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Molecular mechanisms of selenium-Induced spinal deformities in fish

Allison Kupsco*, Daniel Schlenk

Environmental Toxicology Program and Department of Environmental Sciences, University of California-Riverside, Riverside, CA, United States

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ABSTRACT

Selenium toxicity to oviparous vertebrates is often attributed to selenomethionine (SeMet), which can biomagnify through maternal transfer. Although oxidative stress is implicated in SeMet toxicity, knowledge gaps remain in how SeMet causes characteristic spinal deformities. In the present study, we use the Japanese medaka (*Oryzias latipes*) model to investigate the role of oxidative stress, cell death, and the unfolded protein response (UPR) on skeletal gene expression and SeMet toxicity, linking localization of cellular effects to observed abnormalities. Medaka embryos were treated with 2.5 μM or 5 μM SeMet for 24 h at stage 25 (48 h post fertilization). Post treatment, embryos were separated into normal, deformed (mild, moderate or severe), or dead categories. Dichlorofluorescein staining demonstrated oxidative stress in tails of embryos with observable spinal malformations. Furthermore, acridine orange staining for apoptosis identified significantly more dead cells in tails of treated embryos. Gene expression studies for the UPR suggest a potential role for CHOP (c/ebp homologous protein) induced apoptosis deformed embryos after 5 μM SeMet, accompanied by a significant decrease in PDIA4 (protein disulfide isomerase A4) and no change in Dnajb9 (ER DNA J Domain-Containing Protein 4). This expression was distinct from the UPR induced by well-studied ER stress inducer, tunicamycin, which robustly activated CHOP, PDIA4 and Dnajb9. Finally, SeMet treatment significantly decreased transcripts of cartilage development, Sox9 (SRY box 9), while increasing Runx2 in deformed embryos, without altering Twist or Collagen 2a1. Results suggest that oxidative stress, the UPR and cell death play key roles in SeMet induced deformities and altered skeletal development factors.

1. Introduction

Selenium (Se) is an essential micronutrient with a narrow margin between essentiality and toxicity to oviparous vertebrates. Although Se is present naturally in soils, anthropogenic disturbance can release Se into waterways. Free waterborne Se is bioaccumulated at low trophic levels, and integrated into selenomethionine (SeMet), which is then incorporated non-specifically into proteins (Fan et al., 2002). Thus, vertebrate consumption of Se in the diet is often in the form of SeMet. SeMet can be maternally transferred in vitellogenin to developing embryos, where it exerts developmental toxicity causing teratogenesis and mortality. Common abnormalities from Se include spinal deformities, such as lordosis, cranio-facial abnormalities, and fin deformities (Lemly, 1997). In particular, skeletal deformities are characteristic of Se toxicity.

Speculations about the mechanism of toxicity have suggested a role for oxidative stress in Se embryo toxicity (Lavado et al., 2012; Palace et al., 2004; Misra et al., 2012; Arnold et al., 2016). However, most studies were performed with high concentrations of SeMet, only a few link oxidative stress to an adverse outcome, and none have showed it occurring at the sites of malformations. Furthermore, other research suggests that oxidative stress is not the only molecular and cellular disturbance caused by SeMet (Kupsco and Schlenk, 2014).

Oxidative stress has been linked to the unfolded protein response (UPR) in several studies (Cao and Kaufman, 2014). The UPR is an integrated stress response activated by an increase in unfolded proteins in the endoplasmic reticulum (ER), which causes ER stress. The response induces increases in protein folding capacity, translational attenuation, mRNA degradation, and proteolysis (Hetz, 2012). Activation of the UPR occurs when the master regulatory chaperone BiP dissociates from the three branches, ATF6 (Activating transcription factor 6), PERK (PKR-like endoplasmic reticulum kinase) and IRE1 (Inositol requiring enzyme 1), each with some independent function. IRE1 is primarily responsible for degradation of mRNA and proteins. PERK is responsible for translational attenuation and ATF6 for an increase in folding capacity (Hetz, 2012). If the stress remains uncorrected, the UPR will initiate apoptosis via C/EBP homologous protein (CHOP). Apoptosis is pro-

* Corresponding author at: Geology 2460, Environmental Sciences, University of California-Riverside, 900 University Ave, Riverside, CA 92521, United States.
E-mail address: akups001@ucr.edu (A. Kupsco).

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grammed cell death that is also a common outcome from oxidative stress, which may affect developing chondrocytes. The UPR is further connected to skeletal development through the PERK pathway, and PERK and ATF4 (Activating transcription factor 4) knockout models result in osteopenia and reduced bone mineralization and collagens (Zhang et al., 2002; Yang et al., 2004).

In the present study, we further characterized mechanisms of SeMet-induced developmental toxicity with the model organism, the Japanese medaka (*Oryzias latipes*). We investigated the localization of oxidative stress and apoptosis in malformations following SeMet exposure and examined gene expression of UPR and skeletogenesis genes. We chose downstream UPR target genes PDIA4 (protein disulfide isomerase associated 4), which is involved in disulfide bond formation and activated via ATF6; CHOP, which is involved in UPR induced apoptosis through PERK and ATF4; and Dnajb9 (also ERdj4), a chaperone involved in ER associated degradation. Skeletal development is regulated by various chondrogenic and osteogenic factors. In the present study, we focused on SRY-box protein 9 (Sox9), the primary transcription factor inducing chondrogenesis; collagen 2a1 (Col2a1), a collagen secreted in large amounts during chondrogenesis; Runx2 (Runt-related transcription factor 2), a transcription factor critical to osteogenesis; and Twist (Twist-related protein 1), a BHLH transcription factor responsible for repression Runx2-induced osteogenesis until the end of chondrogenesis. We hypothesized that acute SeMet exposure at stage 25 would result in an increase in oxidative stress, and the UPR leading to apoptosis and mis-regulation of cartilage and bone formation.

2. Methods

2.1. Embryo collection and exposures

Japanese medaka (*Oryzias latipes*) were maintained at a ratio of 4:6 males:females, on a 14 h light to 10 h dark cycle at 27 °C. Fish were fed daily a diet of live brine shrimp at University of California-Riverside in accordance with animal use protocols (AUP # 20140002). Embryos were collected daily by removal of egg clutches from females into nets. Embryos were placed into sterile, polystyrene, 100 × 15 mm petri dishes containing de-chlorinated freshwater and rinsed thoroughly. Freshwater quality was monitored with aquarium test strips (Tetra), and was found to have pH 7.8, and to contain approximately 150–300 ppm hardness with 180 ppm alkalinity, with no nitrogen species or chlorine detected. At stage 25 (48 h post fertilization; Iwamatsu, 2004) embryos (15–25/replicate) were separated into controls or treated with 5 μM or 2.5 μM seleno-L-methionine (Sigma Aldrich, 98% purity) for 24 h (until stage 29) in 60 × 15 mm polystyrene petri dishes. Treatments were prepared fresh from 500 μM stock solutions stored in aliquots at −20 °C. During treatments, embryos were maintained in an incubator at 27 °C under a full-spectrum UV light. Previous studies on Japanese medaka treated with 5 μM SeMet for 24 h at stage 25 demonstrated an embryo Se content of 24 μg/g dry weight (Kupsco and Schlenk, 2016). Stage 25 was chosen as the treatment stage because it was specific for Se induced lordosis, and there was a significant correlation between Se content and deformities (Kupsco and Schlenk, 2016). Post treatment, embryos were rinsed and examined under a microscope for deformities. Embryos were characterized as either normal (indistinguishable from controls), mildly deformed (some abnormality in the tip of the tail), moderately deformed (abnormality in the entire tail), severely deformed (abnormalities to tail, body and head), or dead (embryos lacking a heart beat). They were then separated for analysis of oxidative stress, apoptosis, the UPR and skeletal gene expression.

UPR positive controls were also treated for gene expression comparison. Canonical UPR-inducer, Tunicamycin (Tm; a glycosylation inhibitor) (Sigmal Aldrich), was dissolved in a stock concentration of 2 mg/ml in DMSO. Embryos were treated at stage 25 with a 0.2% DMSO control, 4 μg/ml Tm in 0.2% DMSO, 2 μg/ml Tm in 0.2% DMSO and 1 μg/ml Tm in 0.1% DMSO for 24 h (10–15 embryos/replicate). No toxicity was observed in DMSO controls, 1 μg/ml Tm, or 2 μg/ml Tm treatments. Embryos treated with 4 μg/ml Tm displayed small lesions in the end of the tail, similar to the observed deformities following SeMet exposure. Embryos were frozen and pooled for analysis of UPR and skeletal gene expression (n = 6).

2.2. DCFDA staining for oxidative stress

Control embryos, and normal, mildly deformed, moderately deformed, and severely deformed embryos treated with 2.5 μM and 5 μM SeMet were incubated in 10 μg/ml 2′,7′-Dichlorofluorescin diacetate (DCFDA) (97%, sigma Aldrich) for 1hr, on a shaker in the dark at room temperature. Embryos were rinsed 2 × 10min in freshwater and 10 min in 300 μg/ml tricaine. Embryos were imaged with a SPOT Pursuit camera under a green fluorescent filter on a Leica MZIII Pursuit stereoscope and were scored for the presence or absence of staining of the tail or yolk sac (n = 7–20).

2.3. Acridine orange staining for apoptosis

As acridine orange was unable to penetrate the chorion, deformed embryos from 5 μM and 2.5 μM SeMet treatments and controls were dechorionated according to Porazinski et al., 2010. In brief, embryos were rolled on fine grit sandpaper to remove hairs on chorion exterior, then incubated with 1 mg/ml protease for 1 h. Embryos were rinsed and incubated with 20% hatching enzyme for 10–30 min, under observation. Dechorionated embryos were rinsed and incubated in 2 μg/ml acridine orange (0.2% DMSO) for 30 min at room temperature on shaker in the dark. After incubation, embryos were rinsed 2 × 10 min in freshwater and 10 min in 300 μg/ml tricaine. The dechorionation process is harsh due to the use of proteases and thus only controls, and normal and mildly deformed embryos survived for imaging. Embryos were mounted in 4% methylcellulose for visualization with a 488 nm filter on a fluorescent Nikon Eclipse Ti microscope. The number of cells in the tail up to the yolk sac attachment was counted on live embryos. Images were analyzed with NIS Elements AR 3.0 software (n = 20 controls and 8 treated).

2.4. Gene expression analysis

Following deformities analysis, dead embryos were discarded and embryos were pooled into control, normal, or deformed, and frozen for gene expression analysis (n = 10–17). The Lipid Tissue RNeasy kit (Qiagen, Valencia, CA) was used to isolate total mRNA from pooled embryo samples according to the manufacturers instructions. mRNA quality and quantity was assessed with the ND-1000 (Nanodrop, Wilmington, DE). cDNA was prepared with 1 μg mRNA using the Reverse Transcription System (Promega Corporation, Madison, WI), according to the manufacturers instructions.

Primers were designed using Integrated DNA Technologies PrimerQuest software and optimized using PCR Miner (Zhao and Fernald, 2005) (Table 1). EF1α was run as a housekeeping gene. qPCR was performed with the iScript One-step RT-PCR kit with SYBR Green from Bio-Rad (Hercules, CA), omitting the reverse transcriptase, on a MyiQ Thermo cycler (Biorad). The samples were denatured and the polymerase activated at 95 °C for 5 min, then 40 cycles of 10 s at 95 °C and 30 s of 55 °C. Samples were subjected