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ARSENIC EXPOSURE INDUCES THE WARBURG EFFECT IN CULTURED HUMAN CELLS

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Abstract

Understanding how arsenic exerts its diverse, global disease burden is hampered by a limited understanding of the particular biological pathways that are disrupted by arsenic and underlie pathogenesis. A reductionist view would predict that a small number of basic pathways are generally perturbed by arsenic, and manifest as diverse diseases. Following an initial observation that arsenite-exposed cells in culture acidify their media more rapidly than control cells, the report here shows that low level exposure to arsenite (75 ppb) is sufficient to induce aerobic glycolysis (the Warburg effect) as a generalized phenomenon in cultured human primary cells and cell lines. Expanded studies in one such cell line, the non-malignant pulmonary epithelial line, BEAS-2B, established that the arsenite-induced Warburg effect was associated with increased accumulation of intracellular and extracellular lactate, an increased rate of extracellular acidification, and inhibition by the non-metabolized glucose analog, 2-deoxyglucose. Associated with the induction of aerobic glycolysis was a pathway-wide induction of glycolysis gene expression, as well as protein accumulation of an established glycolysis master-regulator, hypoxia-inducible factor 1 α . Arsenite-induced alteration of energy production in human cells represents the type of fundamental perturbation that could extend to many tissue targets and diseases.

Keywords

Warburg effect; Arsenite; Glycolysis; Hypoxia-inducible factor 1

Introduction

Arsenic toxicity to humans has been known for centuries, but human arsenic toxicology remains enigmatic. The contamination of water, food, and air by inorganic arsenic is unparalleled in its scope and in the scale of its deleterious impact on human health. Recent epidemiological findings in Bangladesh suggest that one in five deaths may be attributable to arsenic exposure (Smith *et al.*, 2000; Argos *et al.*, 2010; Flanagan *et al.*, 2012). Globally, controversy persists regarding the extent to which drinking water limits (typically 10 ppb–50 ppb) are sufficiently protective against excess cancer risk (Chen *et al.*, 1988; Chen *et al.*, 1992; Smith *et al.*, 1998; Simeonova and Luster, 2004). While efforts aimed at reducing

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arsenic exposure have met with some success, interventional research to mitigate arsenic toxicity is in early stages and of unknown effectiveness.

A substantial obstacle in moving these areas forward is the fundamental puzzle of the mechanism or mechanisms of arsenic toxicity. This is complicated by the diversity of arsenic-associated diseases. Arsenic exposure in humans is associated with increased risk for various cancers (Smith *et al.*, 1992; Smith *et al.*, 1998), obstructive lung disease (Amster *et al.*, 2011), atherosclerosis (Simeonova and Luster, 2004), diabetes (Navas-Acien *et al.*, 2006), and neuropathy (Smith *et al.*, 1992; Smith *et al.*, 1998; Simeonova and Luster, 2004; Navas-Acien *et al.*, 2006; Vahidnia *et al.*, 2007). Molecular target diversity is no less complex. Arsenic studies in experimental models have defined a wide range of perturbed pathways, including cellular redox systems (Sinha *et al.*, 2013), DNA damage repair (Andrew *et al.*, 2006), autophagy (Bolt *et al.*, 2010), apoptosis (Huang *et al.*, 1999), and protein homeostasis (Bolt *et al.*, 2012).

Greater definition of key cellular processes perturbed by arsenic would benefit fundamental toxicology, public health, and regulatory standard setting. The reductionist viewpoint would hold that disruption of a small number of fundamental, central cellular processes may underlie many of the diverse effects resulting from arsenic exposure. In the course of *in vitro* studies of arsenic-induced malignant transformation, it was observed that cultured cell lines exposed chronically to arsenite at non-cytotoxic concentrations appeared to acidify the extracellular culture media more rapidly than the counterpart control cells, despite comparable proliferation rates. The possibility that this increased extracellular acidification was due to lactate efflux generated interest in bioenergetic metabolism as a potential target of arsenite.

The perturbation of energy production and transfer by arsenic represents the type of fundamental cellular process that could impact diverse downstream cellular pathways typical of those associated with arsenic toxicity in various models. This possibility has longstanding literature support. Work by Sir Rudolph Peters and others identified vicinal dithiols in oxidative phosphorylation enzymatic systems as inactivation-targets of trivalent arsenicals (Peters, 1955; Aposhian, 1989). Samikkannu *et al.* reported that inorganic trivalent arsenicals inhibit pyruvate dehydrogenase complex activity (Samikkannu *et al.*, 2003). Lee *et al.*, reported that low micromolar exposures of sodium arsenite in a keratinocyte cell line resulted in an accumulation of several proteins involved in glycolysis (Lee *et al.*, 2005). Trivalent inorganic arsenic has been demonstrated to target mitochondria in a manner that may be relevant to its carcinogenicity (Echaniz-Laguna *et al.*, 2012; Zhang *et al.*, 2012). Chronic, low dose exposure of a pulmonary epithelial cell line (BEAS-2B) causes a marked induction in mitochondrial gene expression that is associated with the acquisition of malignant characteristics (Stueckle *et al.*, 2012). Autophagy, a process tightly linked to cellular energy balance, is perturbed by arsenic (Bolt *et al.*, 2010). Taken together, a substantial foundation of research over the past several decades supports further examination of the impact of arsenic exposure on cellular energy metabolism. This report describes a novel, generalized effect of arsenite on cellular energy metabolism: the induction of aerobic glycolysis associated with increased extracellular acidification and the accumulation of lactate.

Materials and Methods

Reagents

Sodium arsenite (dissolved in Milli-Q H₂O), and 2-deoxy-D-glucose (2-DG, dissolved in Milli-Q H₂O) were purchased from Sigma Aldrich (St. Louis, MO).

Cell culture

BEAS-2B, an adenovirus-immortalized, human bronchial epithelial cell line (ATCC, Manassas, VA), was cultured with the BEGM BulletKit (Lonza, Walkersville, MD). RWPE-1, a papilloma virus-immortalized, human prostate epithelial cell line (ATCC), was cultured with the Keratinocyte serum-free medium kit (Life technologies, Carlsbad, CA). A549, a human pulmonary epithelial carcinoma cell line (ATCC), was cultured in Dulbecco's Modified Eagle Medium supplemented with 10% v/v FBS and 1% antibiotic-antimycotic. Primary human urothelial cells (HUC, ScienCell, Carlsbad, CA) were cultured with the Urothelial Cells Medium (ScienCell). Human dermal fibroblasts (HDF) were cultured with the Medium 106 kit, with the recommended low serum growth supplement (Life technologies). Two million cells were seeded to 75 cm² culture flasks and subcultured when 90% confluence was reached. Trypsin-EDTA (0.25%) was used to remove cells from culture flasks for sub-culturing. All cells were incubated under 5% CO₂ at 37°C during culture. Separate groups for each exposure condition were assayed for viability using trypan blue exclusion.

Lactate measurement

Cell media and intracellular L-lactate levels were measured using the L-lactate assay kit from Biomedical Research Service Center (Buffalo, NY) according to the manufacturer protocol. To minimize potential variability introduced by cell culture density, 48 hr prior to analysis, cells were re-seeded to 60 mm cell culture dishes at identical density. For extracellular lactate determination, 1 ml of supernatant media was collected directly from the culture. Samples were deproteinized by polyethylene glycol precipitation (25% w/v PEG-8000) and clarified by centrifugation at 20,000g for 5 min (Ingham, 1984). For intracellular lactate determination, cells were washed twice with ice cold Phosphate buffered saline (PBS), scraped off the dishes in 100 µl Milli-Q water (4 °C), vortexed vigorously and immersed in a 100°C water bath for 10 min. The cell lysates were then deproteinized and analyzed as described above. To verify "in-house" lactate measurements, randomly selected samples were sent for lactate measurement to an independent clinical laboratory.

Antibodies and immunoblot analysis

Primary antibodies were used at the following dilutions: hypoxia-inducible factor 1 α -HIF-1A, 1:250 and α -tubulin, 1:1000 (Santa Cruz Biotechnology, Santa Cruz, CA); secondary antibody goat anti-rabbit IgG-HRP and goat anti-mouse IgG-HRP (Santa Cruz Biotechnology) were always used at 1:5000 dilution. After treatment, cells were washed twice with PBS, and lysed in sample buffer [10% glycerol, 100 mM DTT, 50 mM Tris-HCl (pH 6.8), 2% SDS]. Samples were then denatured in 90°C dry bath for 5 min. After sonication, protein concentration was measured by Pierce 660 nm Protein Assay (Thermo Scientific, Rockford, IL). Equal concentrations of each sample (final concentration 0.1% v/v bromophenol blue) were subjected to SDS-polyacrylamide gel electrophoresis and immunoblot analysis. Immunoblots were visualized by addition of chemiluminescence substrate (Thermo Scientific) and quantified using a GeneGenome5 imaging system (Syngene, Frederick, MD).

mRNA isolation and quantification

BEAS-2B Cells were washed twice with PBS. Total RNA was isolated using RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer protocol. RNA (500 ng) was used in cDNA synthesis (RT² First Strand Kit, Qiagen). Quantification of mRNA for genes involved in glucose metabolism was performed using Glucose Metabolism PCR Arrays (Qiagen) according to the manufacturer protocol. Real-time PCR was performed with the

StepOnePlus Real-Time PCR system (Life technologies). Data analysis utilized the web-based RT² Profiler PCR Array Data Analysis (Version 3.5 Qiagen).

Extracellular acidification rate measurement

Extracellular acidification rate (ECAR) was measured using XF96 analyzer (Seahorse Bioscience, North Billerica, MA). BEAS-2B cells were seeded into a XF96 cell culture microplate at a density of 30,000 cells/well, 24 hr prior to analysis. Immediately prior to analysis, the cell culture media was exchanged for XF assay media (unbuffered, 2.5 mM glucose). ECAR was measured at 0, 8, 16, and 24 min in assay media. Immediately after ECAR measurement, assay media was removed and cells were processed for protein quantification, which was used to normalize the ECAR values from each well.

Statistical analyses

For data containing two comparison groups, unpaired t-tests were used to compare mean differences between control and treatment group at a significance threshold of $P < 0.05$. For data containing three or more groups, univariate ANOVA, followed by Tukey's post hoc tests, were used to compare mean differences of groups at a significance threshold of $P < 0.05$. Comparison of arsenic-exposed cells to control cells for their ECAR, measured at multiple time points over 24 minutes, was performed using repeated measures ANOVA analysis. GraphPad Prism version 6.0 for MAC (GraphPad, La Jolla, CA) was used for all statistical analyses.

Results

Arsenic exposure increases lactate level and extracellular acidification

To determine if lactate production is induced by arsenic, BEAS-2B cells were exposed to sodium arsenite concentrations from 0 to 4 μM for 7 days, after which cell lysates were subjected to lactate analysis. Arsenite exposure increased the intracellular concentration of lactate in a dose-dependent fashion (Figure 1A). To understand how general the arsenite-induced lactate accumulation was, several different human cells and cell lines (BEAS-2B, RWPE-1, A549, HUC, HDF) were tested for arsenite-induced intracellular lactate accumulation, as well as in the extracellular media (Figure 1B). In every cell type tested, intracellular and extracellular lactate was elevated by arsenite exposure, suggesting that this is a generalized effect of arsenite exposure. To corroborate these findings, ECAR was measured in BEAS-2B cells (Figure 1C). ECAR measurement over the course of 24 min demonstrated a significant increase in extracellular acidification in 2-week arsenite-exposed cells, compared to controls.

Arsenic-induced lactate is glucose dependent

To establish that arsenite-induced increase in lactate was the result of glycolysis, the inhibitory activity of the non-metabolized glucose analog, 2-DG, on lactate accumulation was tested (Figure 2A). Doses under 2mM of 2-DG were found to have no effect on BEAS-2B cell viability (data not shown). Extracellular lactate levels in BEAS-2B cell culture media decreased with 2-DG exposure in a dose-dependent manner. Based on the initial dose-response experiment, control cells and BEAS-2B cells that had been exposed to arsenite for 14 days were exposed to 2-DG (1 mM) for 48 hr (Figure 2B). The inhibition of glycolysis by 2-DG essentially abrogated the increase in lactate caused by arsenite exposure. Taken together, the data support an induction of aerobic glycolysis caused by arsenite.

Arsenic-induced glycolysis is associated with a coordinated induction of gene expression in the glycolytic pathway

Arsenic-induced glycolysis was evaluated at the transcriptional level by RT-PCR. A coordinated induction of genes coding for enzymes involved in glucose metabolism was observed in arsenite-treated BEAS-2B cells at 2 weeks and 4 weeks of 1 μ M sodium arsenite exposure (Figure 3B, Supplemental Table 1). Arsenite exposure at 1 μ M for 1 week in BEAS-2B resulted in decreased mRNA (compared to passage-matched control cells) for 3 of the 25 glycolysis-related genes, enolase 3 (ENO3), hexokinase 3 (HK3), phosphoglycerate kinase 2 (PGK2). One gene, glucokinase (GCK) was induced in the 1 week group. Paradoxically, expression of ENO3, HK3, and PGK2 increased in the 2 week samples, and were all significantly induced at 4 week exposures (Figure 3B, Supplemental Table 1). In the 2 week, 1 μ M sodium arsenite exposed BEAS-2B, 14 of the 25 glycolysis related genes, fructose biphosphate aldolase C (ALDOC), 2,3-bisphosphoglycerate mutase (BPGM), enolase 1 (ENO1), enolase 3 (ENO3), galactose mutarotase (GALM), glucose-6-phosphate isomerase (GPI), pyruvate dehydrogenase kinase, isozyme 2 (PDK2), pyruvate dehydrogenase kinase, isozyme 3 (PDK3), pyruvate dehydrogenase kinase, isozyme 4 (PDK4), phosphoglycerate mutase 2 (PGAM2), phosphoglycerate kinase 1 (PGK1), phosphoglucomutase 1 (PGM1), and pyruvate kinase, liver and RBC (PKLR) were induced (Figure 3B, Supplemental Table 1). At 4 weeks of 1 μ M sodium arsenite exposure, 17 of the 25 glycolysis related genes, fructose biphosphate aldolase B (ALDOB), ALDOC, BPGM, ENO1, enolase 2 (ENO2), ENO3, GALM, GPI, hexokinase 3 (HK3), PDK2, PDK3, PDK4, PGAM2, PGK1, phosphoglycerate kinase 2 (PGK2), PGM1, PKLR were all significantly induced, compared to passage-matched control cells (Figure 3A and 3B, Supplemental Table 1). Exposure of cells to 1 μ M arsenite for the durations used in this study did not result in increased cytotoxicity as measured by trypan blue exclusion (data not shown).

The apparently coordinated induction of glycolysis genes suggested the possibility of a master regulator controlling the induction. Among the best-characterized transcription factors in this context are the hypoxia inducible factor (HIF) family members. The RT-PCR arrays used to analyze gene expression in this study target 14 HIF-1A-responsive genes (Semenza *et al.*, 1994; Krones *et al.*, 2001; Jean *et al.*, 2006; Kim *et al.*, 2006; Martin-Rendon *et al.*, 2007; Hamaguchi *et al.*, 2008; Lu *et al.*, 2008; Pelletier *et al.*, 2012; Baumann, 2013). Intriguingly 9 out of 14 HIF-1A-responsive genes were induced in the 2 week 1 μ M sodium arsenite exposed cells, and 10 out of 14 HIF-1A-responsive genes were induced in the 4 week 1 μ M sodium arsenite exposed cells, including ALDOC, ENO1, ENO2, GPI, PDK2, PDK3, PDK4, PGK1, PGM1, and PKLR (Figure 3B, Supplemental Table 1). To test a possible role for HIF-1A in arsenite-induced glycolysis, HIF-1A protein level was evaluated by immunoblot analysis (Figure 3C and 3D), which revealed an arsenite-induced accumulation of HIF-1A protein.

Discussion

Primary cells, immortalized cells, and malignant cell lines exposed to 1 μ M (75 ppb) arsenite, accumulate both intracellular and extracellular lactate. In the pulmonary epithelial cell line, BEAS-2B, the arsenite-induced lactate accumulation was inhibited by 2-DG exposure, establishing that the lactate was derived from glucose via glycolysis. Extracellular lactate accumulation was associated with an increased extracellular acidification rate, which is consistent with the co-transport of hydrogen ions with lactate in monocarboxylate transporters. The glycolysis observed at one week of arsenite exposure does not appear to be associated with altered expression of glycolysis-related genes. One possible explanation is that allosteric regulation of the glycolysis pathway may dominate early arsenite exposure. Allosteric regulation of glycolysis is well-established (Porporato *et al.*, 2011). Alternatively, the well-established capacity of trivalent arsenicals to inactivate oxidative phosphorylation

enzyme complexes could divert pyruvate toward glycolytic pathways (Aposhian, 1989). In contrast to the earlier time points, between 2 and 4 weeks of arsenite exposure there is a substantial induction of both glycolytic genes and glycolysis-regulatory genes, suggesting that increased glycolysis in longer-term arsenite exposures is supported by a higher quantity of glycolysis pathway members. One gene family in particular, the pyruvate dehydrogenase kinase family, is responsible for the inactivating, post-translational modification of mitochondrial pyruvate dehydrogenase, with a resultant shift of pyruvate to glycolytic metabolism (Gudi *et al.*, 1995; Kolobova *et al.*, 2001). A significant induction of three pyruvate dehydrogenase kinase family members in arsenite-exposed BEAS-2B cells was observed in this study.

Glycolysis is a coordinated pathway that is under the control of master regulatory genes, most notably the hypoxia-responsive transcription factors of the HIF family (Yeung *et al.*, 2008). A statistically significant increase of HIF-1A protein levels in BEAS-2B cells exposed to arsenite for 2 weeks was observed, and HIF-1A protein remains elevated at 8 weeks of 1 μ M arsenite exposure (data not shown). In its well-characterized role as a hypoxia-responsive factor, HIF-1 α is constitutively transcribed, but degraded in an oxygen-dependent process that is interrupted during hypoxic conditions (Semenza, 2001; Yu *et al.*, 2001). It has been well-established that several metals are capable to stabilize HIF-1A protein levels (Li *et al.*, 2006). In addition to hypoxia, however, HIF-1A protein levels can be increased by exposure to arsenic (reviewed in (Ke and Costa, 2006). Our data suggest that a potential consequence of the modulation of HIF-1A by arsenite may be alterations in cellular energy metabolism, but more work is needed to establish the HIF-dependence of arsenite-induced glycolysis.

The data presented in this report suggest that a general consequence of extended *in vitro* exposures to inorganic arsenic at 75 ppb is a perturbation of glucose catabolic pathways that results in increased glycolysis. This increased glycolytic rate is occurring under oxygen replete conditions, a phenomenon first characterized by Otto Warburg in the 1920s, subsequently dubbed the Warburg effect (Warburg, 1956). To our knowledge this is the first report of an environmental toxicant exposure inducing the Warburg effect.

The induction of glycolysis by arsenite occurs in diverse cell types *in vitro*, including primary cells of diverse embryonic origin (human urinary bladder epithelial, dermal fibroblasts), immortalized non-malignant cells (prostate epithelial, pulmonary epithelial), and malignant cells (lung epithelial carcinoma). This suggests that the ability of arsenite to induce glycolysis is unlikely to be extremely specific to particular cell types and may occur at the level of fundamental biochemical pathways. This is intriguing in light of the diversity of cellular targets represented in arsenic-associated human disease, including the association between chronic arsenic exposure and the risk of several human cancers. The induction of aerobic glycolysis during the acquisition of malignancy is an area of longstanding interest in carcinogenesis research. A widely held hypothesis is that the Warburg effect is a metabolically supportive phenotype for the increased demands associated with growth, proliferation, and invasion of cancer cells (Vander Heiden *et al.*, 2009). While less efficient than oxidative phosphorylation in terms of ATP molecules produced per molecule of glucose, glycolysis has a higher turnover rate and has the additional advantage of producing molecular intermediates that can be used in biosynthetic pathways (Najafov and Alessi, 2010). Equally interesting is the possibility that in cancer development, induction of glycolysis in neighboring stromal cells may result in their production of extracellular lactate that is then taken up and consumed by the transformed cells, a scenario dubbed the “reverse Warburg” effect (Pavlidis *et al.*, 2009; Pavlidis *et al.*, 2010). Thus, a plausible case could be made for arsenite-induced glycolysis in a permissive role in carcinogenesis either by the Warburg effect or by a “reverse Warburg” effect.

Energy metabolism and its modulation is an intense area of scientific focus, with publications associating perturbed catabolic and anabolic pathways with diseases ranging from cancer to diabetes to atherosclerosis to developmental defects. Futures studies should shed light on the extent to which arsenic toxicology explains the overlap between diseases associated with arsenic exposure and those associated with disrupted energy metabolism.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used

2-DG	2-Deoxy-D-glucose
As	Arsenite
HIF-1A	Hypoxia inducible factor-1 alpha subunit

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Highlights

Chronic arsenite exposure induces aerobic glycolysis, dubbed the “Warburg effect”.

Arsenite-induced Warburg effect is a general phenomenon in cultured human cells of various types.

HIF-1A may mediate arsenite induced Warburg effect.

Figure 1.

Induction of lactate production by arsenite. A) BEAS-2B cellular lactate levels ($\mu\text{mol per million cells}$) from 1 week exposure of 0–4 μM arsenite (Lactate, $\pm$ S.D., from 3 independent replicates). B) BEAS-2B, RWPE-1, A549, HUC, and human fibroblast cellular or culture media lactate levels ($\mu\text{mol per million cells}$) from 1 or 2 week indicated arsenite exposure (Lactate, $\pm$ S.D., from 3 independent replicates). C) Extracellular acidification rate (ECAR, milli-pH units per minute, normalized to cellular protein per well) of BEAS-2B control vs. 2 week 1 μM arsenite exposure (ECAR, $\pm$ S.D., from 12 replicates). * $P < 0.05$, *** $P < 0.0001$.

Figure 2.

Inhibition of Lactate production by 2-DG. A) Lactate levels in BEAS-2B culture media after 72 hour 2-DG dose response (% Lactate, $\pm$ S.D., from 6 replicates). B) Lactate levels (μ mol per million cells) in BEAS-2B culture media with or without 1 week 1μ M arsenite treatment and 48 hour terminal 2-DG treatment (Lactate, $\pm$ S.D., from 3 independent replicates). * $P < 0.05$

Figure 3.

Glycolysis-related gene expression in BEAS-2B. A) Diagram of the glycolysis pathway labeled with induction (red arrow) or repression (green arrow) of transcription at 4 week 1 μ M arsenite exposure in Beas-2. B) Gene expression heatmap (Mean of 3 independent replicates) of glycolysis-related genes. "HR": HIF-1A-responsive gene. * $P < 0.05$ compared to control. C) HIF-1A immunoblot of BEAS-2B treated with or without 1 μ M arsenite for 2 weeks. "Tub": α -tubulin. D) Immunoblot quantification, HIF-1A normalized to α -tubulin (+/-S.D. from 3 independent replicates). * $P < 0.05$