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Monomethylarsonous acid, but not inorganic arsenic, is a mitochondria-specific toxicant in vascular smooth muscle cells

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Arsenic exposure has been implicated as a risk factor for cardiovascular diseases, metabolic disorders, and cancer, yet the role mitochondrial dysfunction plays in the cellular mechanisms of pathology is largely unknown. To investigate arsenic-induced mitochondrial dysfunction in vascular smooth muscle cells (VSMCs), we exposed rat aortic smooth muscle cells (A7r5) to inorganic arsenic (iAs(III)) and its metabolite monomethylarsonous acid (MMA(III)) and compared their effects on mitochondrial function and oxidative stress. Our results indicate that MMA(III) is significantly more toxic to mitochondria than iAs(III). Exposure of VSMCs to MMA(III), but not iAs(III), significantly decreased basal and maximal oxygen consumption rates and concomitantly increased compensatory extracellular acidification rates, a proxy for glycolysis. Treatment with MMA(III) significantly increased hydrogen peroxide and superoxide levels compared to iAs(III). Exposure to MMA(III) resulted in significant decreases in mitochondrial ATP, aberrant perinuclear clustering of mitochondria, and decreased mitochondrial content. Mechanistically, we observed that mitochondrial superoxide and hydrogen peroxide contribute to mitochondrial toxicity, as treatment of cells with MnTBAP (a mitochondrial superoxide dismutase mimetic) and catalase significantly reduced mitochondrial respiration deficits and cell death induced by both arsenic compounds. Overall, our data demonstrates that MMA(III) is a mitochondria-specific toxicant that elevates mitochondrial and non-mitochondrial sources of ROS.

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1. Introduction

Exposure to arsenic has been associated with cancer, diabetes, cardiovascular disease, and metabolic syndrome (Singh et al., 2011; Maull et al., 2012, Kim et al., 2015a, 2015b, Mohammed Abdul et al., 2015). It has been suggested that arsenic alters common pathways such as those involved in oxidative stress and inflammatory signaling, which underlie a variety of arsenic-associated diseases (States et al., 2009, Straif et al., 2009, Mo et al., 2011, Kim et al., 2015a, 2015b). The generation of ROS in particular is a major pathological mechanism induced by arsenic and is involved in the pathogenesis of cancer (Shi et al., 2004), insulin resistance (Padmaja Divya et al., 2015), metabolic syndrome (Ando and Fujita, 2009), and cardiovascular disease (States et al., 2009).

Arsenic has been shown to induce oxidative stress in numerous cell lines, including human vascular smooth muscle cells (VSMCs) (Lynn et al., 2000, Lantz and Hays, 2006; Naranmandura et al., 2011; Calatayud et al., 2014; Pan et al., 2014), by causing the release of iron from ferritin (Ahmad et al., 2000), or by altering antioxidant activity and increasing inflammation (Flora, 2011). Additional tissue pathology in the vascular endothelium caused by exposure to arsenic includes an increase in oxidative stress caused by increased NADPH oxidase activity in VSMCs and endothelial cells, leading to an increase in superoxide and hydrogen peroxide levels (Lynn et al., 2000), and a vicious cycle of dysregulated calcium and nitric oxide signaling. Arsenic-induced ROS couples with nitric oxide (NO) producing peroxynitrite (Bunderson et al., 2002) and decreases the bioavailability of NO to vascular endothelium and smooth muscle, which likely contributes to cardiovascular complications. ROS have also been shown to result in cell damage and cell death in numerous cell lines and models of arsenic-induced disease pathology (Li et al., 2010; Shi et al., 2010; Gu et al., 2016; Aposhian et al., 2003; Abhyankar et al., 2012).

Inorganic arsenic (iAs(III)) undergoes biomethylation in the liver to form the toxic intermediate metabolites monomethylarsonous acid (MMA(III)) and dimethylarsonous acid (DMA(III)). These trivalent organic arsenic species have demonstrated elevated toxicity relative to

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iAs(III) in several studies (Styblo et al., 2000; Cohen et al., 2007; Calatayud et al., 2013) and may be responsible for many of arsenic's pathological effects. Indeed, cells with higher rates of arsenic metabolism show increased susceptibility to oxidative damage to DNA and induction of tumorigenesis (Styblo et al., 2000, Sakurai et al., 2006). These organic arsenic species can also promote cytotoxicity and tissue pathology by decreasing the levels of several antioxidants. For instance, in comparison to iAs(III), much lower concentrations of MMA(III) over shorter periods of time can alter the activity of glutathione peroxidase and catalase, induce expression of stress proteins and metallothioneins, and increase ROS levels (Calatayud et al., 2013). Other proposed mechanisms for the enhanced toxicity of MMA(III) include greater rates of cell permeation and accumulation (Wang et al., 2015), differences in subcellular distribution, and selective toxicity towards subcellular compartments such as the mitochondria (Naranmandura et al., 2011).

Several studies have investigated the effects of arsenic on VSMCs, which comprise the medial cell layer of vascular arteries and are known to be exposed to arsenic through contact with blood plasma. In vivo, it has been reported that iAs(III) increases blood pressure through calcium sensitization in VSMCs (Lee et al., 2005a, 2005b), and that exposure to MMA(III) at high doses induces VSMC dysfunction by impairing voltage-sensitive Ca^{2+} channels and reducing vascular reactivity to contractile agonists. In addition to inducing apoptosis of VSMCs (Lee et al., 2005a, 2005b, Bae et al., 2008; Martin-Pardillos et al., 2013), arsenic trioxide has been shown to induce apoptosis in human coronary smooth muscle cells (Luan et al., 2009), microvascular cells (Suriyo et al., 2012), human umbilical vein endothelial cells (Shi et al., 2010), and intestinal endothelial cells (Calatayud et al., 2013). However, some studies have demonstrated a proliferative effect of arsenic on VSMCs (Soucy et al., 2004) and endothelial cells (Barchowsky et al., 1999), presumably by activating mitogenic signals and inducing the release of vascular endothelium growth factor. Bimodal effects of MMA(III) have also been demonstrated. Lim et al. reported that MMA(III) elicited higher vasopressor responses at low doses while suppressing vasoconstriction at high doses in vivo (Lim et al., 2011). Collectively these data suggest that the variable effects of these arsenical species may be caused by differences in concentrations and exposure duration, differences in cell culture conditions, tissue preparations of ex vivo models of arsenic toxicity, and cell type analyzed in each study.

As previously mentioned, mitochondria may be a primary target of arsenic in several tissues. Indeed, exposure to arsenic is associated with loss of mitochondrial membrane potential in human pulmonary cell lines (Han et al., 2008), and reduced ATP content in rat liver mitochondria (Hosseini et al., 2013). In addition, MMA(III), but not iAs(III), can selectively target and inhibit mitochondrial complexes II and IV in isolated mitochondria from rat liver (Naranmandura et al., 2011), and induce ROS levels in intact mitochondria in human epithelial cells (Calatayud et al., 2013). The effects of arsenic species on mitochondrial structure/function and mechanisms of toxicity in VSMCs however, remain to be elucidated.

Our study is the first to comparatively analyze the effects MMA(III) and iAs(III) on mitochondrial structure and function in immortalized rat aortic smooth muscle A7r5 cells, a tissue culture model of VSMCs. The doses of iAs(III) and MMA(III) used in this study are in accordance with environmental exposures of at-risk populations reported in several studies (Lerda, 1994; Warner et al., 1994; Gebel, 2001; Wang et al., 2002) and have been used in previously published studies (Styblo et al., 2000, Naranmandura et al., 2011; Calatayud et al., 2013). In this study, we report that MMA(III), but not iAs(III), promotes mitochondrial dysfunction, metabolic and morphological alterations, and oxidative stress compared to untreated VSMCs. We also found that MMA(III)-mediated induction of ROS preceded the loss of mitochondrial content and cell death. Overall, our data support a conceptual model that suggests that MMA(III) impairs mitochondrial function by eliciting mitochondrial and non-mitochondrial sources of ROS that contribute to cytopathology of VSMCs.

2. Materials

Dulbecco's Modified eagle's media (DMEM), trypsin-EDTA solution (0.25% Trypsin, 0.02% EDTA), fetal bovine serum (FBS), antibiotic-antimycotic (ABAM) (100×), and Amplex® Red Hydrogen Peroxide/Peroxidase Assay kit were purchased from Invitrogen (Carlsbad, CA). Trypan Blue solution (0.4%), RIPA buffer, inorganic arsenic (As_2O_3) (iAs(III)), 4′,6-diamidino-2-phenylindole (DAPI) nuclear stain, sodium pyruvate solution, sodium hydroxide pellets, methyl iodide, hydrochloric acid, sulfur dioxide, protease inhibitor cocktail, adenosine 5′ -triphosphate (ATP) disodium salt hydrate, dithiothreitol (DTT), catalase from bovine liver, and Bradford Assay Kit were obtained from Sigma-Aldrich (St. Louis, MO). GlutaMax (GIBCO) and MitoSOX (Molecular Probes) were purchased from Thermo Fisher Scientific (Waltham, MA). MnTBAP chloride hydrate was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The CellTiter-Glo® Luminescent Cell Viability Assay kit and the CellTiter 96® AQueous One Solution Cell Proliferation Assay kit were obtained from Promega (Madison, WI). The XF Cell Mito Stress Test Kit was purchased from Seahorse Biosciences (Billerica, MA). The broad-spectrum caspase inhibitor Z-VAD-FMK was purchased from Enzo Life Sciences, Inc. (Farmingdale, NY).

The following antibodies were used: mouse anti-OXPHOS (Abcam), rabbit anti-β-tubulin (Abcam), rabbit anti-Tom20 (Santa Cruz Biotech), rabbit anti-cleaved caspase-3 (Cell Signaling Technology), rabbit IgG and mouse IgG secondary antibodies conjugated to horse radish peroxidase (GE Healthcare Bio-Sciences, Pittsburgh, PA), and Alexa 568-conjugated donkey anti-rabbit IgG secondary antibody (Molecular Probes, Eugene, CA).

Diiodomethylarsine (CH_3AsI_2), a precursor of monomethylarsonous acid (MMA(III)), was synthesized in the Angermann lab as described by Millar et al. (1960). In brief, arsenic(III) oxide (As_2O_3) and sodium hydroxide were combined in a solution, to which ethyl alcohol and 1.6 M methyl iodide were added. The resulting solution was cooled in an ice bath under continuous stirring for 20 h, and evaporated to dryness under vacuum. A fraction of the solid generated was diverted, reconstituted in ethanol, and recrystallized 2× from ethanol as disodium methanearsonate ($Na_2(CH_3AsO_3)$; 'MMA(V)'), which presented as white crystals melting at 161 °C. The remainder of the residue was dissolved in water, to which concentrated hydrochloric acid, sulfur dioxide, and potassium iodide (KI) were added, generating a precipitate which was then filtered out, washed twice, and discarded; the lower layer of diiodomethylarsine (the diiodo- salt of MMA(III)) was subsequently separated from the filtrate and recrystallized 2× from methanol. The final product was washed with ice water, vacuum dried, and the resultant yellow crystals were verified as diiodomethylarsine by capillary melting point determination at 30 °C (Millar et al., 1960). Purity of MMA(III) was further confirmed via 400 MHz ^1H NMR spectroscopy on a Varian 400-MR instrument (Fig. S1). Diiodomethylarsine crystals were stored at −80 °C. MMA(III) working solutions were made fresh as needed by dissolving diiodomethylarsine in ultrapure water, storing at 4 °C, and using within 24 h. Arsenic compounds (iAs(III) and MMA(III)) are carcinogens and skin irritants. Health Precautions: Proper precautions should be taken to avoid inhalation or direct skin contact by wearing proper laboratory attire including a laboratory coat, safety goggles, and nitrile gloves when handling these compounds.

3. Methods

3.1. Vascular smooth muscle cell cultures

Immortalized rat aortic smooth muscle cells (A7r5) were generously provided by Dr. Dean Burkin (Department of Pharmacology, University of Nevada School of Medicine). Cells were seeded in polystyrene tissue culture treated T-75 flasks (Sigma-Aldrich) in DMEM containing 4500 mg/L glucose and 100 mg/mL sodium pyruvate supplemented

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