). Mitochondrial diseases may be inherited or induced and result in a variety of pathologies, including diabetes mellitus, various neuropathies and myopathies (33,34).

ROS can perturb the function of mitochondria, the principal source of oxygen radicals under normal conditions. The role of ROS in arsenic toxicity has been shown by suppression of ROS generation. Pretreatment of myoblasts with N-acetylcysteine (NAC) before sodium arsenite treatment resulted in suppressed lipid peroxidation, ER stress, caspase cascade activity and apoptosis (35). Arsenic is known to alter mitochondrial membrane potential and disrupt electron transport, particularly by altered activity of complex I (36). Altered activity of complex I and III of the electron transport in

particular is associated with an increase in mitochondrial ROS (37,38). Isolated rat hepatocytes show decreased complex I activity when treated with sodium arsenite (39). With ROS playing an important role in the occurrence of many diseases and pathologies and mitochondria being a large source of intracellular ROS, it is important to investigate and link the production of mitochondrial ROS to arsenic toxicity. Previous studies strongly suggest the alteration of mitochondria function plays a key role in the development of various diseases and systemic effects associated with arsenic.

Chapter 2: **EXPERIMENTAL MATERIALS AND METHODS**

CELL CULTURE

Human liver-derived cell lines Hep3B and HepG2 were from the American Type Culture Collection (Manassas, VA). Cells were cultured in modified Eagle's medium supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin, 2mM L-glutamine. Mouse primary hepatocytes were plated and grown in Williams' Medium E supplemented with 10% FBS and 100 units/ml penicillin and streptomycin for 3 hours before replacing with serum-free Williams' Medium E. For arsenic treatment, culture medium was replaced with fresh media containing the appropriate concentration of sodium arsenite dissolved in water and incubated at 37°C for 16 hours. All chemicals were from Sigma-Aldrich Corporation (St Louis, MO) unless otherwise stated.

LIVE CELL IMAGING

HepG2 cells were cultured overnight in chamber slides in increasing concentrations of arsenic. Culture medium was replaced with PBS containing 5 µM mitoSOX (Invitrogen), 1µM mitoTracker green (Life Technologies), and 0.5 µM Hoechst 33342 (Invitrogen) and incubated at 37°C for 15 minutes before washing 3 times with PBS. Slides were mounted and viewed at excitation/emission of 510/580 nm (mitoSOX), 490/516 nm (mitoTracker green) and 350/450 nm (Hoechst).

ISOLATION OF PRIMARY HEPATOCYTES

Primary human hepatocytes were isolated by the Cell Isolation Core at University of Kansas Medical Center as described by Xie, et al. (40).

CYTOTOXICITY ASSAY

In 96-well plates, HepG2 cells, primary mouse or human hepatocytes were seeded with 15,000 cells/well and kept at 37°C for one day. Then, media was replace with media containing sodium arsenite in water in a dilution series or with vehicle for 2 days. We assessed cytotoxicity by using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide assay as described (28,41). Briefly, 1 mg/ml MTT was added to each well and incubated at 37°C for 2-4 hours until color developed. Media was removed and replaced with 0.04M HCl in isopropanol. The plate was mixed on a shaker for 15 minutes and read absorbance at 590 nm. IC_{50} values were calculated based off absorbance and arsenite concentration.

REAL-TIME PCR AND WESTERN ANALYSIS

RNA isolation, Reverse transcription and Real-Time PCR analysis. RNA was isolated using TRIZOL® reagent (Invitrogen, Grand Island, NY). One µg of RNA was used for reverse transcription using iScriptTM cDNA synthesis kit, following the manufacturer's protocol (BioRad, CA). Real-time PCR was performed using CFX384TM Real Time PCR System (BioRad, CA) as described previously using primer sets specific for the mouse or human genes (42) GCK, HK-2, PFK, GCLC, GCLM, GSS, GSTP1, GSTT1, actin, or GAPDH.

For Western analysis, cell lysates were prepared in 1x RIPA buffer with protease inhibitors (Roche Applied Science) and 15 µg of total protein was analyzed by polyacrylamide gel electrophoresis (PAGE). Polyclonal primary antibodies were used to detect SOD2 (Abcam; Cat #ab13533, Cambridge, MA), Catalase (Cell Signalling, Cat #8841, Danvers, MA), GAPDH (Cell Signaling, Cat #2118), Actin (Sigma-Aldrich, Cat #A5441, St Louis, MO), HO-1 (Enzo Life Sciences, Cat #ADI-SPA-895, Farmingdale, NY), OXPHOS cocktail (Abcam, mouse #ab110413, human #ab13533). Immunoreactive proteins were detected using polyclonal goat antirabbit horseradish peroxidase IgG secondary antibodies (Thermo Scientific, Waltham, MA) and visualized using Supersignal™ chemiluminescent horseradish peroxidase substrate (Thermo scientific, MA). Densitometric analysis was performed using ImageJ analysis software (NIH).

COMPLEX I AND PYRUVATE DEHYDROGENASE ACTIVITY ASSAY

Complex I and PDH activity was measured using the Complex I Enzyme Dipstick Assay kit or PDH Enzyme Dipstick Assay kit from abcam (Cambridge, UK) as per the protocol. 50 µg protein was immuno-captured using a dipstick with anti-human or anti-mouse complex I or PDH antibody. Activity was measured by densitometry using ImageJ (NIH).

MITOCHONDRIA STRESS TESTS

Oxygen consumption rate (OCR) was measured in real-time, in an XF24x3 Extracellular Flux Analyzer (Seahorse Bioscience, Billerica, MA). HepG2 cells or mouse primary hepatocytes were seeded in XF24-well plates (50,000 cells per well in 100 µl). After 6 h, 400 µl media with sodium arsenite was added to achieve a final concentration of 0, 2.5, 5 and 10 µM arsenic (n=5 wells). Cells were further incubated overnight at 37°C, 5% CO2. The XF24 sensor cartridge was hydrated with 1 ml calibration buffer per well overnight at 37°C. The sensor cartridge was loaded with oligomycin (1 µM, port A), carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP, 0.5 µM, port B) and rotenone plus antimycin-A (1 µM each, port C) to measure the bioenergetic profile. Cells were washed twice with pre-warmed XF assay medium containing 25 mM glucose and incubated in XF assay medium at 37°C without CO^2. Once the sensor cartridge was equilibrated, the calibration plate was replaced with the cell plate to measure OCR using mitoStress application.

ATP LEVEL MEASUREMENT BY HPLC

Extraction of ATP from cells: Six-well plates seeded with 1 x 10^6 cells were treated with different concentrations of arsenic for 16 hr. Cells were lysed with 0.5 ml of 0.5M perchloric acid on ice for 5 min. The extraction mixture was centrifuged at 16,000 x g for 10 min at 4°C. Three hundred microliters of supernatant was quickly neutralized to ~pH 6.0 with 4M potassium dichromate. The neutralized supernatant was incubated for 30 min on ice to precipitate potassium perchlorate which was removed by centrifugation at 16,000 x g for 10 min at 4°C. The supernatant was stored at -80°C until further analysis.

ATP Standards: ATP standards were made in 0.5M perchloric acid neutralized with potassium dichromate as described above. Fifty microliters of sample was used for the HPLC analysis. ATP standards were linear for the dilution range used in this study (0.5 µM – 1 mM).

HPLC analysis: The HPLC instrument (Schimadzu Instruments Inc., USA) was equipped with an LC-20AD pump system and SPD-M20A diode array detector was used. Supelcosil™ LC-18 150 x 4.6 mm column (5µm particle size) was used for separation of analytes. HPLC separation was achieved using gradient elution. Mobile phase A and B consisted of 0.1M monopotassium phosphate and 0.008M tetrabutylammonium hydrogen sulfate dissolved in deionized water and adjusted to pH= 6.0. Two percent acetonitrile was added to mobile phase A while mobile phase B consisted of 30% acetonitrile. The elution program was as follows: 2.5 min 100% A, 0% B; 3.5 min 90% A, 10% B; 1 min 80% A, 20% B; 5 min 60% A, 40% B; 8 min 0% A, 100% B and 10 min 100% A, 0% B. Flow rate of the mobile phase was 1 ml/min, while

the injection volume was 50 µl. The autosampler was set at 4°C and the column was maintained at 30°C. ATP in the samples was identified by comparison with the retention time of the standard (~7 min), while the concentration was determined using an external standard method.

Chapter 3: **RESULTS**

3.1 VIABILITY OF HUMAN AND MOUSE PRIMARY HEPATOCYTES AND HUMAN HEPATOMA CELLS AFTER EXPOSURE TO INORGANIC SODIUM ARSENITE

Although much information is available on the toxicity profile of arsenic in cultured cell lines, very little is known about the toxicity profile of arsenic in human and mouse primary hepatocytes. Thus, in these studies we first established the IC_{50} values for sodium arsenite in human and mouse primary hepatocytes and compared it to the toxicity profile in human hepatoma HepG2 cells. Mouse and human primary hepatocytes and HepG2 cells were treated with increasing concentrations of sodium arsenite (0 – 100μM) for 16 hours. At the end of treatment viability was evaluated by the MTT assay as described in the methods. Human primary hepatocytes were significantly more resistant to sodium arsenite treatment compared to either mouse primary hepatocytes or human hepatoma HepG2 cells. The order of resistance increased with mouse primary hepatocytes < HepG2 cells < human primary hepatocytes (Table 1). Sodium arsenite was also non-lethal to all three cell types up to a concentration of 20 μM for a maximum exposure time of 16 h. Based on these initial observations an exposure regimen of 0 – 20 μM for 16 h was considered as non-lethal to both primary hepatocytes and human hepatoma cells, and was employed as the treatment regimen in the rest of the studies.

Cells	IC_{50}
HepG2	40 ± 8 µM
MPH	36 ± 4 µM
HPH	65 ± 7 µM

Table 1. Sodium arsenite IC_{50} values of human hepatoma cells (HepG2), mouse and human primary hepatocytes. HepG2 cells, human primary hepatocytes (HPH) and mouse primary hepatocytes (MPH) were exposed to increasing concentrations of sodium arsenate (0 µM to 100 µM) for 16 h. Arsenate toxicity was evaluated by measuring cell death using the MTT assay as described in methods.

3.2 DOSE AND TIME DEPENDENT EFFECT OF SODIUM ARSENATE ON MITOCHONDRIAL FUNCTION.

Experimental evidence indicates that exposure to arsenic induces oxidative stress. Further, given the prominent role of mitochondria in the generation of reactive oxygen species (ROS), it has been suggested that mitochondria are the major source of ROS production in cells following arsenic exposure. Based on these observations, we hypothesized that mitochondrial dysfunction following exposure to arsenic may be associated with the generation of ROS. To assess this, we evaluated mitochondrial function in mouse primary hepatocytes and hepatoma cells in response to the non-lethal concentrations of sodium arsenate described above. Mitochondrial function was evaluated using the Seahorse extracellular flux analyzer, which allows for real-time measurements of oxygen consumption and provides an overall assessment of the bioenergetics function of the cell. Arsenic decreased both basal and mitochondrial respiratory rate in a dose dependent manner (Figure 2A, C and D and Figure 3A, B). In addition, we found that arsenic dose dependently decreased coupling efficiency (Figure 2F and 3D) and spare respiratory capacity (Figure 2E and 3C) and increased proton leak (Figure 2G) in both hepatoma cells (Figure 2) and primary hepatocytes (Figure 3) . These results suggest that even at concentrations as low as 2.5 µM, arsenic treatment results in altered mitochondrial function.

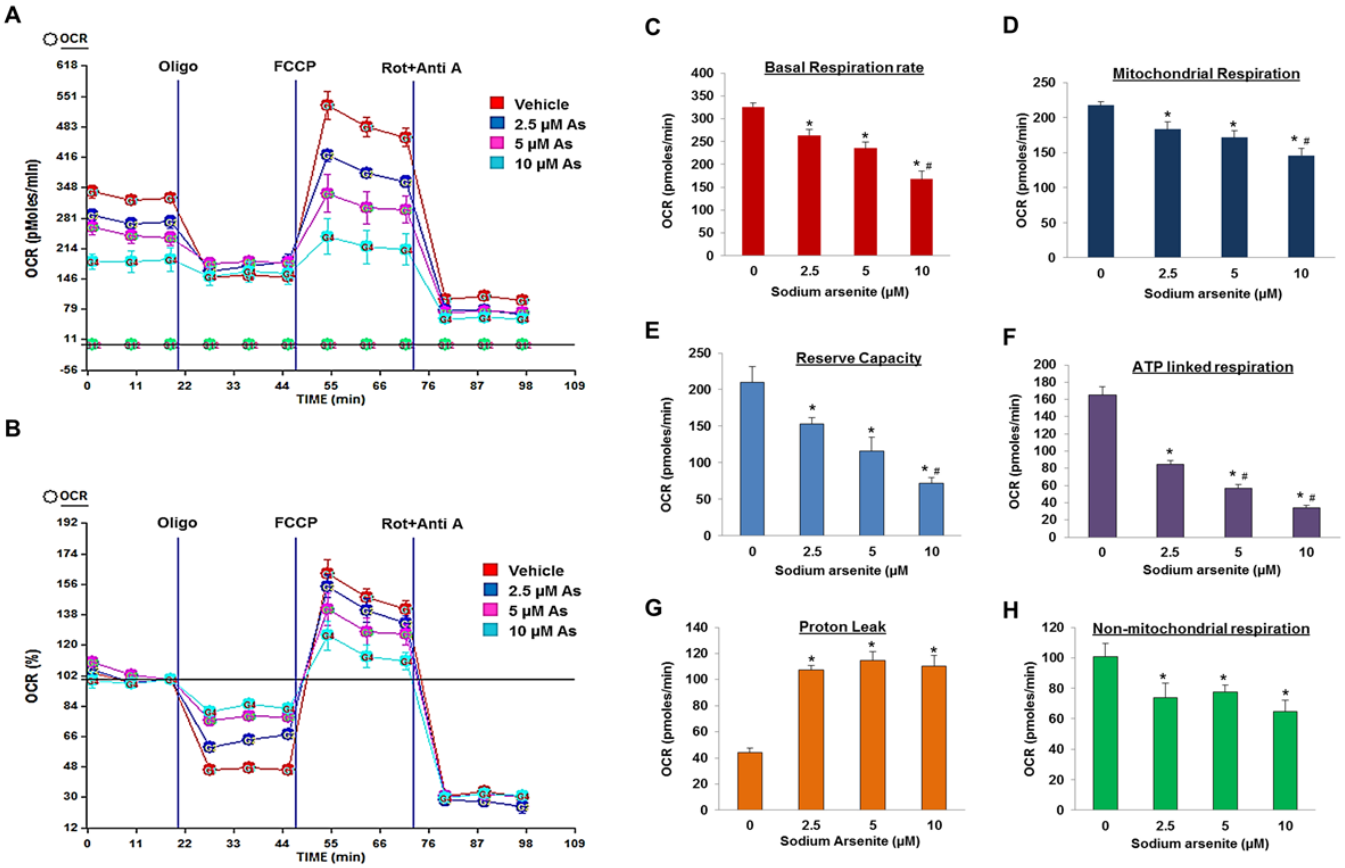


Figure 2. Sodium arsenite decreases mitochondrial coupling and increases proton leak in human hepatoma (HepG2) cells in a dose dependent manner.

OXPHOS was manipulated with injections of oligomycin (1 µM), FCCP (0.5 µM), and rotenone plus antimycin-A (1 µM each). Bioenergetics profile (A) and percent OCR (B) of HepG2 cells treated with 2.5, 5 and 10 µM of sodium arsenite for 16 h. Arsenic treatment altered basal OCR (C), mitochondrial OCR (D), reserve capacity (E), ATP-linked respiration (F), proton leak (G) and non-mitochondrial respiration (H). Reserve capacity was calculated from the difference between the maximal and basal OCR. ATP-linked respiration is the difference between the oligomycin-induced decrease in OCR. Proton leak was the difference between ATP-linked respiration and non-mitochondrial respiration. Non-mitochondrial OCR was determined by injecting rotenone plus antimycin-A simultaneously to fully inhibit the electron transport chain. Results are mean ± SEM. Results are representative of three independent experiments.

n=5 per group. “*” Significantly different from control (vehicle treated), $p < 0.05$. “#” Significantly different from 2.5 µM sodium arsenite treated, $p < 0.05$.

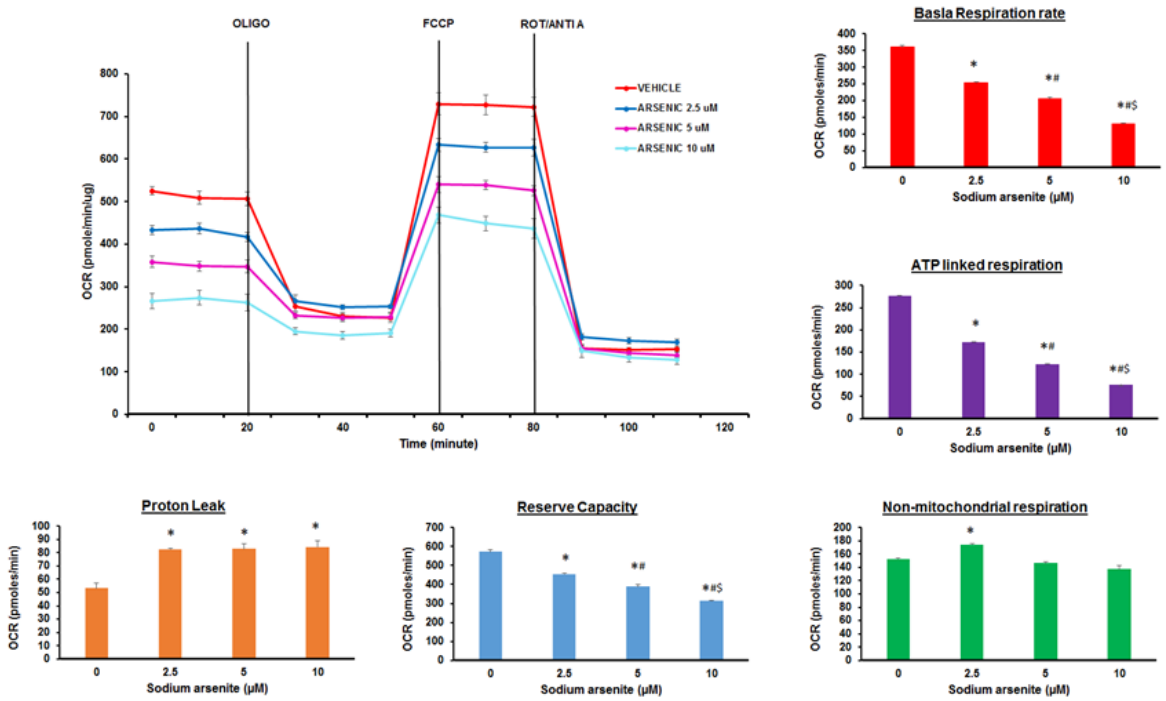


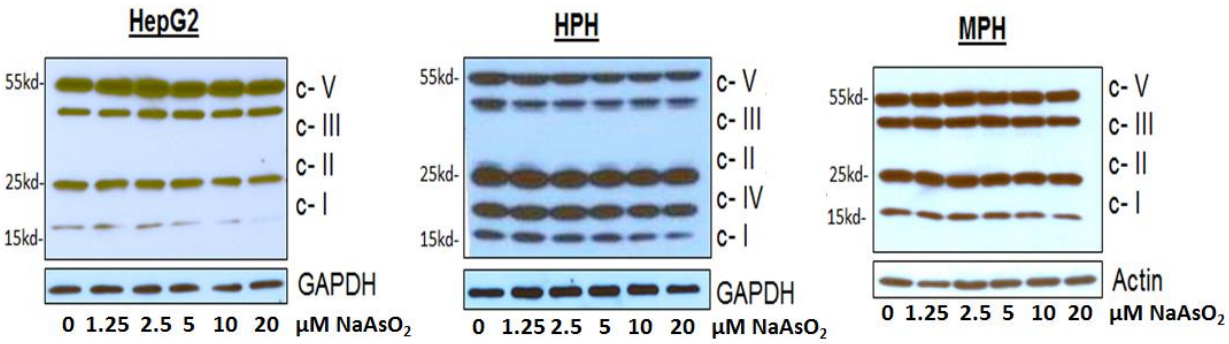
Figure 3. Sodium arsenite decreases mitochondrial coupling and increases proton leak in mouse primary hepatocytes (MPH) in a dose dependent manner.

OXPHOS was manipulated with injections of oligomycin (1 μM), FCCP (0.5 μM), and rotenone plus antimycin-A (1 μM each). MPH cells treated with 2.5, 5 and 10 μM of sodium arsenite for 16 h demonstrated altered basal OCR (B), reserve capacity (C), and ATP-linked respiration (D). Reserve capacity and ATP-linked respiration were calculated as described in Figure 1 for HepG2 cells. Basal respiratory rates decreased with arsenic treatment with statistical significant differences seen only with the initial dose. Results are mean ± SEM. Results are representative of three independent experiments. n=5 per group.

3.3 **EFFECT OF SODIUM ARSENITE ON ELECTRON TRANSPORT CHAIN (ETC) EXPRESSION AND ACTIVITY IN HEPATOCYTES**

Decrease in coupling efficiency and increased proton leak following exposure to sodium arsenite could result either from a decrease in ATP demand, a lack of substrate availability, or damage to the ETC, which would impede the flow of electrons. To explore this further and to understand the mechanism by which arsenic reduces coupling efficiency, we first evaluated the ATP levels in HepG2 cells and in primary hepatocytes treated with arsenic. Arsenic treatment did not significantly affect cellular ATP levels in either HepG2 cell or in primary hepatocytes, suggesting that the decreased coupling efficiency was not due to decreased ATP demand (Figure 5A). We next evaluated the effect of arsenic on the ETC. Alterations to ETC could result either from changes in complex expression and/or activity. Thus, in these studies we assessed both the expression and activity of ETC complexes. At higher concentrations (> 2.5 μM) arsenic decreased complex I expression in a dose dependent manner in all three cell systems (Figure 4A). Arsenic dose dependently inhibited complex I activity in all three cell systems tested (Figures 4B, 4C, and 4D). These results suggest that increased uncoupling in response to arsenic is a result of decreased ETC function. These results are consistent with increased proton leak seen in both hepatoma and mouse primary hepatocytes in response to arsenic.

A


HepG2
55kd-
25kd-
15kd-
c- V
c- III
c- II
c- I
GAPDH
0 1.25 2.5 5 10 20 µM NaAsO2
HPH
55kd-
25kd-
15kd-
c- V
c- III
c- II
c- IV
c- I
GAPDH
0 1.25 2.5 5 10 20 µM NaAsO2
MPH
55kd-
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Actin
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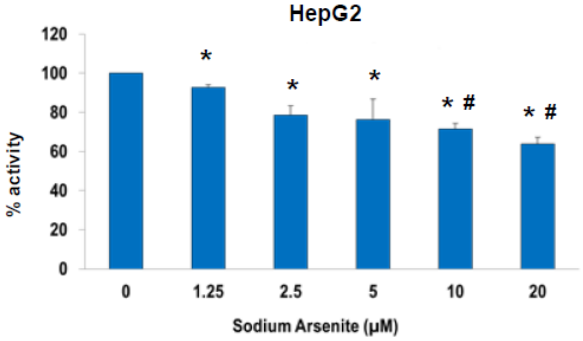
B


HepG2
% activity
120
100
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60
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0
0
1.25
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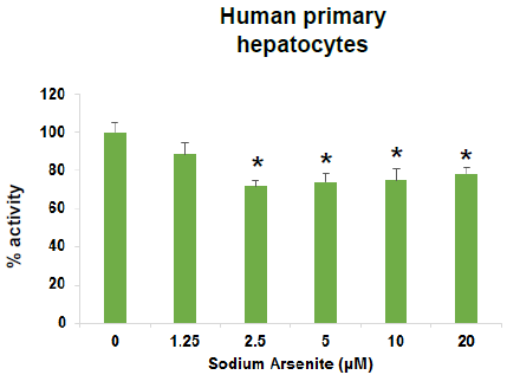
5
10
20
Sodium Arsenite (µM)

C


Human primary hepatocytes
% activity
120
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40
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1.25
2.5
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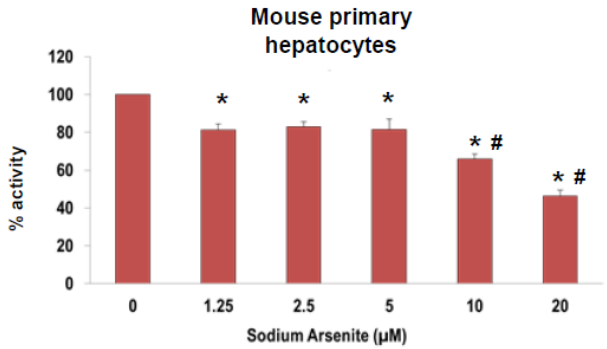
10
20
Sodium Arsenite (µM)

D


Mouse primary hepatocytes
% activity
120
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0
1.25
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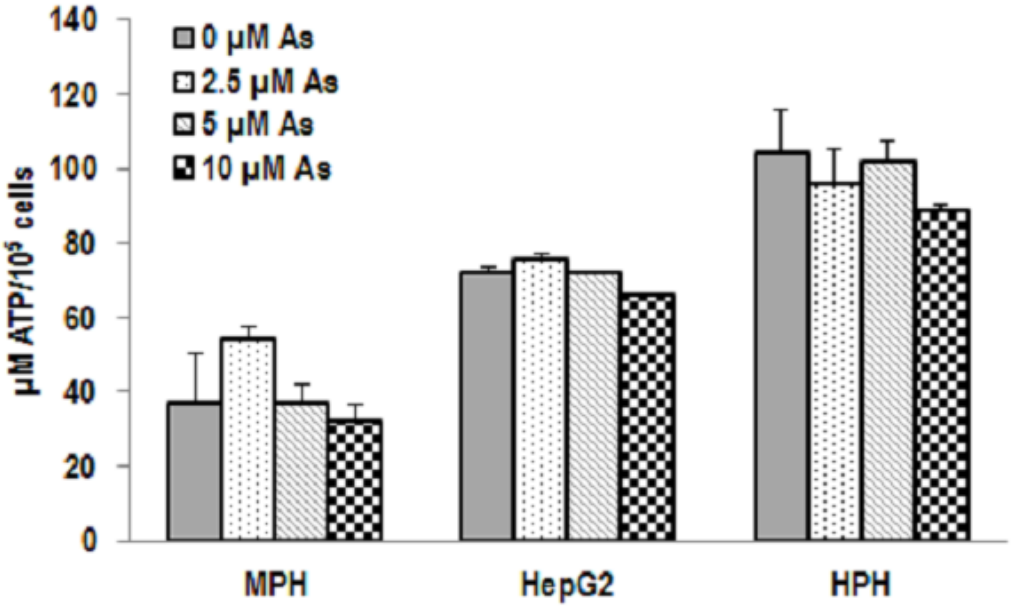
20
Sodium Arsenite (µM)

Figure 4. Sodium arsenite reduces mitochondrial complex expression and activity in a dose dependent manner. (A) Immunoblot analysis of mitochondrial complex subunit proteins in HepG2 cells (left panel), human primary hepatocytes (middle panel, HPH) and mouse primary hepatocytes (right panel, MPH) treated with 1.25, 2.5, 5, 10 and 20 µM of sodium arsenite for 16 h. (B – D) Mitochondrial complex I activity measurement in (B) HepG2 cells (C) human primary hepatocytes and (D) mouse primary hepatocytes treated with increasing concentrations of sodium arsenite for 16 h. Results are mean ± SD. Results are representative of two independent experiments. ;'*' Significantly different from 0 µM arsenite treatment; $P < 0.05$ '#' significantly different from 1.25 µM arsenite treatment; $P < 0.05$

3.4 INCREASED MITOCHONDRIAL OXIDATIVE STRESS AND INCREASED EXPRESSION OF ANTIOXIDANT ENZYMES IN HEPATOCYTES FOLLOWING ARSENIC EXPOSURE.

As mentioned above, exposure to arsenic leads to ROS-mediated oxidative stress. Our results presented in Figure 4 suggest that arsenic exposure also results in mitochondrial dysfunction and increased proton leak. Taken together, these observations suggest that arsenic induced mitochondrial dysfunction and proton leak could play a causative role in the development of arsenic induced oxidative stress. To test this, we evaluated mitochondrial oxidative stress and mitochondrial function simultaneously in cells exposed to arsenic. For these studies we decided to use only HepG2 cells for two reasons 1) the pattern of arsenic induced mitochondrial dysfunction and ETC expression and activity were similar between the cell lines providing an opportunity to test the proof concept of our hypothesis and 2) primary hepatocytes were susceptible to toxicity associated with the triple treatment of mitoSox, arsenic and Hoechst. We observed that at low dose (2.5 μM), arsenic induced detectable mitochondrial oxidative stress as measured by mitoSox fluorescence (Figure 5B). Consistent with these observations, analysis of cellular oxidative stress response evaluated as a measure of antioxidant gene and protein expression demonstrated induction of cellular antioxidant response even at concentrations as low as 1 μM (Figure 6 and Figure 7). Moreover, cellular antioxidant response appeared to directly correlate with mitochondrial dysfunction and mitochondrial oxidative stress. Taken together these results suggest that even at low concentrations arsenic induces mitochondrial dysfunction leading to mitochondrial oxidative stress.

A


0 µM As
2.5 µM As
5 µM As
10 µM As
µM ATP/10^5 cells
140
120
100
80
60
40
20
0
MPH
HepG2
HPH

B

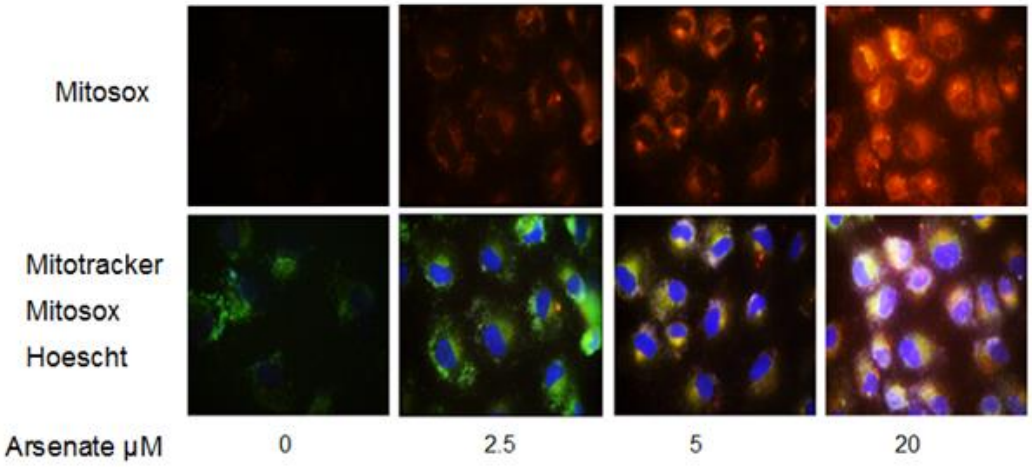
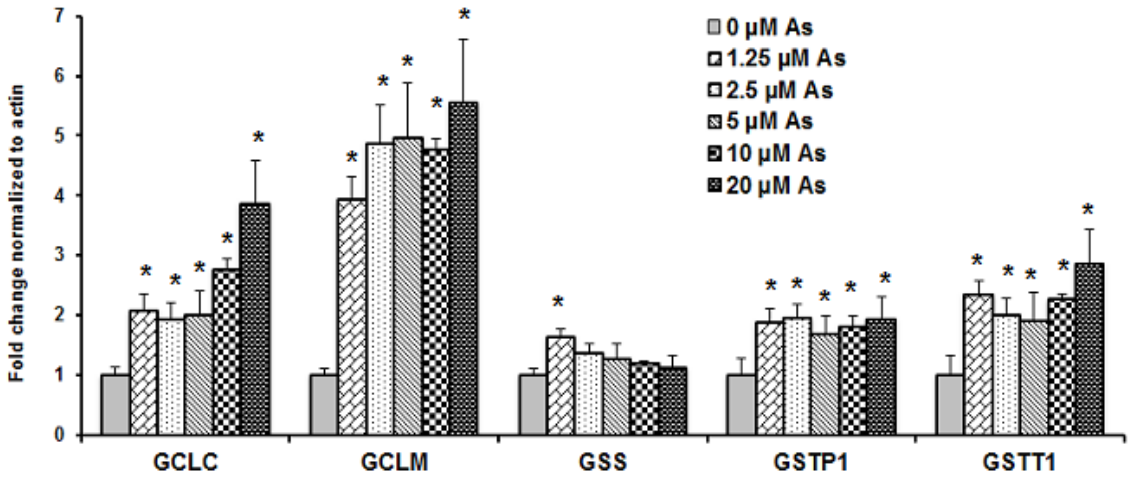

Mitosox
Mitotracker
Mitosox
Hoescht
Arsenate µM
0
2.5
5
20

Figure 5. Sodium arsenate does not affect cellular ATP production but increases mitochondrial oxidative stress in a dose dependent manner. (A) HepG2 cells, human primary hepatocytes (HPH) and mouse primary hepatocytes (MPH) show no change in cellular ATP levels with increasing concentration of arsenite. (B) HepG2 cells and treated with 2.5, 5 and 20 µM of sodium arsenite for 16 h were incubated with MitoTracker® , MitoSOX™ and Hoechst for 20 minutes to label mitochondria (green), mitochondrial superoxide (red) and nuclei (blue) respectively. Results are representative of three independent experiments.

A


Fold change normalized to actin
0 μM As
1.25 μM As
2.5 μM As
5 μM As
10 μM As
20 μM As
GCLC
GCLM
GSS
GSTP1
GSTT1

B

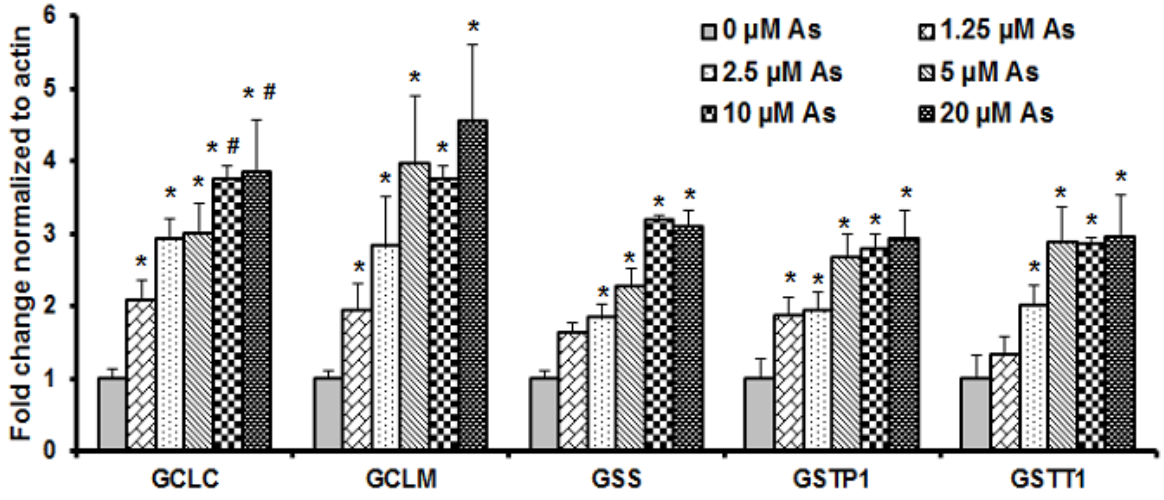
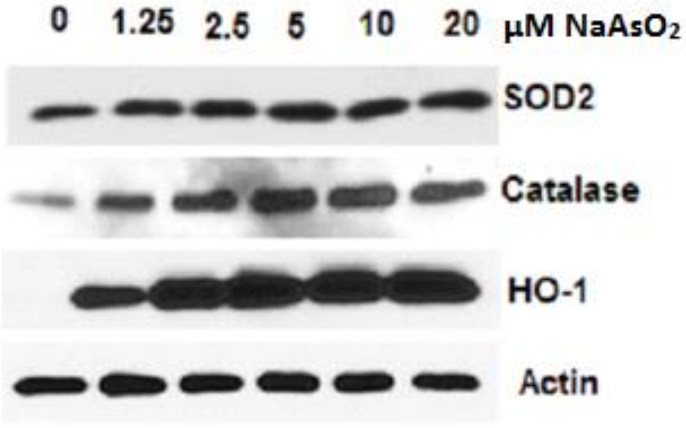

Fold change normalized to actin
0 μM As
1.25 μM As
2.5 μM As
5 μM As
10 μM As
20 μM As
GCLC
GCLM
GSS
GSTP1
GSTT1

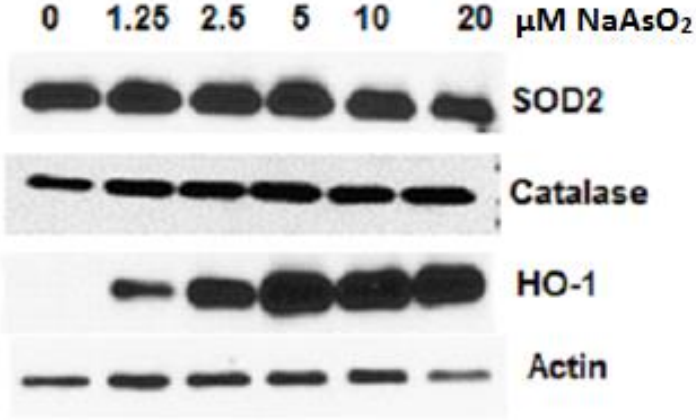
Figure 6. Sodium arsenite induces antioxidant gene expression in a dose dependent manner.

Real-time PCR analysis of antioxidant gene expression in (A) HepG2 and (B) human hepatocytes exposed to increasing concentration of sodium arsenite. Results are representative of three independent experiments. '*' Significantly different from 0 μM arsenite treated samples; $P < 0.05$; '$^{\#}$' significantly different from 1.25 μM arsenite treated samples: $P < 0.05$. GCLC, Glutamate-cysteine ligase, catalytic subunit, GCLM Glutamte-cysteine ligase, regulatory subunit, GSS, Glutathione sythetase, GSTP1, Glutathione-S-transferase pi 1, GSTT1, Glutathione-S-transferase theta 1.

A


0 1.25 2.5 5 10 20 μM NaAsO2
SOD2
Catalase
HO-1
Actin

B


0 1.25 2.5 5 10 20 μM NaAsO2
SOD2
Catalase
HO-1
Actin

C

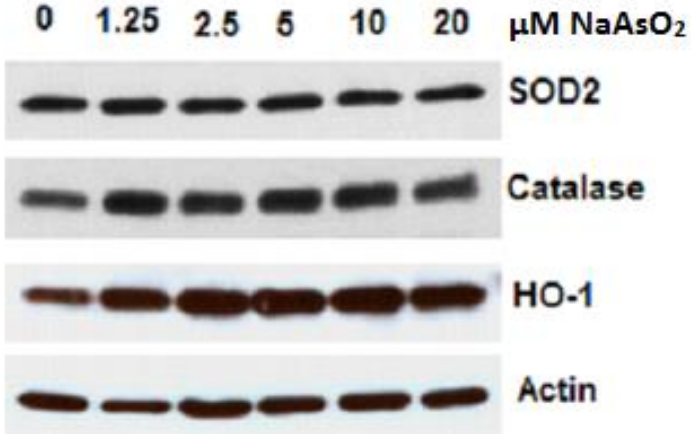
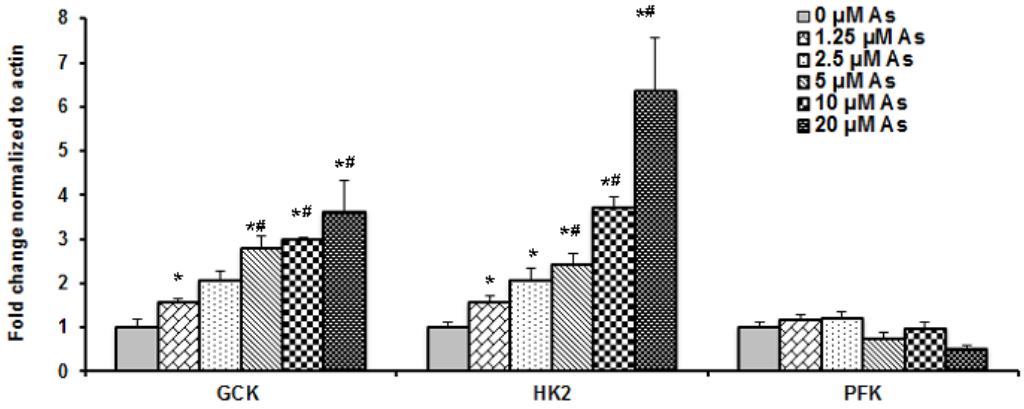

0 1.25 2.5 5 10 20 μM NaAsO2
SOD2
Catalase
HO-1
Actin

Figure 7. Sodium arsenite induces antioxidant protein expression in HepG2 cells and human and mouse primary hepatocytes. Antioxidant protein expression in (A) HepG2 (B) human primary hepatocytes and (C) mouse primary hepatocytes treated with increasing concentrations of arsenite. Antioxidant protein expression was measured in 15 µg of total lysate using protein specific antibodies. Actin is used as loading and negative control in these experiments. Heme-oxygenase 1 (HO-1) is used as a positive control. Results are representative of three independent experiments. SOD2, Superoxide Dismutase 2.

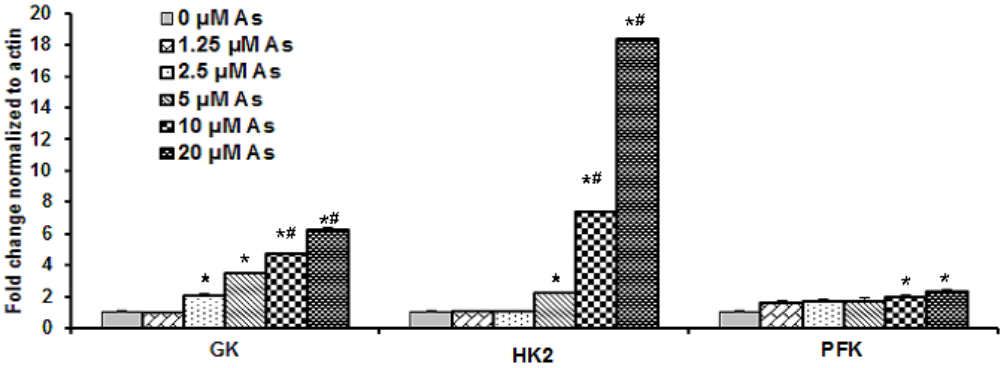
3.5 Increased activation of glycolysis pathway in hepatocytes in response to arsenic treatment.

Decreased mitochondrial function in response to arsenic in theory should result in decreased cellular ATP levels. However, in our studies (Figure 5A) we found no significant difference in ATP levels between arsenic treated and untreated cells. These results prompted us to test whether cells reprogram their metabolism more towards glycolysis in response to arsenic to compensate for the decreased mitochondrial function. To test this, we measured mRNA levels of glycolytic enzymes in response to arsenic. In both HepG2 hepatoma cells and the human and mouse primary hepatocytes, arsenic treatment resulted in increased expression of Glucokinase (GK), Hexokinase (HK) and Phosphofructokinase (PFK), the three key enzymes involved in regulating glycolysis (Figure 8).

A


Fold change normalized to actin
0 μM As
1.25 μM As
2.5 μM As
5 μM As
10 μM As
20 μM As
GCK
HK2
PFK

B


Fold change normalized to actin
0 μM As
1.25 μM As
2.5 μM As
5 μM As
10 μM As
20 μM As
GK
HK2
PFK

C

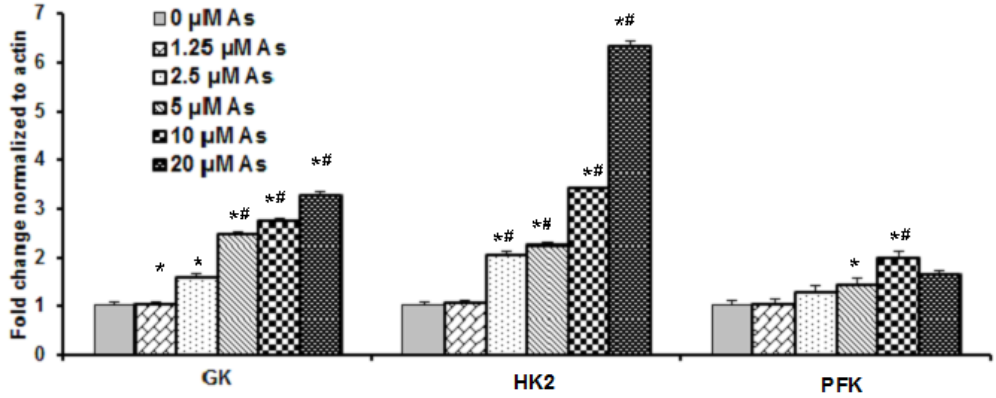

Fold change normalized to actin
0 μM As
1.25 μM As
2.5 μM As
5 μM As
10 μM As
20 μM As
GK
HK2
PFK

Figure 8. Sodium arsenite reprograms mitochondrial metabolism from oxidative phosphorylation to glycolysis. Real-time PCR analysis of glucokinase (GK), Hexokinase (HK) and Phosphofructokinase (PFG) in (A) HepG2 (B) human primary hepatocytes and (C) mouse primary hepatocytes exposed to increasing concentration of arsenite. '*' Significantly different from 0 µM arsenite treatment; $P < 0.05$; '$^{\#}$' Significantly different from 1.25 µM arsenite treatment; $P < 0.05$. Results representative of three independent experiments.

Chapter 4: **DISCUSSION AND FUTURE DIRECTIONS**

4.1 CONLCUSIONS

Arsenic is a major health concern globally, with over 100 million people exposed to unhealthy levels of inorganic arsenic through contaminated drinking water. Extensive studies have been made in understanding the mechanisms of arsenic carcinogenesis with the goal of designing prevention and intervention strategies and in assigning appropriate levels of risk to exposure. Risk assessment has been difficult, as many epidemiological studies suggest that populations can be affected by arsenic-associated pathologies sometimes at levels below the current WHO recommendation. Much progress has been made in the way of equipment capable of removing detectable levels of arsenic and other heavy metals from drinking water. However, financial, material, and logistical constraints stand in the way of proper water purifying equipment becoming ubiquitous in areas with high levels of arsenic. Given these issues, it is equally important to understand the mechanisms of arsenic toxicity so that prevention and treatment of diseases from arsenic exposure have more successful outcomes.

The difficulty in understanding the pathogenesis of these diseases lies in the myriad of cellular processes that are altered or disrupted by arsenic. Disruption of signaling pathways, cellular proliferation, DNA repair and methylation, interaction with thiol and phosphate groups, and generation of ROS are all influenced by arsenic species (13). This makes it difficult to elucidate which disrupted process, or processes, result in which diseases. While genotoxic effects may be clearly implicated in carcinogenesis, production of ROS is a common factor in most, if not all diseases associated with

arsenic. Therefore further studies of the sources of ROS and how they are produced is a worthwhile effort in investigating mechanisms of arsenic toxicity.

Our studies suggest that mitochondria are the primary source of ROS. Sodium arsenite treatment dose-dependently increased mitochondrial superoxide, and consistent with this, arsenic exposure resulted in altered mitochondrial bioenergetics including decreased respiratory reserve capacity and increased uncoupled respiration leading to increased proton leak. We also confirmed previous reports that complex I of the electron transport chain is inhibited with increasing concentrations of arsenic, suggesting an additional avenue of mitochondrial dysfunction (39). To cope with these stressors, we see an induction in catalase at the protein level which shows that at non-toxic levels, cells cope with arsenic through protective mechanisms against oxidative stress (28). It has previously been shown that pre-treatment with catalase alleviates arsenic-induced ROS in human bladder cells, and that inhibition of catalase increases micronuclei from arsenite (43).

Reports indicate that glycolytic rates are increased and key regulatory enzymes are induced in human cells exposed to arsenic (44). In addition to mitochondrial dysfunction, we also found that mRNA of key glycolytic regulatory enzymes GCK and HK-2 is induced. This suggests that cells attempt to cope with energy demands by inducing glycolysis. Further studies to explore how glycolytic rates and regulation change with arsenic are another potential avenue of investigation.

An interesting and well-studied link between glycolysis and oxidative phosphorylation is the effect of arsenic on pyruvate dehydrogenase. Arsenic is known to allosterically

inhibits this enzyme (21,25,45). We showed that oxidative phosphorylation is also decreased by arsenic. If mitochondria are unable to keep up with energy demands and the entry point of pyruvate into the TCA cycle is blocked, this could explain the induction of glycolytic enzymes to keep up with the need for ATP. Disruptions in metabolism are well-established in the pathogenesis of metabolic diseases such as diabetes mellitus, and may provide insights into future therapies.

Studying the mechanisms of toxicity by inorganic arsenic species is a complex task, as arsenic interacts with a large number of targets to disrupt a large number of cellular processes. A common factor whether studying arsenic's chronic carcinogenic or non-carcinogenic effects is the production of ROS. ROS itself reacts with multiple targets, giving it a prime role in genotoxic effects, protein and enzyme inhibition, and lipid peroxidation. Understanding where arsenic is producing this ROS is therefore an important area of investigation. Previous studies combined with our own find that arsenic affects mitochondrial function. When mitochondrial function was measured, we found that basal respiration, ATP linked respiration, reserve capacity, and non-mitochondrial respiration were all decreased significantly. Concurrently, proton leak increased. When mitochondrial complex function was measured, complex I was inhibited. Complex I inhibition is associated with the generation of ROS (37,38). Co-staining mitochondrial superoxide and mitochondria showed that a large portion of the superoxide staining overlapped with mitochondria. These data suggest that superoxide production is sourced from mitochondria, and ROS causes further damage to mitochondria to inhibit their function. In turn, these malfunctioning mitochondria can

produce more ROS causing a loop of increasing ROS and decreasing oxidative phosphorylation.

4.2 FUTURE DIRECTIONS

From these results, there are more studies to be conducted. In future studies we would seek to confirm that superoxide is primarily sourced from mitochondria. The hepatoma cell line HepG2 stained clearly with mitoSOX® and mitotracker fluorescent dye, but was difficult to visualize in isolated human and mouse hepatocytes. A genetic approach to fluorescently label mitochondria would eliminate some of the cytotoxic effects from using these dyes.

Our experiments were conducted *in vitro*, with either cancer cell lines (HepG2 and Hep3B) or isolated human and mouse hepatocytes. Conducting our studies of mitochondria function and superoxide localization in an animal model is crucial to moving to a full understanding of arsenic toxicity in humans. A mouse model would also provide a more accurate representation of chronic exposure to arsenic in humans. In addition, mitochondrial function studies in cell lines from other tissues prone to arsenic-induced carcinogenesis (skin, lung, and bladder) would provide strong evidence for dysfunctional mitochondria as the source for ROS in arsenic toxicity. These experiments in cell lines would also provide a strong rationale for examining these tissues in a rodent model.

We intend to further this study in the near future by examination of DNA damage in hepatocytes after arsenic treatment. Both nuclear and mitochondrial DNA are prone to damage by ROS, although to varying degrees. If ROS is primarily sourced from mitochondria, one could hypothesize that mitochondrial DNA would initially sustain more damage than nuclear DNA. Directly measuring DNA integrity and adduct formation

would be telling as to which cellular compartment is exposed to higher levels of ROS. An indirect but important marker is measuring expression and induction of DNA repair enzymes. These experiments would build on our study of mitochondrial dysfunction as a source of ROS production.

An area we did not explore in these studies was the effect of cellular antioxidant systems in the prevention and resolution of damage from arsenic. A future direction of our lab is to test if adding exogenous antioxidants or inducing antioxidant enzymes would prevent damage or restore function to mitochondria. Similarly we could see if inhibiting antioxidants would exacerbate superoxide levels and mitochondria function. These studies have been conducted by others to measure protein and DNA damage, but not mitochondrial function.

In areas where high arsenic levels in water are endemic, it is crucial to not only develop ways to purify water supplies but to understand the ways by which arsenic exerts its toxic effects. By further characterizing the mechanisms of arsenic toxicity we can provide the basis for prevention and treatment of diseases and symptoms caused by arsenic.

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