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Methylation of Inorganic Arsenic by Murine Fetal Tissue Explants

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Abstract

Although it is generally believed that the developing fetus is principally exposed to inorganic arsenic and the methylated metabolites from the maternal metabolism of arsenic, little is known about whether the developing embryo can autonomously metabolize arsenic. This study investigates inorganic arsenic methylation by murine embryonic organ cultures of the heart, lung, and liver. mRNA for AS3mt, the gene responsible for methylation of arsenic, was detected in all of embryonic tissue types studied. In addition, methylated arsenic metabolites were generated by all three tissue types. The fetal liver explants yielded the most methylated arsenic metabolites (~7% of total arsenic/ 48 hr incubation) while the heart, and lung preparations produced slightly greater than 2% methylated metabolites. With all tissues the methylation proceeded mostly to the dimethylated arsenic species. This has profound implications for understanding arsenic-induced fetal toxicity, particularly if the methylated metabolites are produced autonomously by embryonic tissues.

Keywords

arsenicals; metabolism; development

Introduction

Several epidemiological studies show increase in human fetal and infant death as a result of chronic exposure to arsenic (Ahmad et al., 2001; Milton et al., 2005). Pregnant mice exposed to high levels of arsenic also results in higher rate of fetal resorptions (Wang et al., 2006), which are usually related to severe structural heart malformations. Exposure to high arsenic concentrations during key developmental periods in a mouse model predisposes animals to cancer (Nohara et al., 2012; Waalkes et al., 2003).

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Declaration of Interest

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Many human populations are exposed to a range of arsenic concentrations in the parts per billion (ppb) ranges over protracted periods of time including fetal development and early life. These are critical developmental periods for heart, lung, and liver, potentially making the development of these organ systems susceptible to effects of arsenic. In this regard, our laboratory reported mice exposed to 100 ppb As(III) in drinking water *in utero* (ED 6-term) developed non-alcoholic fatty liver disease as adults (Sanchez-Soria et al., 2014). In addition, others have shown that in ApoE^{-/-} mice, *in utero* drinking water exposure (ED 8-term) to 49 parts per million (ppm) arsenite resulted in an inflammatory state in the adult liver (States et al. 2012) as well as increased atherosclerosis (Srivastava et al. 2007).

The bioprocessing of inorganic arsenic (iAs) occurs through biochemical reduction and methylation steps producing detectable methylate species including monomethylarsonous acid [MMA(III)], dimethylarsonous acid [DMA(III)], monomethylarsonic acid [MMA(V)], and dimethylarsinic acid [DMA(V)] (Challenger, 1951; Dheeman et al., 2014). The methylation of arsenic by arsenite methyltransferase (As3mt) is an important pathway to eliminate the metal (Ding et al., 2012). Although MMA(V) and DMA(V) are generally less cytotoxic than As(III), MMA(III) and DMA(III) are more cytotoxic than As(III) (Styblo et al., 2000).

The efficiency in arsenic methylation is increased in the first trimester of folate supplemented pregnant women independent of plasma folate levels (Gardner et al., 2011) and iAs and its metabolites cross the placental barrier into the fetus (Concha et al., 1998). This early first trimester increase in methylation and elimination of iAs suggests a maternal protective mechanism to limit exposure of the fetus to arsenic toxicity. Furthermore, if arsenic methylation is suppressed in pregnant mice, then there is an increase in fetal toxicity (Lammon et al., 2003). It is generally believed that the developing fetus is principally exposed to iAs and the methylated metabolites from the maternal metabolism of arsenic (Concha et al., 1998). However, whether embryonic tissues can process arsenic and if this is an efficient process is unknown. The localized concentrations of the more toxic arsenic metabolites, such as MMA(III), could have immediate and persistent toxic effects during organogenesis.

This study investigates general arsenic methylation by fetal organ cultures from the heart, lung, and liver: the earliest organs to form during embryogenesis. Dorsal murine embryonic fibroblasts (MEF) were used as controls as they have been shown to generate methylated arsenic metabolites after dosing with As(III) at an efficiency of approximately 1.45% (of total arsenic) after 48 hr (Bach et al., 2014).

Materials and Methods

2.1 Animals

Swiss Webster mice were purchased from Harlan Laboratories (Madison, WI.). Pregnant female mice were euthanized and all cells and tissues surgically isolated from E12 embryos as previously described (Camenisch et al., 2002); observation of the vaginal plug was considered E0.5 with verification of developmental stage by somite number. Target tissues were surgically removed in sterile 1× PBS and rinsed several times. One mg of embryonic

heart, lung, and liver and MEF were explanted onto hydrated type I collagen gels. MEF were prepared in DMEM culture medium by serial passage through sterile 18 gauge and 25 gauge syringes: single cell suspensions were cultured, allowing cells to adhere and all experiments were performed in passage number 6 or less. A total of 4 explants (or 4 mg of tissue) were placed on collagen gels in one well of four well plate (Nunc/ Fisher Scientific cat#176740) for culture and treatment conditions following a 6 hr attachment period. Cultures were incubated at 37°C with 5% CO₂ and 95% humidity. All animal use and experimental protocols followed University of Arizona Institutional Animal Care and Use Committee regulations and remained in accordance with institutional guidelines.

2.2 Arsenic dosing and speciation

Whole embryonic heart, liver, embryonic fibroblasts and lung organ cultures were performed on hydrated rat tail type I collagen (BD Biosciences, Bedford MA) as previously described (Camenisch et al., 2002). Cultures were exposed to 100, 375, and 750 ppb sodium arsenite [As(III)] and concentrations were validated by inductively coupled plasma mass spectrometry (ICPMS) by the Arizona Laboratory of Emerging Contaminants (ALEC) which is part of the University of Arizona Superfund Research Program. After 48 hr, culture media was removed and filtered using PES centrifugal filters with a 10K Dalton molecular weight limit (VWR cat#82031-348). Collagenase (StemCell Technologies cat#07902) treatment dissolved the collagen and samples were separated into cellular and aqueous phases by centrifugation. The aqueous gel fraction was filtered using PES centrifugal filters and the cell fraction subjected to nitric acid digestion (200 µl) for 1 hr at room temperature. This digested sample was added to 1 ml of double-distilled arsenic free water, and filtered as above. The cell fraction contained no detectable arsenic. All samples were stored at -20°C until analyzed.

2.3 Expression of As3mt in fetal tissues

E12 heart, lung, and liver tissue was microdissected from 12 day old embryos along with tails for MEFs (5 organs were pooled for replicates with an n=4) and immediately subjected to RNA isolation with TRIzol® according to the manufacturer's protocol (Life Technologies, Grand Island, NY, USA). 1 µg RNA was used to generate first strand cDNA using the Transcriptor First Strand cDNA Synthesis kit (Roche, Indianapolis, IN, USA). Real-time Polymerase Chain Reaction (qPCR) was performed using the TaqMan Master Primer-Probe System (Roche, Indianapolis, IN, USA). 40S Ribosomal Protein 7 (Rps7) was used as a housekeeping gene. The primer sequences for the genes of interest, and the corresponding proprietary fluorescein labeled probes were obtained from Roche's Universal ProbeLibrary (Table 1).

2.4 Determination of arsenic compounds

Arsenic speciation was performed by ALEC using a protocol adapted from Milstein et al., (Milstein, 2003). Since immediate access to ALEC was not available, absolute detection of MMA(III) and DMA(III) was not attempted. The samples from the incubations were analyzed for total MMA and DMA. Briefly, arsenic speciation was analyzed with high performance liquid chromatography (HPLC) coupled to ICPMS using anion exchange column; ammonium carbonate gradient on Agilent 7700x. Organic arsenic species was

discriminated by the coupled hydride generation in order to ensure analysis of inorganic arsenic forms. Quality assurance for arsenic speciation includes use of certified reference materials such as Arsenic Species in Frozen Human Urine (NIST 2669, National Institute of Standards and Technology, Gaithersburg, MD, USA). For each batch run, three samples are spiked with low to mid-range standards to monitor arsenic recovery. The detection of arsenic species is $\pm 10\%$ by this HPLC-ICPMS method in the ALEC core.

2.5 Statistical Significance

To determine significance, a one-way ANOVA followed by a Tukey's multiple comparison test was used utilizing Prism6 (GraphPad, La Jolla, CA, USA) for comparisons between groups: the reported p-value is the multiplicity adjusted p-value corrected for multiple comparisons.

Results

3.2 As3mt was expressed in all 3 tissue explants and the embryonic fibroblasts (Figure 1). Expression in the heart was significantly higher than in all other tissues. A lower, similar expression was observed in the lung, liver, and MEF. Because As3mt is expressed in every tissue, it was expected that all tissues would possess some capability to autonomously methylate arsenic.

3.1 To determine whether these select embryonic tissues can metabolize inorganic arsenic into methylated forms, primary tissues were exposed for 48 hr to various concentrations of As(III) and arsenic species determined. Media alone, cells alone, and gels alone produced no detectable levels of arsenite or metabolites (data not shown). The hydrated type I collagen gel incubation conditions (without tissue explants or cells) were capable of oxidizing a substantial portion of the As(III) to As(V) (data not shown), so no studies were performed test the ability of the tissue explants or cells to oxidize/reduce iAs.

All tissue explants produced both MMA and DMA metabolites at the highest dose - 750 ppb (Table 2). The values (ppb) are the combined arsenic species in the media supernatant and within the collagen gels. At the lower concentration of substrate [As(III)], MMA was not always detected but DMA was formed at all substrate concentrations and increased as the substrate concentration increased (Table 2).

The MEF in this study were able to methylate ~2% of the total arsenic to methylated metabolites (Figure 2). This is consistent with the 1.45% methylation rate detected previously in MEF (Bach et al, 2014). Compared to all examined tissue types, the liver explants generated the highest levels of methylated arsenic forms - ~7% of total arsenic (Figure 2). Lung and heart explants produced about the same amount of methylated metabolites as the MEF at ~ 2% of total arsenic. In all cases there was more DMA generated than MMA, indicating a predisposition to form the methylated metabolite that is the major urinary metabolite following arsenic exposure.

It is important to note that not all embryonic cell types converted As(III) to methylated metabolites. Primary murine epicardial cells were tested in this culture system and did not

produce any detectable levels of MMA or DMA even at the highest concentrations of As(III) (Table S1).

Discussion

Chronic exposure to arsenic is linked to a number of diseases as well as adverse pregnancy outcomes (Aschengrau et al., 1989; Milton et al., 2005). This current study shows that embryonic tissues can methylate arsenic producing the metabolites, MMA and DMA. This is the first report of embryonic heart, lung, and liver producing methylated species of arsenic. Both DMA and MMA were detected in the liver, heart, and lung explant cultures with liver producing almost 3 fold more methylated metabolites. This can provide new insight to observations such as those made by Tsang et al., (2012) showing detection of MMA and DMA in E18 fetal livers after maternal exposure. Previously it was unknown what, if any, capacity the fetal liver had to methylate arsenic, but the present data suggest that at least some of these metabolites are produced by the developing liver. Although there is indirect exposure to methylated forms produced maternally, this study identified a direct tissue production of arsenic metabolites during fetal exposure to inorganic arsenic. In addition, As3mt expression was detected in all examined tissues bolstering the argument that these fetal organs are capable of generating methylated metabolites of arsenic.

The heart, lung and liver are all target organs for toxicity by arsenic (Abernathy et al., 1999; Mandal and Suzuki, 2002). Smith's group studying the region II area of Chile, where there was high arsenic exposure (1958–1970), found a very significant increase in cardiovascular diseases (Yuan et al., 2007). They reported increased mortality from acute myocardial infarction by 10-fold in the population exposed during development. This was the first report revealing protracted developmental exposure to arsenic and cardiovascular disease in adulthood. Our group has also demonstrated that developmental exposure to 100 ppb As (III) led to adult incidence of non-alcoholic fatty liver disease (Sanchez-Soria et al., 2014). The mechanisms of arsenic-induced cardiovascular, lung, and liver diseases are not clear and the risks and biological effects associated with arsenic ingestion at lower levels commonly found in the U.S. remain ambiguous especially for *in utero* exposures.

This current study reveals that embryonic heart, lung, and liver tissue are capable of metabolizing arsenic as methylated metabolites in a whole organ explant culture system. This suggests that the more toxic metabolites may contribute to developmental changes in these target organs that predispose to disease in adulthood. Future studies are needed for detection of arsenic *in situ*, it will be important to determine the accumulation of various arsenic species in fetal tissues from exposure during development. The present study is just a first step identifying fetal capacity to methylate arsenic; we propose that it will be beneficial for others to examine the full profile of fetal metabolites across a range of doses and exposure profiles in various fetal tissues.

Conclusions

Developmental exposure to arsenic is implicated in adult disease states in multiple organ systems. Up until the present work, it was unknown if embryonic tissue contributed to the

generation of methylated arsenic metabolites, or if metabolites were solely attributed to maternal metabolism of arsenic. This study demonstrates that in a whole organ explant system, heart, lung, and liver are capable of generating methylated metabolites of arsenic and express the gene As3mt which is necessary for arsenic metabolism.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. As3mt Gene Expression in Fetal Tissue

As3mt is expressed in all 4 tissues examined via RT-qPCR. Expression in the heart is significantly higher than in all other tissues. There is a significant difference between expression in the MEF and the lungs. The longest horizontal line represents the mean and the error bars represent the standard deviation.

$p \leq 0.001$ = * Compared to No Cell; # Compared to Heart; † Compared to Lung; ‡ Compared to Liver; \$ Compared to MEF

Figure 2. Biotransformation of Arsenic in Fetal Tissue Explants

Combined total percentage of organic arsenic species in the media supernatant and within the collagen gels as a percentage of total arsenic detected for all three doses tested as determined by HPLC coupled to ICPMS. All tissues generate a significantly greater proportion of methylated metabolites than the no cell controls and the liver generated more than all other tissues examined. The longest horizontal line represents the mean and the error bars represent the standard deviation.

Table 1

Primers and Probes for RT-qPCR

The genes examined are arsenite methyltransferase (As3mt) and 40S Ribosomal Protein 7 (Rps7).

Gene	Left (5'-3')	Right (5'-3')	Probe	NCBI RefSeq
As3mt	TGCAGAAATGTACACGAAAGACG	AGCCGCTCAGGAAACAGTC	76	NM_020577.2
Rps7	AGCACGTGGTCTTCAITGCT	CTGTCAGGGTACGGCTTCTG	101	NM_011300.3

Table 2**Arsenic Metabolism by Murine Fetal Tissue Explants**

Arsenic speciation after 48 hr dosing of As (III) at 100, 375, or 750 ppb in embryonic heart, lung, liver, and MEFs as determined by HPLC coupled to ICPMS. Methylated metabolites are found at all doses in all tissues examined. MMA and DMA values are reported independent of oxidation state and the values represent the total ppb species concentration detected in the supernatant and the collagen gel combined.

Tissue	As (III) Dose (ppb)	Arsenic Speciation after 48 hr (ppb)				
		Total iAs	As (III)	As (V)	MMA	DMA
Heart	100	90.6	40.8	49.8	0.0	2.5
	375	343.9	171.7	172.1	0.0	10.4
	750	703.9	395.1	308.8	1.1	13.0
Lung	100	86.1	50.7	35.4	0.0	2.2
	375	326.3	176.9	149.3	0.2	8.0
	750	689.8	399.0	290.8	1.7	14.3
Liver	100	71.4	36.3	35.1	0.0	4.8
	375	269.3	155.4	114.0	0.0	21.1
	750	579.2	343.8	235.4	3.7	34.2
MEF	100	87.9	45.9	42.0	0.0	2.0
	375	306.6	161.9	144.7	0.1	6.4
	750	660.6	437.1	223.5	1.9	15.0