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Arsenic Toxicity and Mitochondrial Metabolism in Astrocytes

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ARSENIC TOXICITY AND MITOCHONDRIAL METABOLISM IN ASTROCYTES

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Abstract

The various cells that support neurons in both the central and peripheral nervous systems are known collectively as neuroglia, or glia. Astrocytes, a type of glial cell found exclusively in the central nervous system (i.e. the brain and spinal cord), are particularly abundant and serve many functions. One of the most important is the role these cells play in the brain as a defense against harmful substances, many of which can be found in the environment and make their way into the human body as xenobiotic substances. Elemental arsenic in its inorganic form occurs naturally throughout the Earth's crust, but poses a major health risk worldwide when it is found at elevated levels particularly in groundwater used as a source for domestic or agricultural use. Exposure to arsenic has been shown to cause a number of adverse health effects, from cardiovascular and liver damage to neurological dysfunction, though much less is known about its effects in the brain. Astrocytes are likely to play a protective role in the form of a barrier capable of detoxifying inorganic arsenic molecules carried to the brain via the circulatory system. However, the mechanism(s) by which the process of detoxification occurs has yet to be fully elucidated. Using primary astrocyte cultures, the present study focused on the role of glutathione, glucose metabolism, and mitochondria in minimizing the adverse neurological effects of xenobiotic arsenic in astrocytes. Results show that the introduction of arsenic compounds to cultures of astrocytes stimulates changes in intracellular glucose metabolism, altering flux of glucose-derived carbons through the glycolytic and oxidative pathways. The diversion has been indicated to result in an increased anaplerotic synthesis of glutathione, an important intracellular tripeptide for xenobiotic defense. In addition, results suggest that mitochondria rely on the input of acetyl-CoA, which, surprisingly, alludes to the importance of fatty acid metabolism

as the alternate carbon source for acetyl-CoA in the mitochondrial contribution to detoxification of arsenic via glutathione synthesis.

Key words: Astrocytes, glutathione, arsenic toxicity, Veterinary Science, School of Veterinary Medicine and Biological Sciences

Introduction

Astrocytes as primary defense against xenobiotics

Glial astrocytes in the central nervous system (CNS) play a crucial role, performing a variety of functions in support of neurons. Astrocytes help to facilitate a stable intracellular and extracellular environment by maintaining redox status via glutathione (GSH) synthesis, balance energy metabolism via glycolytic and mitochondrial activity, and function in neuronal signaling pathways via production, release, and reuptake of neurotransmitter molecules (1). Ultimately, these dynamic cells constitute the primary defense against xenobiotic threat in the form of a protective layer of cells known as the blood-brain barrier, which serves to screen the blood before it reaches highly sensitive neurons. Cellular processes extending from the cell bodies of astrocytes wrap around capillaries and arterioles transporting blood carrying nutrients, drugs, and potentially harmful xenobiotic substances from the circulatory system into the CNS. These processes form a virtually continuous layer between the bloodstream and the brain that allows astrocytes, functioning as intermediaries, to take up and metabolize blood contents (5). In the case of harmful xenobiotics, this action helps to reduce the negative impact on neural function through metabolic detoxification.

Astrocytes and glutathione

GSH, a tripeptide ubiquitous in the body's cells, is most commonly identified in an antioxidant capacity as an important reducing agent under conditions of oxidative stress. It undergoes an oxidation-reduction cycle, in which GSH is oxidized to glutathione disulfide (GSSG). In astrocytes, it is also exported via multidrug resistance-associated protein 1 (MRP1) for subsequent cleavage and uptake by neurons as cysteine, a precursor which is then resynthesized to glutathione. Its role in xenobiotic detoxification in astrocytes, however, is

less understood. It has been shown in previous studies that biotransformation occurs via glutathione-S-transferase enzymes to convert arsenic taken up by astrocytes into various metabolites (3). Another mechanism that has been suggested is the formation of complexes with inorganic arsenic, thus rendering them less toxic, though this ability as well as the ability to then export the complexed GS-As adducts via MRP1 have yet to be confirmed in astrocytes (3).

Glucose metabolism in astrocytes

Most cells in the body, especially in the brain, rely on glucose metabolism for energy production and survival. Astrocytes, too, rely on these pathways; this is due to the fact that glucose readily crosses the blood-brain barrier, and is normally present in stable concentrations in circulation (4). The major pathways of glucose metabolism are glycolysis and oxidative phosphorylation. In astrocytes these pathways have been shown to be compartmentalized in distinct regions in the cell: oxidative metabolism, carried out by mitochondria, takes place in the cell body, while glycolysis is carried out in the cellular processes due to the inability to house mitochondria, which are approximately twice the diameter of the processes themselves (4). Oxidative phosphorylation is the primary source of adenosine triphosphate (ATP), the main energy molecule of cellular metabolism, with a much larger net yield (32 ATP) compared to glycolysis (2 ATP), yet the cell bodies of astrocytes comprise only 2% of their volume (4). In conditions of xenobiotic stress, astrocytes increase glucose flux through both of these pathways (4), though to what extent and for what reason each is involved in the process of detoxifying arsenic is has not been extensively shown in astrocytes.

Arsenic

Arsenic poses a pervasive health risk around the world, contributing to a number of high-priority and potentially fatal diseases, including cardiovascular disease and cancer, and has more recently been implicated as a risk factor of neurological disorders. It is found throughout the Earth's crust primarily as a natural geologic component, as well as as a result of anthropogenic activity. Most significantly, however, arsenic is found in groundwater, with concentrations at their highest and most catastrophic levels particularly in the regions of Southeast Asia, India, and China (5). In the US, groundwater contamination from arsenic is particularly prominent in the west and central regions, where it can be detected at levels meeting or exceeding the maximal safe permissible level of 10 ug/L established by the World Health Organization in 2011(6). The most common forms of inorganic arsenic detected in groundwater are the pentavalent [arsenate, As(V)] and in reduced environments, trivalent [arsenite, As(III)] species (7). These species are represented by sodium arsenate ($NaAsO_2$) and arsenic trioxide (As_2O_3), respectively.

Inorganic arsenic compounds are toxic to humans and animals due to their ability to disrupt the normal function of metabolic processes and a great number of enzymes (8). Historically, anthropogenic use of arsenic compounds has ranged from cosmetics to the production of chemical pesticides and herbicides. Exposure through these sources can result in acute toxicity, though in coming to understand the many adverse health effects of arsenic its use in such activities has largely been discontinued, whereas chronic exposure to arsenic as a result of groundwater contamination continues to pose substantial risk (8). Adverse effects of chronic arsenic exposure include increased risk of multiple types of cancer, diabetes, and blackfoot disease. Neurological conditions include impaired cognitive

development and neuropathy (8), though the etiology on a cellular level are as yet largely unexplored.

Prior Research in the Franco Lab

The direction of the current study was guided in part by findings previously achieved in the Franco lab, which served as the basis for investigation of the role of mitochondrial glucose metabolism and GSH in detoxification of harmful xenobiotics in astrocytes.

A. Increase in glutathione prior to cell death

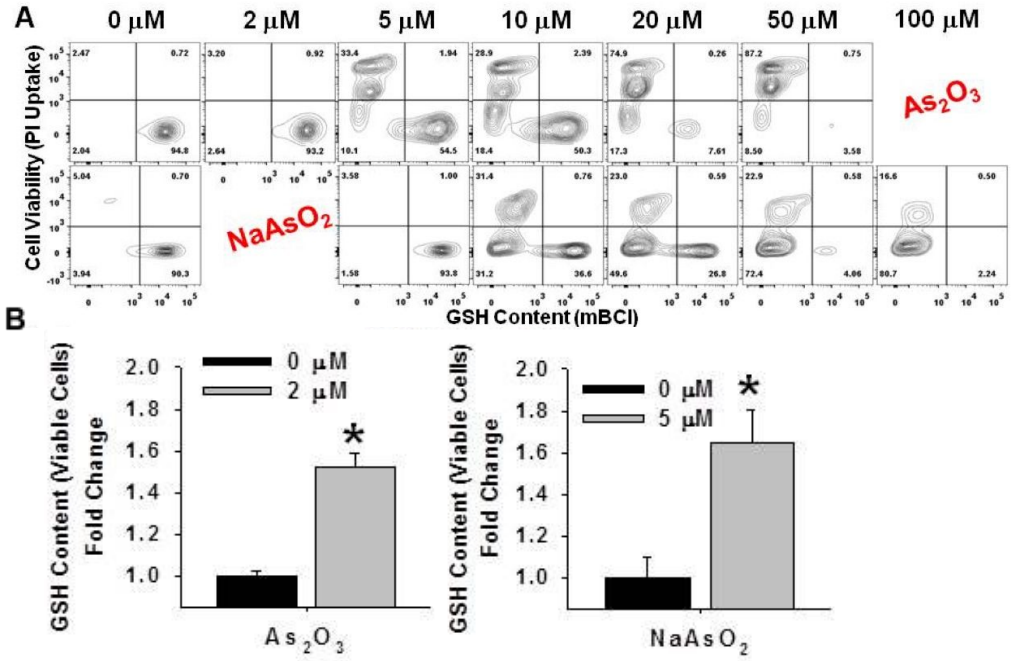


Figure 1. Increase in intracellular glutathione content in astrocytes prior to cell death. Cells treated with $NaAsO_2$ and As_2O_3 at 0, 2, 5, 10, 20, 50, and 100 µM for 48h. Cells were harvested and analyzed as indicated in Methods and Materials. Astrocytes exhibit increased GSH content at sub-lethal levels of arsenic, shown as a result of flow cytometry (A) and in statistical analysis of these sub-lethal concentrations (B). * $p < 0.05$

Astrocytes were treated with increasing concentrations of $NaAsO_2$ and As_2O_3 in order to investigate conditions of arsenic exposure that lead to an increase in intracellular GSH content. It was found that at sub-lethal concentrations of each of the arsenic treatments (2 µM for As_2O_3 and 5 µM for $NaAsO_2$; Figures 1A and B), GSH content increased approximately

1.6-fold. At greater concentrations, cell membranes were compromised and levels of cell viability decreased. This demonstrated that just prior to cell death, intracellular GSH production was likely being upregulated.

B. Arsenic treatment results in a reduced intracellular environment

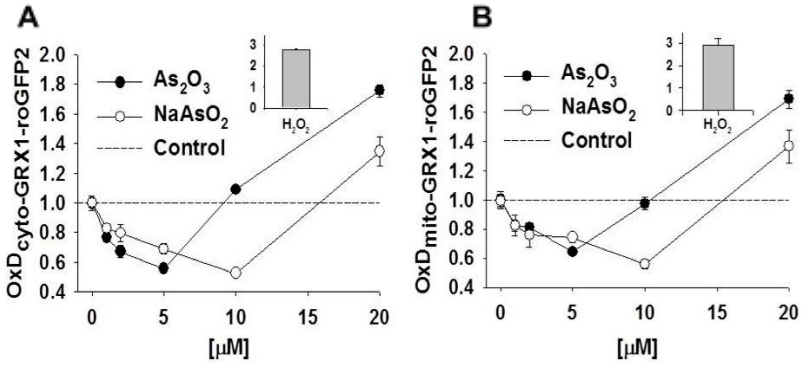
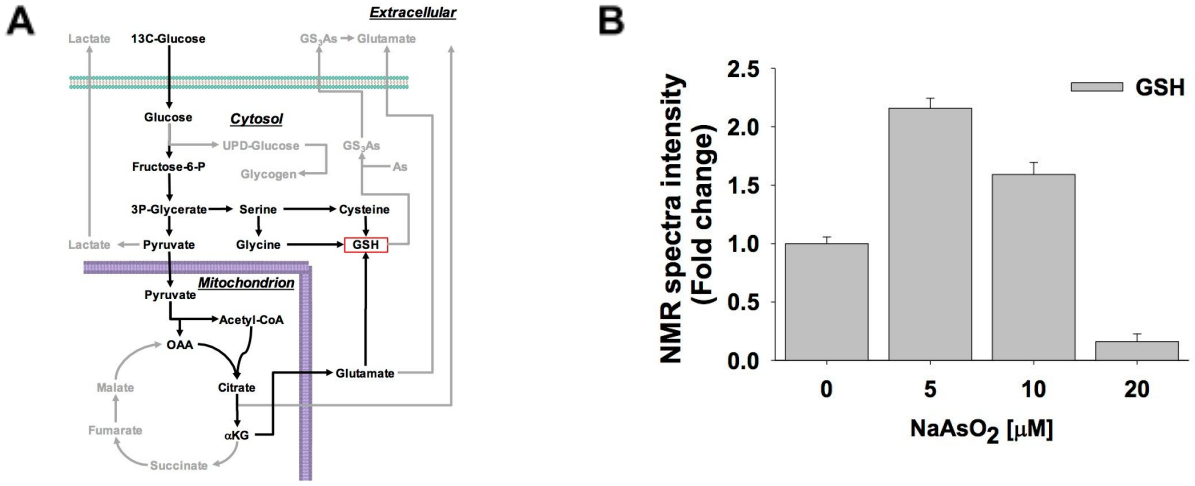


Figure 2. Treatment with inorganic arsenic compounds causes a reduced intracellular environment in astrocytes. Cells were treated with concentrations of 0, 5, 10, 15, and 20 μM for 48h and analyzed as described previously (10). At concentrations of 5 μM for As_2O_3 and 10 μM for $NaAsO_2$, the intracellular redox status showed a reduced environment (A and B).

Upon treatment with increasing concentrations of As_2O_3 and $NaAsO_2$, analysis of oxidation-reduction (redox) status showed the resulting intracellular environments in both the cytoplasm and mitochondria (Figures 2A and B, respectively) to be reduced, again reaching maxima at similar respective sub-lethal concentrations. This indicates the presence of a higher concentration of the reduced form of glutathione, GSH, versus to its oxidized form, GSSG, and suggests the role of GSH in detoxification to involve a metabolic pathway other than redox cycling as this would instead deplete GSH content as it is converted to GSSG.

C. Glutathione synthesis via cytosolic and mitochondrial glucose metabolism



Rose, J., et al. (9)

Figure 3. Glutathione is synthesized via both glycolytic and oxidative metabolism. Cells were incubated in media containing labeled 13C-glucose. After 48h, cells were harvested and analyzed using NMR spectroscopy. Glycine and cysteine from glycolysis and glutamate from oxidative phosphorylation were utilized in the synthesis of GSH. (A) Following treatment with arsenic, separate cell cultures exhibited increased intracellular GSH content (B). * $p < 0.05$

Metabolomic analysis by nuclear magnetic resonance (NMR) spectroscopy allowed investigators to track the pathways into which extracellular glucose was incorporated in astrocytes (Figure 3A). Cells were treated with labeled 13C-glucose in culture media, which was then taken up and metabolized, where metabolic substrates and products containing the labeled isotope could be identified. The results of the NMR analysis revealed that astrocytes rely both on glycolysis in the cytoplasm and oxidative phosphorylation in the mitochondria to synthesize glutathione. It was shown that cataplurotic glutamate exported from mitochondria was combined with glycine and cysteine (from glycolysis), highlighting the importance of mitochondrial activity in the biosynthesis of GSH from glucose. NMR analysis also indicated the same increase in GSH content following sub-lethal treatment at 5 μM $NaAsO_2$ that signals a potential upregulation of GSH synthesis as astrocytes undergo xenobiotic threat (Figure 3B).

Hypothesis

In light of these previous results, the need for further study into the role of GSH synthesis via mitochondrial glucose metabolism, as well as the mechanism and significance of the role of GSH in the detoxification of xenobiotics, is addressed in the present study. It is hypothesized that i) the upregulation of glutathione in the intracellular environment is the result of increased biosynthesis, involving increased mitochondrial activity, and ii) that the resulting GSH is then directly complexed with the toxic xenobiotic compound for export from the cell. Furthermore, in the previous findings it was observed that both arsenic compounds (i.e. As_2O_3 and $NaAsO_2$) generated similar toxic effects, though higher concentrations of $NaAsO_2$ were required to achieve the same response (Figures 1A and B, 2A and B). Due to ease of acquisition and handling safety it was elected to perform further experiments using $NaAsO_2$ only, adjusting concentrations accordingly.

Methods and Materials

Astrocyte cell culture

Primary rat astrocytes were cultured in Dulbecco's Modified Eagle's Medium (DMEM)/Ham's F-12 Nutrient Mixture (GIBCO or Hyclone) supplemented with 10% fetal bovine serum (FBS, Atlanta Biologicals), 2 mM L-glutamine (Hyclone), 1% streptomycin/penicillin (Hyclone) at 37°C under 5% atmospheric CO_2. Cells were cultured in 12-well culture plates for 48 hours, at the end of which they reached monolayer confluency, verified by microscope. Cells were treated as indicated in the figures. Control conditions included the appropriate vehicle. Treatments started with fresh media.

Cell viability determination

Loss of cell viability was determined using flow cytometry (FACS, Fluorescence Activated Cell Sorting) by measuring propidium iodide (PI, 1 µg/ml, Life Technologies or Sigma-Aldrich) and monochlorobimane (mBCl, M) as markers for plasma membrane integrity loss. PI was detected using BluFl3 or BluFl5 and mBCL was detected using VioFl2 in a BDFACSort (Cytek-DxP-10 upgrade). Data were analyzed using FlowJo 7.6.5 software. Total cell count set to 10,000. At least 4 experimental replicates were performed.

Statistical analysis

Collected data were analyzed according to statistical criteria by using paired or unpaired t-test, two-way ANOVA, and the appropriate parametric or non-parametric normality post-test using SIGMA-PLOT/STAT package. A probability value of $p<0.05$ was considered as statistically significant. Data were plotted as mean values of at least 4 experimental replicates ± standard error of the mean (SE) using the same statistical package for data analysis. Flow cytometry graphs presented are representative of at least 4 experimental replicates.

Results and Discussion

A. Blocking glutathione synthesis potentiates cell death

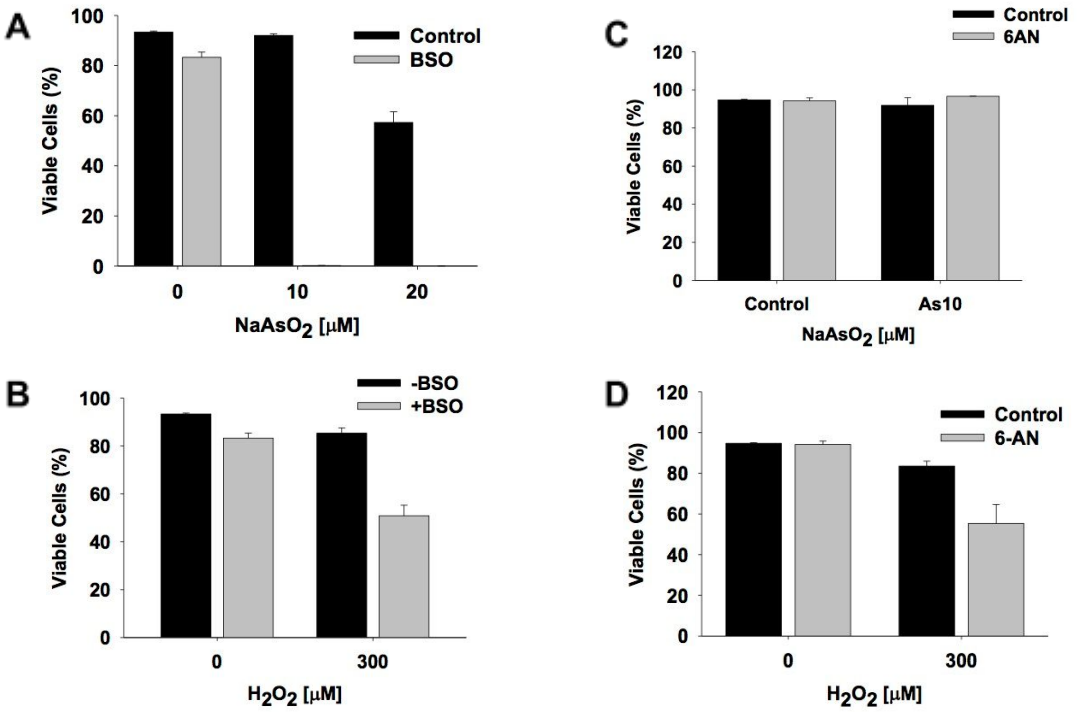


Figure 4. Inhibition of GSH synthesis results in cell death. Astrocytes treated as indicated in figures for 48h. Cells experience complete loss in viability after treatment with 500 μM BSO and 10 μM $NaAsO_2$ (A) compared to modest decrease in viability with 300 μM H_2O_2. Cells treated with 6AN were not affected in the presence of $NaAsO_2$ (C) compared to decreased viability with 300 μM H_2O_2. * $p < 0.05$

In order to investigate whether it is the biosynthesis of glutathione, rather than redox cycling, that is most significantly responsible in the detoxification of arsenic, primary astrocyte cultures were treated with 0 μM, 10 μM, and 20 μM of $NaAsO_2$ and separately with 0 μM and 300 μM hydrogen peroxide (H_2O_2, an agent of oxidative stress that induces redox cycling), both with or without buthionine sulfoximine (BSO, 500 μM), an inhibitor of glutathione synthesis (Figures 4A and B; see Figure 10, Appendix for inhibitor action diagram and list of compounds used) . Cultures were collected and analyzed as indicated in Methods and Materials. Treatment with arsenic in the presence of BSO resulted in a complete loss of cell viability, compared to the reduction in viability following treatment with H_2O_2. Therefore, while oxidative stress may have a moderate effect when cells are treated with a

known oxidizer, the comparatively greater necessity of generating GSH for metabolic response when astrocytes are treated with arsenic is clear.

In a second set of conditions to establish the priority of GSH synthesis as opposed to redox cycling, astrocytes were treated with 0 μM and 10 μM $NaAsO_2$ and separately with 0 μM and 300 μM H_2O_2 with or without 6-aminonicotinamide (6AN, an inhibitor of the pentose phosphate pathway, PPP; see Figure 10, Appendix) (Figures 4C and D). Again, a similar comparison can be made. The PPP is in part responsible for replenishing nicotinamide adenine dinucleotide phosphate (NADPH), and when this pathway is inhibited, the ability of the cell to recycle oxidized glutathione, GSSG, back to its reduced state is impaired. Cell viability was not impacted under conditions of arsenic treatment, indicating that response depends little on the redox status of the cell but instead on an alternative metabolic pathway. These results suggest that indeed astrocytes rely on glutathione synthesis as opposed to recycling as the primary response to arsenic toxicity.

B. Glutathione conjugation and export as a complex with arsenic

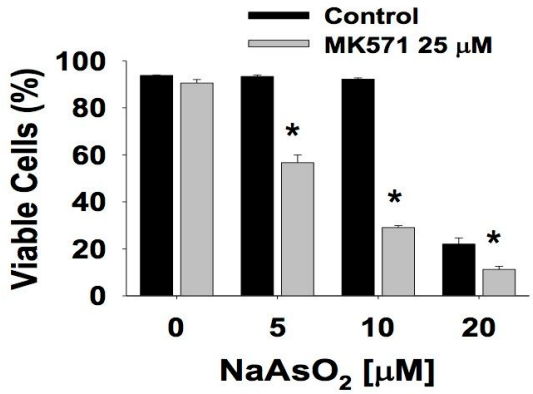


Figure 5. GSH forms a complex with arsenic compounds before export from the cell. Cells were treated with 0, 5, 10, and 20 μM $NaAsO_2$ and 25 μM MK571 for 48h. As treatment concentrations increased a concentration-dependent decrease in viability was observed. * $p < 0.05$

To examine the hypothesis of complex formation between arsenic and GSH for export from the cell, astrocytes were treated with concentrations of 0 μM, 5 μM, 10 μM, and 20 μM

of $NaAsO_2$ with or without 25 μM MK571, an inhibitor of the MRP1 transport protein that is responsible for the export of GSH compounds from the cell (Figure 5). Following incubation and analysis (see Methods and Materials), results show a concentration-dependent decrease in cell viability compared to control conditions. This supports the hypothesis that GSH does form adducts with xenobiotic arsenic for export from the cell, which, when this action is inhibited, would cause an accumulation in the intracellular environment, decreasing cell survival.

C. Mitochondrial glucose metabolism plays an important role

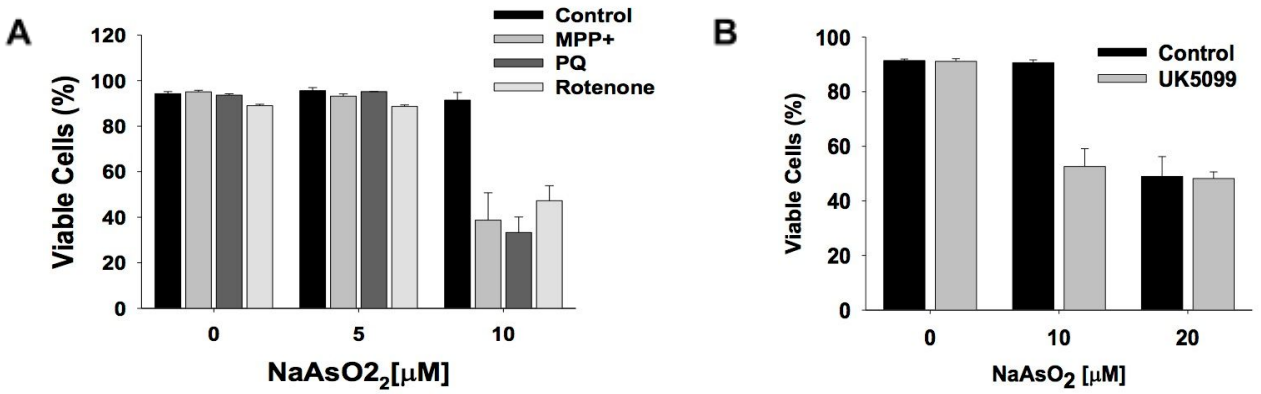


Figure 7. Glucose metabolism carried out in mitochondria of astrocytes are important in xenobiotic defense. Cells were treated with 0, 5, and 10 μM $NaAsO_2$ and either 10 μM MPP^+, 250 μM of rotenone, or 50 μM PQ, or under control conditions, for 48h. At 10 μM $NaAsO_2$, cells experienced decrease in survival (A). Under separate conditions cells were treated with 0, 10, and 20 μM $NaAsO_2$ with or without UK5099 for 48h. These cells also experienced a decrease at 10 μM $NaAsO_2$. * $p < 0.05$

Cells were treated with 0 μM, 5 μM, and 10 μM concentrations of $NaAsO_2$ and concurrent treatment with one of three inhibitors of the electron transport train, used to block mitochondrial function: 10 μM of 1-methyl-4-phenylpyridinium (MPP^+), 250 μM of rotenone, or 50 μM of paraquat (PQ) (Figure 7A). At 10 μM, the capacity of astrocytes treated with all three compounds to respond adequately to arsenic was decreased significantly, while the control remained viable at this concentration. The electron transport train is linked to the TCA cycle by redox cycling of reducing agents $FADH_2$ and NADH. By

preventing this system from supporting the TCA cycle, which generates glutamate via α-ketoglutarate for GSH synthesis, astrocytes are thus subject to arsenic toxicity.

Cell cultures were also treated separately under the same $NaAsO_2$ conditions but with or without 5 μM UK5099, an inhibitor of MPC1 transport protein, which imports pyruvate into the mitochondria for conversion into oxaloacetate and subsequent incorporation into the TCA cycle (Figure 7B). Here it is also evident by the decrease in cell survival at 10 μM of $NaAsO_2$ that the metabolism of glucose carried out by the mitochondria is vital to maintaining cell viability in astrocytes in response to arsenic exposure.

D. Mitochondrial conversion of pyruvate to oxaloacetate vs. acetyl-CoA in mitochondria

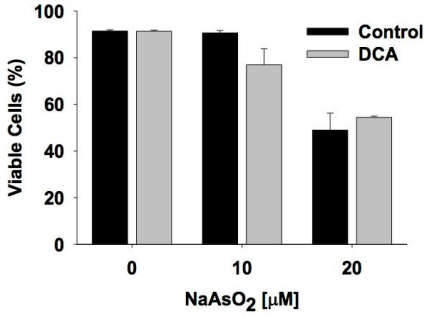


Figure 8. Pyruvate conversion to oxaloacetate vs. acetyl-CoA. Cells were treated with 0, 10, and 20 μM $NaAsO_2$ for 48h with or without DCA. There was a moderate reduction in cell viability at 10 μM $NaAsO_2$ compared to the control. * $p < 0.05$

Astrocyte cultures were treated with 0 μM, 5 μM, and 10 μM of $NaAsO_2$, with or without 5 μM of dichloroacetate (DCA) (Figure 8), which inhibits the dephosphorylation of pyruvate dehydrogenase (PDH) via pyruvate dehydrogenase kinase (PDK). By preventing the dephosphorylation of PDH enzyme, more pyruvate is channeled into the production of acetyl-CoA for input into the TCA cycle. Alternatively, under normal circumstances, pyruvate may also be converted to oxaloacetate by pyruvate carboxylase (PC), another TCA

intermediate. The importance of this second pathway for pyruvate that has been taken up into the mitochondria was explored in addition to the central hypothesis given the fact that conversion to oxaloacetate does not occur in neurons, and may be key in the ability of astrocytes to serve in their defense when harmful xenobiotic compounds such as arsenic are introduced. It was speculated that causing the preferential conversion of pyruvate instead to acetyl-CoA may result in a decrease in cell survival when treated with arsenic.The results of this experiment indicate that at higher levels of arsenic, cell viability decreases slightly; however, cells were still able to maintain high levels of viability despite the diversion in mitochondrial glucose metabolism. Thus, without exploring pyruvate conversion to oxaloacetate further, it cannot be concluded that this contributes especially to astrocytes' capacity to respond to arsenic exposure while maintaining viability.

E. Free fatty acids support mitochondrial glucose metabolism

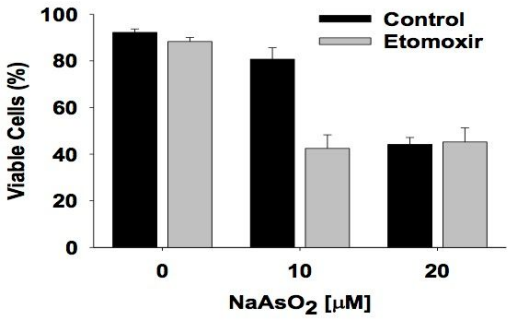


Figure 9. Fatty acid oxidation supports astrocyte response to arsenic exposure. Cells were treated with 0, 10, and 20 μM $NaAsO_2$ with or without 200 μM etomoxir. At 10 μM $NaAsO_2$, cell viability was reduced significantly. * $p < 0.05$

Acetyl-CoA is also provided by the cytoplasmic conversion of free fatty acids to acyl carnitine, which is then transported into the mitochondria and metabolized to acetyl-CoA. The importance of this source of acetyl-CoA was also explored as a potential metabolic mechanism through which astrocytes defend against xenobiotic threat. Astrocytes were

treated with concentrations of 0 μM, 5 μM, and 10 μM of $NaAsO_2$, with or without 200 μM of etomoxir, which inhibits carnitine palmitoyltransferase (CPT1), an enzyme necessary in the conversion of fatty acid molecules into acyl carnitine. . It was found that by inhibiting the contribution of this source of acetyl-CoA to the TCA cycle, cell viability was reduced. This effect was not observed at the same level of arsenic treatment without CPT1 inhibition. This suggests that fatty acid oxidation, therefore, may be an important source of acetyl-CoA during detoxification of arsenic and other xenobiotics.

Conclusions

Astrocytes are vital for the defense of the central nervous system against the threat posed by environmental sources of toxicity. Arsenic is a ubiquitous environmental toxicant, and has been implicated in a number of adverse health effects resulting from chronic exposure. While some of these effects have been relatively well explored, effects on the central nervous system have been less so, particularly with reference to specific mechanisms of action and the metabolic processes involved. The series of experiments in this study in which astrocytes were exposed to inorganic arsenic and inhibitors of various metabolic pathways suspected to play a significant role in astrocyte response to such xenobiotics have attempted to elucidate these processes. As a result, support has been further given to the hypothesis that i) the upregulation of GSH biosynthesis is necessary, involving increased mitochondrial metabolic activity, as opposed to redox activity in the cytoplasm, and ii) that the resulting GSH is then directly complexed with the toxic xenobiotic compound for export from the cell.

In addition, the importance of mitochondrial metabolism in astrocytes' capacity to detoxify xenobiotics was investigated in terms of fatty acid metabolism, resulting in the

indication that fatty acid oxidation and the contribution of acetyl-CoA to the TCA cycle as a result of this pathway is a potentially significant part of the response. Further exploration of these pathways is needed to confirm the degree to which fatty acid oxidation contributes, though these results are promising.

Appendix

Diagram of experimental inhibitor compounds

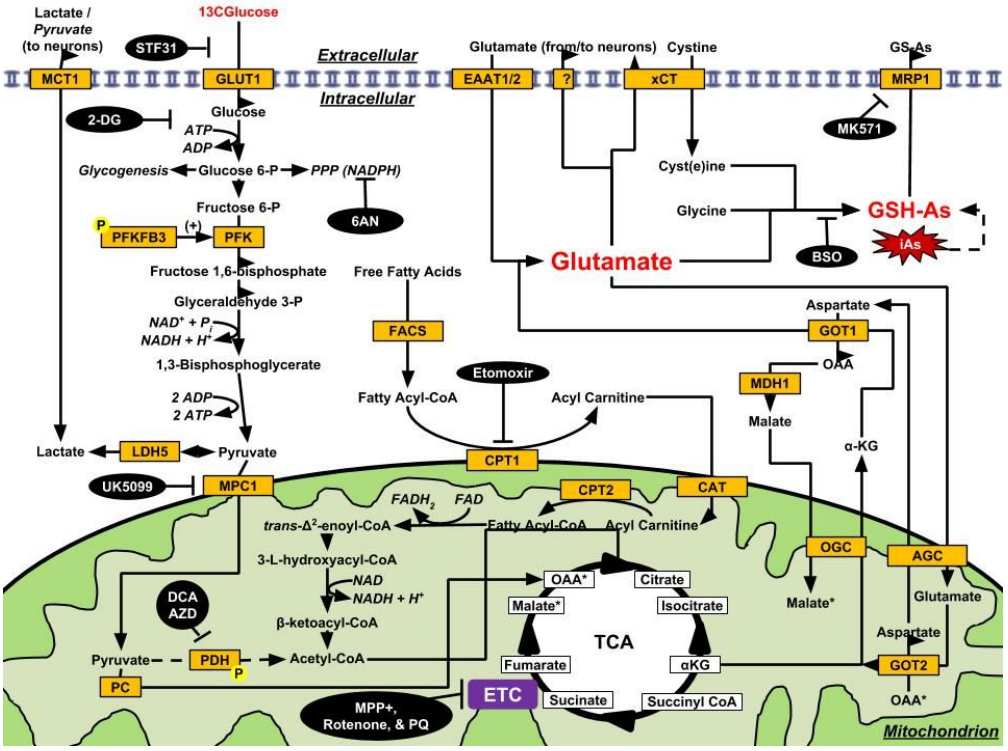


Figure 10. Rose, J., et al. (9)

List of experimental compounds

6AN (Alfa Aesar) - blocks pentose phosphate pathway, leading to depletion of NADPH and thus rendering GSH-GSSG redox recycling unavailable
BSO - blocks glutathione biosynthesis by preventing incorporation of component amino acids
DCA - inhibits PDK, allowing more pyruvate to be channeled into the production of acetyl-CoA
Etomoxir - blocks CPT1, inhibiting fatty acid oxidation to TCA input acetyl-CoA in mitochondria
MPP$^+$ (Sigma-Aldrich) - blocks ETC at Complex I
PQ (Acros Organics) - blocks ETC at Complex I
Rot - blocks ETC at Complex I
MK571 - blocks MRP1 transport, preventing the export from the cell of the GSH-As complex
UK5099 - blocks MPC1 transport, inhibiting pyruvate transfer into the cell for incorporation into the TCA cycle by oxaloacetate

Acknowledgements

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I would also like to extend my sincere thanks to the University of Nebraska and the funding received through the Undergraduate Creative and Research Experience (UCARE) program, without which support this research opportunity would not have been possible.

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