Proteomic Analysis of Arsenic-Exposed Zebrafish (Danio rerio) Identifies Altered Expression in Proteins Involved in Fibrosis and Lipid Uptake in a Gender-Specific Manner

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The zebrafish (Danio rerio) was used to investigate protein expression in the liver following arsenic exposure. Several disorders have been linked to arsenic exposure, including cancer, diabetes, and cardiovascular disease. The mechanisms of arsenic toxicity are poorly understood. Prior studies have described altered gene expression, inflammation, and mitogenic signaling in acute or chronic exposure models. A proteomic approach was employed to investigate arsenic-induced alteration in the zebrafish liver proteome following a 7-day exposure to 50 ppb sodium arsenite. Over 740 unique proteins were identified, with fewer than 2% showing differential expression. Molecular pathway analysis software identified lipid metabolism and transport as potential molecular targets. Immunoblots were used to confirm protein expression changes, whereas qPCR was employed to investigate gene expression changes. Overall, 25 proteins were differentially expressed in a gender-specific manner, 11 in males and 14 in females. Of these 25, a single protein, hydroxysteroid dehydrogenase like 2, showed decreased expression in both males and females following arsenic exposure. These findings indicate that protein expression is altered following arsenic exposure. The changes presented here seem to be most prevalent in lipid transport and metabolic pathways, suggesting a potential increase in fibrosis in males and decreased lipid accumulation and uptake in females.

Key Words: arsenic; zebrafish; proteomics; liver.

Numerous epidemiological studies have demonstrated that exposure to arsenic is associated with an increased incidence of lung, bladder, and skin cancer, as well as diabetes and cardiovascular disease (Erraguntla et al., 2012). Arsenic is present in drinking water throughout the world; levels in the ppm have been recorded in many locations (NRC, 2001). In New England, particularly Maine and New Hampshire, many private wells have arsenic levels that exceed the U.S. Environmental Protection Agency’s 10 ppb action level (Ayotte et al., 2003). Increased rates of lung and bladder cancer are seen in individuals exposed to greater than 64 mg/l (640 ppb) arsenic in ground water in Taiwan and China (Guo, 2011; Yunus et al., 2011). In several countries, including Taiwan and Mexico, arsenic exposure has also been linked to an increased incidence of type 2 diabetes (Del Razo et al., 2011; Guo, 2011). These epidemiological studies demonstrate a correlative relationship between chronic exposure to low levels of arsenic and disease development.

The mechanisms of arsenic toxicity are not well defined. In an attempt to understand the potential pathways involved in arsenic toxicity, a variety of animal models have been developed. Paul et al. (2011) have shown that mice exposed to high concentrations of arsenic (50 ppm) have impaired glucose homeostatic regulatory mechanisms. Chen et al. (2004) demonstrated that arsenic exposure (45 ppm) altered DNA methylation in 120/SvJ mouse liver cells, leading to long-term changes in expression of steroid-related genes, cytokines, and genes involved in the regulation of cell cycle and apoptosis. Human lung tumor cells, acutely exposed to 10 ppm arsenic, showed a mitogenic-like effect through extended epidermal growth factor (EGF) receptor activation (Andrew et al., 2009). The formation of reactive oxygen species (ROS) has also been attributed to arsenic exposure (Chowdhury et al., 2010). Female mice exposed to 10 ppb arsenic demonstrated increased liver steatosis and decreased levels of circulating triglycerides (Kozul-Horvath et al., 2012). These studies suggest that exposure to arsenic at different concentrations can impact multiple molecular pathways.

Recent advances in genomics and proteomics have afforded the research community the tools to investigate how exposure to arsenic affects global gene and protein expression. Zebrafish (Danio rerio) embryos have been used to study
mRNA expression changes following short-term (24 h) exposure to 100 ppb arsenic, resulting in altered immune function and cell cycle regulation (Mattingly et al., 2009). Serum from humans exposed to up to 274 μg/l arsenic in drinking water was subjected to SELDI-TOF analysis. Significant differences in the expression of five proteins were observed among treatment groups (Zhao et al., 2010). Studies investigating protein expression changes following arsenic exposure in several different organisms, including a fresh water ciliate, *Tetrahymena pyriformis*, the bacterium *Klebsiella pneumoniae*, and several plants have shown an overt oxidative stress response (Daware et al., 2012; Tripathi et al., 2012; Zhang et al., 2012). High throughput microarray or protein expression analyses have helped identify several pathways of arsenic toxicity and demonstrate that its action varies based on dose and duration, as well as the model.

The zebrafish has been developed into a robust platform for toxicology research for several reasons, including low maintenance cost, fast generation time, well-defined developmental processes, and sequenced genome. Embryos can be rapidly screened for toxicological effects, whereas adults facilitate tissue-specific studies (Forné et al., 2010). The use of zebrafish to investigate potential endocrine-disrupting compounds was recently reviewed (Segner, 2009). Gene expression changes during tumor growth and progression are conserved between zebrafish and humans (Ung et al., 2009). Mirbahai et al. (2011) investigated alterations in liver-specific DNA methylation at Cpg islands over time following carcinogen exposure. Proteomic-specific approaches to single organs in zebrafish have also recently been described for brain, liver, kidney, muscle, and fin (Abramsson et al., 2010). An investigation into the effects of atrazine exposure for 14 days on the adult zebrafish liver showed 7 out of 600 proteins with greater than 50% change in expression compared with untreated animals (Jin et al., 2012). Many of the molecular interactions described in the zebrafish are similar to those described in mouse and human models.

This study investigates protein expression in the liver of adult male and female zebrafish following exposure to 50 ppb sodium arsenite for 7 days. MALDI-TOF/TOF mass spectrometry was used to investigate protein expression. Follow-up experiments with immunoblots and mRNA expression analysis were used to confirm the protein expression. We hypothesized that we would identify changes in protein expression attributable to the onset of arsenic-associated toxicity.

**MATERIALS AND METHODS**

**Animal treatment.** AB strain adult zebrafish, between 4 and 8 months old, were maintained at 28°C on a 14/10 light/dark cycle and used in accordance with standard care practices at the University of Maine Zebrafish Facility. Fish were fed standard lab diet every other day (Hikari, Hayward, CA). Fish were exposed to 0 or 50 ppb NaAsO₂ (Sigma, St Louis, MO) for either 4h (quantitative real-time PCR; qPCR) or 7 days (qPCR and protein analyses) in static treatment tanks with 2 l water. Three tanks per gender/treatment were used for both the mass spectrometry and immunoblot protein expression assays; each tank contained five fish (pooled to obtain sufficient tissue). Fish for gene expression analysis by qPCR were housed ten to a tank per gender/treatment; upon harvest, livers from two fish were pooled to generate five biological replicates. Treatment water (0 or 50 ppb NaAsO₂) was renewed twice daily by siphoning water off, followed by the addition of fresh treatment water. No dead animals were observed during the exposure period.

**RNA and protein extraction.** RNA was extracted from liver tissue pooled from two fish and homogenized by micropestle following the manufacturer’s protocol (RNAqueous, Ambion, Austin, TX) followed by Turbo DNase treatment (Ambion). RNA concentration was determined by UV spectrophotometry, and integrity was determined using a Bioanalyzer 2100 (Agilent, Santa Clara, CA). For protein expression, liver tissues from five fish were pooled and homogenized by micropestle in 8M urea in PBS, pH 7.6, with complete protease inhibitor (Roche, Indianapolis, IN). Liver homogenate was centrifuged for 10 min at 16,000 x g, 4°C, the supernatant extracted, and protein concentration measured using BCA Protein Assay (Thermo Fisher, Rockford, IL).

**Protein expression analysis.** Sample preparation and analysis for three biological replicates followed methods described by Ernoult et al. (2009) with the following modifications. Protein aliquots (100 μg) were reduced with 10mM dithiothreitol (DTT; Sigma) for 1 h at 25°C and alkylated with 50mM iodoacetamide (Sigma) in the dark at 25°C for an additional hour. A second aliquot of DTT was added to quench the iodoacetamide. The urea concentration was reduced to below 0.5M by the addition of 50mM sodium bicarbonate (Sigma), and enzymatic cleavage was carried out with sequencing-grade Trypsin Gold (Promega, Madison, WI) for 12–16 h at 37°C. The peptides were purified by solid phase extraction and differentially labeled using iTRAQ 4-plex reagents (Applied Biosystems, Foster City, CA). The four labeled samples (arsenic-exposed male and female; control male and female) were then combined into one tube, dried, and rehydrated in 1% acetic acid (ThermoFisher, Pittsburgh, PA). Samples were separated on nonlinear immobilized pH gradient (IPG) 10-cm isoelectric focusing strips (pH 3–10; Bio-Rad, Hercules, CA) with the following parameters: equilibration for 12h, 120V for 1 h, gradient to 340V over 3 h, gradient to 1500V over 3 h, gradient to 3120V over 3 h, and held for 40,000 total volt hours. IPG strips were cut into ten 1-cm sections, and peptides were extracted by incubation in 0.01% formic acid (Sigma) for 1 h at room temperature. Extracted peptides were dried and rehydrated in 1% acetic acid and then separated on a C18 column (YMC-Gel, Allentown, PA) with the following parameters: equilibration for 12h, 120V for 1 h, gradient to 340V over 3 h, gradient to 1500V over 3 h, gradient to 3120V over 3 h, and held for 40,000 total volt hours. IPG strips were cut into ten 1-cm sections, and peptides were extracted by incubation in 0.01% formic acid (Sigma) for 1 h at room temperature. Extracted peptides were dried and rehydrated in 1% acetic acid. The samples were then separated on a C18 column (YMC-Gel, Allentown, PA) with a 60-min, 5–40% acetonitrile gradient mixed with α-Cyano-4-hydroxycinnamic acid (Sigma) matrix and spotted onto a standard MALDI plate at 0.1 μl/min and 15 μl/spot. Samples were analyzed using a 4800 plus MALDI TOF/TOF mass spectrometer (AB Sciex, Foster City, CA). Peaks were identified using Protein Pilot 3.0 software, searching the *Danio rerio* protein database (Build 3.63, Uniprot). Only peptides with greater than 90% confidence were used. Protein identification and quantification required the detection of at least one and three peptides, respectively. Protein expression data were assessed following log-2 transformation, where a + 1 value represents a 50% increase, and a − 1 value represents a 50% decrease. Proteins from arsenic-treated animals with greater than 50% change in expression relative to untreated, gender-specific controls were selected for further investigation.

**qPCR analysis.** RNA was reverse transcribed into cDNA using the SuperScript II System (Invitrogen, Carlsbad, CA) with both oligo dT and random primers, as per manufacturer’s recommendations. Primers for gene targets were identified using BLAST primer design (Table 1), and efficiency was determined to be between 90 and 115%. cDNA was then subjected to quantitative PCR using the PerfeCTa qPCR Supermix, low ROX (Quanta, Gaithersburg, MD) and ABI 7500 Real-Time PCR system (Applied Biosystems). Data were normalized to *β-actin* expression and analyzed based on the method defined by Pfaffl (2001), with significance determined by a two-tailed t-test.

**Immunoblots.** Proteins were separated on 10% bis-acrylamide gels, and immunoblotting was performed using standard methods provided by the manufacturer (Life Technologies, Carlsbad, CA). Protein expression was analyzed using the following antibodies: rabbit anti-fatty acid–binding protein (SC50380; Santa Cruz Biotechnology, Santa Cruz, CA), mouse anti-β-actin.
TABLE 1

<table>
<thead>
<tr>
<th>Gene</th>
<th>Expected amplicon</th>
<th>With intron</th>
<th>Forward primer 5′-3′</th>
<th>Reverse primer 5′-3′</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hnf4α</td>
<td>128</td>
<td>1500</td>
<td>CACTACAGAGCATCACCTGGC</td>
<td>AGCGTAGGCTGCCATTTAGC</td>
</tr>
<tr>
<td>Hsd12</td>
<td>128</td>
<td>2227</td>
<td>CCCTCTTGGATACATTGCGTTC</td>
<td>TGGCCCTTGGCAAATACCTCGA</td>
</tr>
<tr>
<td>Pparaγ</td>
<td>130</td>
<td>11,450</td>
<td>AGCGTGAGGTGCTTCATTAGC</td>
<td>AAAACCCCTTGCATCCCTCGA</td>
</tr>
<tr>
<td>Gapdh</td>
<td>140</td>
<td>0</td>
<td>CCGTCTTGAAGAACCTGCAAG</td>
<td>TGGATGAACGGCAATCCACA</td>
</tr>
</tbody>
</table>

Note. *Forward primer spans exon/exon boundary; no product is expected if intron is present.

Protein Expression Analysis

Individual data sets obtained from iTRAQ-labeled peptides were combined and generated a total of 37,989 spectra; from those, 15,933 distinct peptides were identified. Peptides were then assigned to proteins using Protein Pilot 3.0; 745 proteins were identified by at least one specific peptide (Supplementary tables 1A and B). To increase confidence in the proteins identified, at least three specific peptides were used, narrowing the proteins identified to 147. Of these, eleven proteins isolated from livers of male and fourteen from female fish showed differential expression (greater than 50%, relative to controls) (Fig. 1).

Ingenuity Pathway analysis identified two transcription factors, peroxisome proliferator–activated receptor γ (PPARγ) and hepatocyte nuclear factor 4α (HNF4α), both previously associated with arsenic exposure (Nelson et al., 2009; Xue et al., 2011), but not directly detected in our proteomic analysis (Fig. 2).

Immunoblots

To confirm protein expression changes detected by mass spectrometry protein expression analysis, two representative proteins, FABP2 and TPM3, were analyzed by immunoblotting (Fig. 3). FABP2 had 1.6-fold increased expression ($p = 0.008$) in arsenic-treated male fish (Fig. 3), whereas TPM3 had a 1.9-fold increased expression ($p = 0.03$) in arsenic-exposed female fish relative to controls (Fig. 3), supporting the mass spectrometry data.

qPCR

qPCR was used to investigate changes in expression patterns of mRNA of two of the transcription factors identified in the molecular pathway analysis, Ppara and Hnf4α, and Hsd12, whose corresponding protein product was the only one similarly expressed in males and females. mRNA expression was measured at two time points, 4 h and 7 days, following onset of exposure. All transcript levels were normalized to expression.
of glyceraldehyde 3-phosphate dehydrogenase. No significant differences in mRNA expression were detected at either 4 h or 7 days post-exposure for any genes (Fig. 4).

**DISCUSSION**

Several investigations have described how arsenic can alter gene expression or protein function by inducing oxidative stress and increasing mRNA expression in response pathways (Liu et al., 2010; Ruiz-Ramos et al., 2009). EGF receptor–mediated pathways can be activated by arsenic, leading to altered gene expression in mouse lung (Andrew et al., 2009). Zebrafish embryos exposed to 2 or 10 ppb arsenic showed decreased expression of genes involved in the innate immune response (Nayak et al., 2007). In addition, DNA methylation and histone acetylation can be altered by arsenic exposure (Hong and Bain, 2012; Nohara et al., 2010; Reichard and Puga, 2010). These studies describe several mechanisms by which arsenic can influence gene expression.
FIG. 2. Interaction maps show potential connections among differentially expressed proteins in adult male (A) and female (B) zebrafish. Proteins include SCP2, HB1, TPM3, GSTM, RPLP1, HSDL2, RPL18, UQCR1C, and PABPC1A. Shaded boxes represent proteins with altered expression as determined by proteomic analysis: □ cytokine, ○ group or complex, ▴ ligand-dependent nuclear receptor, ◇ peptidase, △ transporter, ◆ chemical/drug, or other entity.
Of the 25 differentially expressed proteins identified in this study, only HSDL2 was found to be decreased in both male and female zebrafish. Arsenic exposure has been shown to affect the enzymatic activity of several different hydroxysteroid dehydrogenase enzymes. Wistar rats were exposed to 400 ppb arsenic for 28 days and showed an approximately 40% decrease in Δ5-3β-hydroxysteroid dehydrogenase levels and a 30% decrease in 17β-hydroxysteroid dehydrogenase activity and decreased circulating estrogen (Chattopadhyay et al., 1999). Liu et al. (2008) examined both a tissue culture and an in vivo model. Primary liver cells from gestation day 13.5 mouse pups exposed to 1 μM sodium arsenite (78 ppb) for 72 h showed an increase in 17β7-hydroxysteroid dehydrogenase RNA expression. In vivo, CD1 mice exposed to 85 ppm arsenic exhibited increased 17β5-hydroxysteroid dehydrogenase enzyme activity in adrenal gland tissue (Liu et al., 2009). Reddy et al. (2011) exposed male Swiss albino mice to 4 ppm arsenic for 35 days and found a decrease in both 3β- and 17β-hydroxysteroid enzyme activity in the testes and decreased sperm mobility and viability. HSDL2 is a member of the short-chain dehydrogenase/reductase (SDR) family and is believed to be involved in lipid metabolism and steroidogenesis (Kowalik et al., 2009; Skogsberg et al., 2008). Decreased expression of HSDL2 in this study may be part of the response to limit production of steroid hormones. It appears that arsenic exposure alters the expression of several hydroxysteroid dehydrogenases in a variety of model systems and may result in the generation of gender-specific effects through steroidogenesis mechanisms.

A number of other proteins were found to be differentially expressed in male, but not female zebrafish, liver following arsenic exposure (Fig. 1). Liver collagen is known to be altered following long-term exposure to arsenic (Ghatak et al., 2011) and is believed to be related to fibrosis. EPDL2, through protein sequence and ontology, is likely involved in cell matrix...
adhesion. A variety of peptidases, but not CPB1 specifically, have been shown to be altered by exposure to arsenic and may be related to the induction of apoptosis (Shen et al., 2011). Decreased FABP1 and FABP2 protein and mRNA expression have been linked to the development of nonalcoholic, fatty liver disease in humans (Charlton et al., 2009). RPSA, also referred to as laminin receptor 1, has not been linked to arsenic exposure but has been linked to preventing fibrosis (Wenzel et al., 2010). Therefore, its decrease would also suggest an increase in fibrosis. RAN, a Ras-related G protein, has not been directly linked to arsenic exposure, but changes in other members of this family are known (Benbrahim-Tallaa et al., 2007). EPHX2 has not previously been associated with arsenic exposure but has a role in inflammatory response in conjunction with members of the cytochrome P450 protein family (Tanaka et al., 2008). Although no data describing the function of NMDMC are available, amino acid sequence analysis suggests that it may have a role in nuclear division. Overall, these observed changes suggest increased fibrosis due to altered cell adhesion and structure and the potential for increased fatty acid transfer and uptake following arsenic exposure in males.

Several proteins were differentially expressed in females, but not in males (Fig. 1). Liver tropomyosin expression is increased during fibrosis (Otogawa et al., 2009). Increased expression of TPM3 has also been reported in cell culture models for hepatocellular carcinoma (Lam et al., 2012). RPL18 regulates protein synthesis, and although it has not previously been associated with arsenic exposure, increased expression is thought to induce cell replication (Kumar et al., 1999). CKMT1 is essential for transferring phosphate groups; increased expression is noted in several in vitro models for prostate cancer, correlated with increased production of ROS and inflammation (Pang et al., 2009). In human kidney cells, decreased RPLP1 gene expression has been shown to decrease cell growth rate (Martinez-Azorin et al., 2008). Although its role in the liver has not been described, data from studies in the kidney suggest that increased RPLP1 may point to higher ribosomal activity and protein translation and/or increased cell replication. EIF4H has previously been identified in zebrafish only as untranslatable mRNA, but protein orthologues in human and mouse have been described as translation initiation factors. Like RPL1, increased expression of EIF4H may increase protein translation in a nonspecific manner. SCP2 is involved in intracellular lipid transfer and saturated fatty acid uptake (Storey et al., 2012). UQRC1 regulates cellular lipid accumulation; decreased expression inhibits uptake from circulation (Kunej et al., 2007). CYP2J is involved in toxicant metabolism and cholesterol synthesis and functions in conjunction with EPHX2 (Xu et al., 2011). GSTM is a member of the glutathione-S-transferase protein family and is involved in carcinogen and heavy metal detoxification. Although other family members have been linked to arsenic exposure, GSTM has not, and its decreased expression is counterintuitive (Goodrich and Basu, 2012). PABPC1A is involved in regulating gene expression by binding to polyadenylated mRNA; it is a common target of viral proteases in that loss of function has a nonspecific effect on mRNA stability (Smith and Gray, 2010). Protein expression for TRP1L has not previously been demonstrated; sequence homology places it in the trypsin peptidase family. As with CPB1 in males, peptidase activity has been associated with apoptosis; unlike CPB1, TRP1L expression was decreased, suggesting an opposite effect in females. Zgc:123333, currently described only by an open reading frame from the zebrafish genome project, has an undetermined function; sequence and gene ontology analysis offer a potential function in carbohydrate metabolism via its hydrolase activity. In summary, proteins with increased expression are involved in protein synthesis and cell division; those with decreased expression would limit lipid uptake and use, destabilize mRNA, and limit detoxification capacity. These findings suggest that female liver may have a general increase in protein synthesis and have a decreased capacity for lipid utilization.

The investigation into mRNA expression using qPCR did not show any significant change in expression following either 4 h or 7-day exposure to arsenic. It has been demonstrated that both 3T3-L1 cells exposed to up to 2μM arsenic trioxide and white leghorn chicken embryos exposed to up to 6.6μM sodium arsenite led to decreased Pparγ mRNA expression (Lencinas et al., 2010; Xue et al., 2011). Hnf4α mRNA expression was decreased in C3H mice exposed perinatally to 85 ppm sodium arsenite (Nelson et al., 2009). The current study used the lower concentration of 50 ppb (0.64μM) arsenic, and change in mRNA expression may not be statistically evident at this exposure, species, age, or tissue type. Hsd12 regulation has not been previously described, and mRNA expression changes were not evident.

The response to arsenic exposure was expected to differ between males and females. These data suggest increased lipid utilization in males but decreased uptake in females. Gender-specific effects have been noted in a number of different species in various tissue types. Microarray analysis of the killifish Fundulus heteroclitus exposed to arsenic showed altered expression of many genes in males and females, but less than 10% of the differentially expressed genes were similar in both genders (Gonzalez et al., 2010). Gender differences in CpG island methylation in mice exposed to arsenic were detected following arsenic treatment (Nohara et al., 2010). Investigations of mouse and human blood cell progenitor cells treated with arsenic also showed gender-specific effects (Ferrario et al., 2008). With the exception of HSDL2, data from the current study describe a gender-specific response to arsenic. Also of note, the interaction networks identified here were gender specific. Overall, both male and female zebrafish show changes in proteins involved in lipid metabolism and transport, but the specific pathways affected may differ. Gender specificity could be due to metabolic needs, different circulating hormones, or activation of endocrine receptors. Overall, we have demonstrated that exposure of zebrafish to low-dose sodium arsenite (50 ppb) resulted in gender-specific responses of the liver proteome. The expression changes described in the male liver are associated with fibrosis, which
is related to dysfunctional lipid metabolism, and may be an early event in arsenic toxicity (Moustafa et al., 2012). Altered proteins in female liver are related to increased cell growth and decreased lipid accumulation. The changes in expression shown here describe mechanisms that have been previously linked to fibrosis and lipid deregulation in humans, which over a prolonged period may lead to decreased liver function.

SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

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