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Sodium arsenite represses the expression of myogenin in C2C12 mouse myoblast cells through histone modifications and altered expression of Ezh2, Glp, and Igf-1

Gia-Ming Hong [a,c], Lisa J. Bain [a,b,*]

[a] Environmental Toxicology Graduate Program, Clemson University, 132 Long Hall, Clemson, SC 29634, USA
[b] Department of Biological Sciences, Clemson University, 132 Long Hall, Clemson, SC 29634, USA
[c] Present address: The University of Chicago, Section of Hematology/Oncology, 900 E. 57th Street, Room 7134, Chicago, IL 60637, USA

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ABSTRACT

Arsenic is a toxicant commonly found in water systems and chronic exposure can result in adverse developmental effects including increased neonatal death, stillbirths, and miscarriages, low birth weight, and altered locomotor activity. Previous studies indicate that 20 nM sodium arsenite exposure to C2C12 mouse myocyte cells delayed myoblast differentiation due to reduced myogenin expression, the transcription factor that differentiates myoblasts into myotubes. In this study, several mechanisms by which arsenic could alter myogenin expression were examined. Exposing differentiating C2C12 cells to 20 nM arsenic increased H3K9 dimethylation (H3K9me2) and H3K9 trimethylation (H3K9me3) by 3-fold near the transcription start site of myogenin, which is indicative of increased repressive marks, and reduced H3K9 acetylation (H3K9Ac) by 0.5-fold, indicative of reduced permissive marks. Protein expression of Glp or Ehmt1, a H3-K9 methyltransferase, was also increased by 1.6-fold in arsenic-exposed cells. In addition to the altered histone remodeling status on the myogenin promoter, protein and mRNA levels of Igf-1, a myogenic growth factor, were significantly repressed by arsenic exposure. Moreover, a 2-fold induction of Ezh2 expression, and an increased recruitment of Ezh2 (3.3-fold) and Dnmt3a (~2-fold) to the myogenin promoter at the transcription start site (−40 to +42), were detected in the arsenic-treated cells. Together, we conclude that the repressed myogenin expression in arsenic-exposed C2C12 cells was likely due to a combination of reduced expression of Igf-1, enhanced nuclear expression and promoter recruitment of Ezh2, and altered histone remodeling status on myogenin promoter (−40 to +42).

Introduction

Arsenic is a toxicant commonly found in water systems around the world. Chronic arsenic poisoning is a global health problem affecting millions of people (Cherry, 2008; McDonald, 2007; Medrano et al., 2010; Wang et al., 2009) which can result in cancer, central nervous system and sensory deficits, effects on development, and neuromuscular deficits (Andrew et al., 2007; Benbrahim-Tallaa and Waalkes, 2007; Kozul et al., 2009; Mohammad et al., 2009). Unfortunately, the mechanisms responsible for these multiple adverse outcomes remain largely unclear and likely are multi-factorial.

Arsenic is also a developmental toxicant. In humans and rodents, arsenic can traverse the placenta and this exposure results in adverse developmental effects, such as increased neonatal death and stillbirths (Agusa et al., 2010; Concha et al., 1998; Markowski et al., 2011; Raqib et al., 2009; von Ehrenstein et al., 2006). In fish, arsenite-exposed zebrafish embryos have reduced survival and delayed hatching, malformations in the spinal cord and heart, and disordered motor axon projections (Li et al., 2009). Recently, the negative effects of arsenic on early embryonic development have been reported (Flora and Mehta, 2009; Stummann et al., 2008). Results from mouse embryonic stem cells indicate that ~2 μM arsenic inhibits cardiac myocyte differentiation and cardiac beating in the embryonic stem cell test (Stummann et al., 2008). Flora and Mehta have observed, using human stem cells, that 1 ppb arsenic reduced the pluripotency of stem cells but also caused a significant down regulation of genes indicative of all the three germ layers (Flora and Mehta, 2009).

Arsenic-mediated adverse effects on muscle differentiation have also been reported. In killifish (*Fundulus heteroclitus*), arsenic exposed parents had offspring with increased trunk curvatures, which was correlated with changes in myosin light chain, type II keratin, tropomyosin, and parvalbumin expression in the hatchlings (Gonzalez et al., 2006). Arsenic exposure to mouse C2C12 myoblasts delayed their differentiation into myotubes, likely due to a reduction in the expression of myogenin (Steffens et al., 2011). In rodent models, arsenic suppresses the regeneration of injured muscles (Yen et al., 2010), alters pulmonary structure and function *in utero* by increasing the smooth muscle actin in the lung (Lantz et al., 2009), and disrupts

* Corresponding author at: Department of Biological Sciences, Clemson University, 132 Long Hall, Clemson, SC 29634, USA. Fax: +1 864 656 0435.
E-mail address: lbain@clemson.edu (L.J. Bain).

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the smooth muscle integrity around the blood vessels in the heart (Hays et al., 2008). Collectively, these results suggest that arsenic acts as a developmental toxicant by affecting the development of the musculature.

The development of skeletal muscle is regulated by several myogenic transcription factors, such as Myo D, myogenin, and myocyte enhancer factor 2 (Mef2). In muscle differentiation, MyoD and Mef2 are early markers, which are expressed during myoblast determination, and they then regulate myogenin, which induces terminal differentiation by converting myoblasts into myotubes (Carvajal and Rigby, 2010; Gianakopoulos et al., 2011; Yokoyama and Asahara, 2011). Moreover, other signaling molecules, such as insulin-like growth factor 1 (Igf-1) and myostatin, regulate myogenin expression *via* the PI3K/AKT pathway during skeletal muscle differentiation (Alzhanov et al., 2010; Artaza et al., 2002; Yang et al., 2007). In addition, chromatin-modifying enzymes also regulate muscle development by epigenetically repressing myogenic transcription factors (Albert and Peters, 2009; McDonald and Owens, 2007; Ohkawa et al., 2007).

Recently, arsenic-induced alterations in DNA methylation and histone modifications have been suggested to play a role in carcinogenesis and the fetal origins of diseases (Zhou et al., 2008, 2009; Arita and Costa, 2009; Baccarelli and Bollati, 2009; Ren et al., 2010). Altered DNA methylation may occur since the pathway for biotransformation of arsenic also relies on methylation (Baccarelli and Bollati, 2009; Ren et al., 2010; Vahter, 2009). To this end, studies have shown that arsenic exposure results in both hypermethylation and hypomethylation at global and gene specific levels, thereby leading to aberrant gene expression. For example, mice exposed to arsenic have reduced p16 expression in lung tumors due to hypermethylation of the p16 gene and in humans, arsenic induces DNA hypermethylation in the promoters of the p53 and p16 genes (Benbrahim-Tallaa and Waalkes, 2007; Chanda, 2006; Salnikow and Zhitkovich, 2008; Zhou et al., 2008). Moreover, a significant relationship between arsenic exposure and promoter hypermethylation of two tumor suppressor genes, PRSS3 and RASSF1A, was identified in a population-based study of human bladder cancer (Marsit et al., 2006). In addition to DNA methylation, arsenic also has a role in histone modification. H3K9 dimethylation (H3K9me2) and H3K9 trimethylation (H3K9me3), both markers of gene silencing, were induced at the global level in human lung carcinoma A549 cells upon exposure to 1 μM arsenic (Zhou et al., 2008). The increased H3–K9 methylation in the arsenic exposed A549 cells was due to the induction of G9a/Ehmt2 (Zhou et al., 2008). G9a heterodimerizes with Glp/Ehmt1 to form a H3–K9 histone methyltransferase (Tachibana et al., 2005). Acetylation of K9 in histone H3 (H3K9 Ac), which represents transcriptional activation, was reduced by 50% at global level in human UROtsa cells upon exposure to arsenic (3 μM) for 7 days (Chu et al., 2011). Moreover, arsenic represses steroid hormone-mediated transcription by disrupting acetylation of K18 in histone H3 (H3K18) at the estrogen-responsive pS2 promoter (Barr et al., 2009). Collectively, these and other reports suggest that arsenic can epigenetically alter gene expression *via* either DNA methylation or histone modifications.

Our previous study indicated that 20 nM sodium arsenite delayed the differentiation of C2C12 mouse myoblast cells by repressing myogenin expression, which was likely due to altered DNA methylation patterns on the myogenin promoter and the decreased nuclear translocation of Mef2 (Steffens et al., 2011). Since the potential regulatory mechanisms responsible for the arsenic-induced delay in muscle differentiation remain largely unclear, the objectives of the present study were to examine whether arsenic-induced abnormal methylation patterns on myogenin promoter would lead to changes in chromatin structure and investigate whether other muscle transcription and growth factors were altered by arsenic exposure. The results indicate reduced Igf-1 expression, coupled with altered histone marks, is likely repressing muscle cell differentiation after arsenic exposure.

Materials and methods

Cell culture

C2C12 myoblasts were maintained in growth medium (GM) consisting of Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, 1% L-glutamine and 1% penicillin/streptomycin solution. Differentiation medium (DM) was DMEM containing 2% horse serum, 1% L-glutamine and 1% penicillin/streptomycin solution (Kubo, 1991). For differentiation studies, 15×10^4 cells were seeded in a 150 mm dish with or without 20 nM arsenic as sodium arsenite ($NaAsO_2$, certified >99.8%; Fisher Scientific, Pittsburg, PA), and then cultured in growth medium for 3 days (GM3). On day 4, the culture medium was changed to differentiation medium (DM) with or without 20 nM arsenic to induce myotube differentiation. Cells were cultured for 2 days (DM2) and then harvested.

Igf-1 mRNA expression

C2C12 cells were cultured with or without 20 nM arsenic as sodium arsenite as described above, harvested at differentiation hour 12, 24, 36, and 48 (n = 3 per group per time point), and total RNA extracted. The mRNA expression of Igf-1 was quantified using RT^2 SYBR Green Supermix (Qiagen) according to manufacturer's instructions. The oligonucleotides used for qPCR were Igf-1 Forward: 5′-GAC CGA GGG GCT TTT ACT TCA-3′, Reverse: 5′-GGA CGG GGA CTT CTG AGT CTT-3′; and Gapdh Forward: 5′-TGC GAC TTC AAC AGC AAC TC-3′, Reverse: 5′-ATG TAG GCC ATG AGG TCC AC-3′. Samples were run in triplicate, and relative gene expression was calculated using the comparative threshold (Ct) method (Livak and Schmittgen, 2001).

Chromatin immunoprecipitation (ChIP)

C2C12 cells were cultured with or without 20 nM sodium arsenite as described above and harvested on differentiation day 2 (DM2) (n = 4 per group per day). Chromatin was extracted and sonicated using a Vibra Cell probe (Sonic and Materials Inc, Danbury, CT) using 20% power output for 120 s and run on a gel to ensure appropriate fragmentation. Both chromatin extractions and immunoprecipitations were performed according to standard protocols (Abcam Inc., Cambridge, MA, http://www.abcam.com/ps/pdf/protocols/x_CHip_protocol.pdf). The antibodies used for ChIP assays were: anti-Ezh2 (#17-622, Millipore Inc., Temecula, CA), anti-Mef2 (sc-313, Santa Cruz Biotech Inc., Santa Cruz, CA), anti-Dnmt3a (IMG-268A, Imgenex Inc., San Diego, CA), anti-Dnmt3b (IMG-184A, Imgenex Inc.), and anti-H3K9 Ac (ab4441, Abcam), -H3K9 Me2 (ab1220, Abcam), and -H3K9 Me3 (ab8898, Abcam). Normal rabbit (sc-2027, Santa Cruz) and mouse IgG (sc-2025, Santa Cruz) were used as negative controls. Quantification of precipitated DNA was performed by qPCR using SYBR green and myogenin promoter-specific primers. The oligonucleotides used for ChIP assays were ChIP 1 Forward: 5′-TAA TCA AAT TAC AGC CGA CGG CCT CC-3′, Reverse: 5′-GCT GCA CAT CAA GAC GTT TCC AGT-3′; ChIP 2 Forward: 5′-CGT CTT GAT GTG CAG CAA CAG CTT-3′, Reverse: 5′-CAT TTA AAC CCT CCC TGC TGG CAT-3′; and ChIP 3 Forward: 5′-GGG TTT AAA TGG CAC CCA GCA GTT-3′, Reverse: 5′-TCA TAC AGC TCC ATC AGG TCG GAA-3′ (see Fig. 2 for specific locations of each ChIP assay). Relative enrichment of the myogenin promoter DNA relative to a matched IgG-antibody control was calculated based on difference in threshold (Ct) values ($2[Ct_{antibody} - Ct_{IgG}]$)(Nelson et al., 2006).

Immunofluorescence analysis of Ezh2, Glp, MyoD, and Igf-1

C2C12 cells were seeded in Lab-tek II 8-well chamber slides (Nunc) at a concentration of 100 cells/well. To determine the expression of Ezh2 and MyoD, cells were cultured with or without 20 nM