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Alterations in Hepatic Gene Expression by Trivalent Chromium in *Fundulus heteroclitus*.

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Cr(III) is the dominant toxicant at some Superfund sites within the United States and therefore we are interested in its effects. Cr(III)'s mechanisms are not well studied or understood because of its low bioavailability. We have attempted to characterize the effects of Cr(III) on gene expression in *Fundulus heteroclitus* (mummichog) liver. The NOEC and LOEC were determined at 32 and 64mg/L, respectively, by measuring growth and mortality after exposing juveniles for 30 days. Secondary adult male exposures were performed at 32mg/L, livers excised, and RNA extracted. Arrays were probed with cDNA from untreated or Cr(III)-exposed adult fish and gene expression was quantified. Cr(III) at 32mg/L altered the expression of 5 genes, including GST τ , GST α , and ALDH4. Ultimately, we anticipate using this gene expression information to ascertain whether Cr(III) is bioavailable at potentially adverse concentrations in contaminated sites.

Introduction

Chromium stably exists in the environment as Cr(III) or Cr(VI). Cr(VI) is more toxic because it is bioavailable and produces radicals during reduction to Cr(III). In turn, the Cr(III) binds macromolecules, forms adducts with thiol groups on proteins, creates DNA adducts, and causes DNA strand breakage (Dayan & Paine, 2001). However, while Cr(III) may be the ultimate toxicant following Cr(VI) exposure, Cr(III) is not as toxic as Cr(VI) because of its low bioavailability (Dayan & Paine, 2001).

Chromium is a primary toxicant at 8% of the Superfund sites, including estuarine sites, such as Shipyard Creek in Charleston, SC and the Piscataqua River in Kittery, ME. Typically both forms of chromium are found at Superfund sites, but one form dominates. Chromium partitions to the sediments. Therefore sediment-associated organisms such as mummichogs are exposed to high levels, as they are in direct contact with contaminated sediments and ingest other sediment dwelling organisms. However, not much is known about the molecular effects of chromium on aquatic organisms.

cDNA arrays were developed for mummichogs following subtractive hybridization or differential display of livers excised from Cr(VI), Cr(III), arsenic, or polycyclic aromatic hydrocarbon (PAH) exposed mummichogs. Thus, the array is targeted for determining differential expression caused by chromium, arsenic, or PAHs in mummichogs. Overall, the array contains 270 duplicate spots, including thirteen blanks or plasmid controls. A hybridized array is shown in Figure 1. Information on the individual genes found on the array can be found on the Gene Expression Omnibus (GEO-GPL2535) or www.mummichog.org.

We used the mummichog arrays developed in our lab to determine Cr(III)-induced changes in hepatic gene expression. The information generated by the array may help determine the mechanism, or bioavailability of Cr(III). In this study, we treated adult mummichogs at the

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NOEC (32 mg/L), and used cDNA arrays to determine alterations in gene expression. In the future, we anticipate using the gene expression profile to determine the dominant bioavailable chromium species at estuarine Superfund sites.

Material and Methods

Exposure of mummichogs to Cr(III)

All animal research was performed in accordance with IACUC approved protocols. Mummichogs were housed at 25°C in a 14/10 light/dark cycle at 18‰ salinity. Juvenile mummichogs (<48hrs old) were exposed to CrCl₃*6H₂O in 500ml tanks (6 fish/tank) for 30 days in static renewal tests at concentrations of 0 to 64mg/L Cr(III) (1.23mM), and growth was monitored according to USEPA protocol to determine the NOEC and LOEC (USEPA, 2002). Total chromium in the media was determined using a Perkin-Elmer 4300 DV ICP-OES (Shelton, CT). Adult mummichogs (n=10) were exposed to Cr(III) at 0 or 32mg/L (0.62mM) (NOEC) individually in 4 L tanks for 7 days. At the conclusion of the exposures, livers were excised and placed in TRI-Reagent® for RNA isolation (Sigma Chemical Co, St. Louis, MO), and stored at -80°C. Total chromium in the media and the remaining tissues was measured by ICP-OES.

Gene Expression

Custom nylon arrays were generated by EcoArray, L.L.C. (Alachua, FL) using 233 unique genes isolated by differential display or subtractive hybridization during this study and other studies in which fish were exposed to Cr(III), Cr(VI), pyrene, anthracene, As(III), or collected from polluted sites (Chapman, Roling, Bingham, Herald & Baldwin, 2004; Roling, Bain, & Baldwin, 2004). Pooled liver mRNA from fish exposed to 0 or 32mg/L Cr(III) was subjected to subtractive hybridization using the PCR-Select cDNA Subtraction kit (Clontech, Palo Alto, CA). Twenty-seven genes from the adult livers were isolated during this process and added to the array.

[³³P]Probes were prepared from RNA by reverse transcription (Chapman et al., 2004) and hybridized overnight as described previously (Larkin, Folmar, Hemmer, Poston & Denslow, 2003). Arrays were washed, exposed to an intensifying screen, visualized with a BioRad® FX Imager (Hercules, CA)(Figure 1), and quantified using Invitrogen ResGen™ Pathways™ software (Carlsbad, CA) (GEO GSE2807). Spot intensities were normalized to the mean intensity of all spots on the array and fold differences calculated. Verification of select genes was performed by Q-PCR, and normalized to 16S rRNA using a MyiQ Single-Color Real-Time PCR Detection System (Bio-Rad, Hercules, CA) (Roling et al., 2004). QPCR primers (annealing temperatures): 16sF-5'-attgattcccgtgcagaa-3'; 16sR-5'-tgtggcaggattcaattgt-3' (52°C); ADH4F-5'-gccgtagaacctcctgtag-3'; ADH4R-5'-catcatcgtgaccaaagacc-3' (58°C); GSTr3F-5'-ttcctgctcaggaccgtga-3'; GSTr3R-5'-cagcgtccatcttccttc-3' (59°C); GSTμF-5'-tatgtgcggagagactgagg-3'; GSTμR-5'-tcacaaagccgtttctgaag-3' (60°C).

Results and Discussion

Juvenile mummichogs were exposed to Cr(III) to determine the NOEC and LOEC. The LOEC concentration of Cr(III) (64mg/L) caused 100% mortality within 19 days with 50% mortality by day 8. The next lowest concentration of Cr(III) tested (32 mg/L) had no effect on mortality or growth. Cr(III) concentrations in the media were measured every 24 hours during the first set of media changes (Table 1a). Measured Cr(III) concentrations were significantly lower than nominal concentrations because Cr(III) readily forms Cr(OH)₃ in water at neutral pH and then precipitates (Rai, Eary, & Zachara, 2005).

Adult males were exposed to Cr(III) at the NOEC-32mg/L (Table 1b) and gene expression in the liver was determined since chromium bioaccumulates in the liver (Calamari, Gaggino, & Pacchetti, 1982). We use adult male mummichogs in our field studies because (1) males and females show different gene expression profiles, and (2) adult male fish are easily identified in the field from females and immature males because of their obvious markings. Therefore, we used adult males in our laboratory studies at our predetermined NOEC, so that we would be able to compare the gene expression profile and chromium body burdens from laboratory-exposed fish to fish at polluted sites. Interestingly, concentrations measured in adult mummichogs at 32mg/L (3.9 ± 0.6 mg/kg) were similar to concentrations measured in adult mummichogs exposed to Cr(VI) at the LOEC (3mg/L) (2.5 ± 0.2 mg/kg) and NOEC (1.5mg/L) (1.3 ± 0.1 mg/kg) (unpublished).

Cr(III) down-regulated several genes in adult male livers including aldehyde dehydrogenase 4 (ALDH4), several glutathione S-transferases (GSTs), and an EST (Table 2). ALDH4 and the GSTs protect the cell from cellular and oxidative stress (Hartley, Ruth, & Petersen, 1995). All three GSTs on the array were down-regulated by Cr(III), including GST τ (-1.51 array, -1.76 QPCR), GST α (-1.53 array), and GST μ (-2.73 array ($p=0.055$), -3.83 QPCR ($p=0.025$)). We added the GST α gene from winter flounder because it was down-regulated by Cr(VI) in winter flounder (Chapman, et al., 2004) and rat (Gunaratnam & Grant, 2001). Since this gene was from winter flounder and the mummichog sequence is not known, it could not be confirmed by Q-PCR. However, the fact that this gene comes from a different genera (*Pleuronectes*) adds some uncertainty to this data.

GST τ was also down-regulated by Cr(III). The GST τ promoter region contains NF- κ B and AP-1 response elements (Leaver, Wright, & George, 1997), and Cr(III) has been shown to inhibit binding of NF- κ B and AP-1 to these response elements *in vitro* (Shumilla, Wetterhahn, & Barchowsky, 1998). Similarly, 2-D gels followed by MS-Maldi indicated a down-regulation of GST τ in winter flounder injected intraperitoneal with Cr(III) at 0.4mg/kg (unpublished data). Significant fragments of this down-regulated protein in winter flounder (ASWPPTWLESPQGQDM, VLNESYAACMYLESQF, MADVIYYSWKVPEAER, DMTLLWGSPPCWR) matched a theta class GST from plaice (gi|232216).

Ultimately, the gene expression profile of Cr(III) exposed mummichog will be compared to the gene expression profile of mummichogs from the Superfund site at Shipyard Creek. We anticipate using these arrays to assess the bioavailability of toxicants and efficacy of remediation at chromium-contaminated Superfund sites.

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Figure 1.
A mummichog cDNA array probed with ^{33}P labeled hepatic cDNA from mummichogs exposed to Cr(III).

Table 1
Nominal and measured chromium concentration

Juvenile. (A) and adult (B) mummichogs were exposed to Cr(III) for 30 or 7 days, respectively in a static renewal test.

(A)				
Nominal (mg/L)	0 hrs	Measured Water Concentration (mg/L) ^a		Fish Weight (mg)
		24 hrs	48 hrs	
0	nd	nd	nd	124.9 ± 5.1
2	1.46 ± 0.10	0.80 ± 0.18	1.05 ± 0.10	119.7 ± 3.9
4	2.87 ± 0.23	0.80 ± 0.16	2.82 ± 0.40	116.3 ± 2.6
8	6.22 ± 0.42	3.23 ± 0.71	4.55 ± 0.85	123.6 ± 4.8
16	14.60 ± 1.58	5.82 ± 0.56	10.71 ± 1.48	150.9 ± 14.4
32	32.98 ± 0.63	6.54 ± 0.80	5.60 ± 1.41	136.7 ± 3.7
64	70.93 ± 2.36	26.82 ± 3.66	13.38 ± 2.42	nm
(B)				
Nominal (mg/L)	0 hrs	Measured Water Concentration (mg/L) ^a		48 hrs
		24 hrs		
0	nd	nd		nd
32	21.34 ± 0.50	5.40 ± 0.75		2.00 ± 0.63

nd – Not Detected

nm – Not measured (100% mortality)

^aWater concentrations were measured at 24 hour intervals during one static renewal.

Data are presented as mean ± SE (juvenile n = 5–6; adult n = 10).

Table 2
Differentially expressed genes following Cr(III) exposure.

Identity	Array ^a	Q-PCR ^b	Accession
ADH4	-2.61 [*]	-1.76 [*]	AY735158
Cyp2X	-1.81 [*]	pa	AY225104
GST A3	-1.53 [*]	pa	AY156727
GSTr3	-1.51 [*]	-1.83 [*]	AY725219
EST – bb93	1.50 [*]	np	CX700379

^a Mean spot change in spot intensity compared to controls when averaging duplicate spots on array (n = 4).

^b Q-PCR confirmation of changes in gene expression after normalization to 16s rRNA (n = 10).

pa – winter flounder (*Pleuronectes americanus*) gene: Winter flounder primers did not work well with *F. heteroclitus* cDNA.

np – not performed

* indicates a significant difference as determined by Students t-test ($p \leq 0.05$)