BioMetals (2006) 19:225–235
DOI 10.1007/s10534-005-5670-x

Comparative effects of direct cadmium contamination on gene expression in gills, liver, skeletal muscles and brain of the zebrafish (*Danio rerio*)

Patrice Gonzalez, Magalie Baudrimont, Alain Boudou & Jean-Paul Bourdineaud*
*Laboratoire d'Ecophysiologie et Ecotoxicologie des Systèmes Aquatiques (LEESA), Université Bordeaux, 1/UMR CNRS 5805, Place du Dr Peyneau, Arcachon, 33120, France; *Author for correspondence (Tel: +33(0)556-22-39-26; Fax: +33(0)556-54-93-83; E-mail: jp.bourdineaud@epoc.u-bordeaux1.fr)*

Received 22 December 2004; accepted 18 April 2005

Key words: bioaccumulation, cadmium, *D. rerio*, gene expression, real-time PCR

Abstract

The effects of cadmium (Cd) on gene expression were examined in four organs (gills, liver, skeletal muscles and brain) of the zebrafish. Adult male fish were subjected to three different water contamination pressures over periods of 7 and 21 days: control medium (C_0: no Cd added) and two contaminated media (C_1: 1.9 ± 0.6 μg Cd l^{-1}, and C_2: 9.6 ± 2.9 μg Cd l^{-1}). Fourteen genes involved in antioxidant defences, metal chelation, active efflux of organic compounds, mitochondrial metabolism, DNA repair and apoptosis were selected and their expression levels investigated by quantitative real-time PCR. Cadmium concentrations were determined in the four organs and metallothionein (MT) protein levels investigated in brain, liver and gills. Although skeletal muscle was a poor Cd-accumulating tissue, many genes were up-regulated at day 7: *mt1*, *cyt*, *bax*, *gadd* and *rad51* genes. Three additional genes, *c-jun*, *pyc* and *tap*, were up-regulated in muscles at day 21 whereas *bax*, *gadd* and *rad51* had returned to basal levels. Surprisingly, *mt1* and *c-jun* were the only genes displaying a differential induction after 21 days in liver, although this organ accumulated the highest cadmium concentration. In brain, only *mt1*, *mt2* and *c-jun* genes were up-regulated after 21 days. In gills, the highest response was observed after 7 days, featuring the differential expression of oxidative stress-response *hsp70* and mitochondrial *sod* genes, along with genes involved in mitochondrial metabolism and metal detoxification. Then, after 21 days, the expression of almost every genes returned to basal levels while both *mt1* and *mt2* genes were up-regulated.

Introduction

Cadmium (Cd) is a non-essential metal whose dispersion in the environment has increased over the past decades due to its widespread industrial use as a colour pigment in paints, in electroplating and galvanising, in batteries, etc. It is also a by-product of zinc and lead mining. Numerous field studies have shown varying degrees of contamination of aquatic systems, via direct or indirect Cd inputs (Hutton 1983; Guinée *et al.* 1999). In spite of its toxicity, little is known about the effects of Cd on genetic and biochemical adaptive responses of aquatic species under chronic and long-term exposure. Toxicological studies at cellular level have shown that Cd inhibits the mitochondrial electron transfer chain and induces reactive oxygen species (ROS) production (Wang *et al.* 2004). Cd-promoted oxidative stress leads to DNA damage and apoptotic cell death (Bagchi *et al.* 2000; Chan and Cheng 2003). Different mechanisms could be involved in Cd detoxification. Among these, one relies on low molecular weight and cystein-rich proteins called metallothioneins (MT) which are able to bind seven metal ions (zinc or cadmium) in mammals (Chan *et al.* 2002). The promoters of MT genes contain several metal regulatory elements (MRE) which are bound by a

metal-activated transcription factor called MTF-1 (Heuchel *et al*. 1994). Another mechanism is based on active efflux via pumps belonging to the ATP-binding-cassette (ABC) transporter family. ABC transporters are membrane proteins that use ATP as an energy source and are able to extrude xenobiotics and metals by active efflux (Dassa and Bouige 2001; Achard *et al*. 2004).

In this paper, we investigated the effect of three Cd exposures via the direct route (control, 17 and 86 nM or 2 and 10 $\mu g\ l^{-1}$ in the water) on gene expression in gills, liver, skeletal muscles and brain from adult *Danio rerio* fish after two exposure durations: 7 and 21 days. The zebrafish constitutes an outstanding fish model in which to study *in vivo* cellular responses following Cd exposure, since its genome has been recently reported, and its gene sequences are therefore available in databases (http://www.ensembl.org). In order to investigate Cd effects, 14 genes were selected encoding for proteins involved in antioxidant defences, metal chelation, active efflux of xenobiotics, mitochondrial metabolism, DNA repair and apoptosis. Three of these genes are known to be involved in the oxidative stress response: cytoplasmic and mitochondrial superoxide dismutases (*sod*(*Cu*/*Zn*) and *sod*(*Mn*)) and the heat shock protein *hsp70* genes. The mitochondrial metabolism was investigated using the pyruvate carboxylase (*pyc*), the cytoglobin (*cyt*), and the cytochrome C oxidase (*coxI*) subunit I genes. The *cyt* gene was selected because of its up-regulation by hypoxia and the O_2 supplying function of cytoglobin (Schmidt *et al*. 2004). Three other genes are assumed to be involved in detoxification mechanisms: the two MT genes reported for *D. rerio* (*mt1* and *mt2*) and a gene belonging to the (ABC) transporter family (*tap*). The *tap* gene was chosen according to its homologies with multidrug-resistance transporters like the human *MDR1* gene (Gottesman and Pastan 1993), the *leishmania LtpgpA* gene (Dey *et al*. 1996) or the yeast *YCF1* gene (Li *et al*. 1997). *LtpgpA* and *Ycf1* pump not only xenobiotics but also arsenic and cadmium, respectively. Three genes involved in Cd-induced apoptotic mechanisms were selected: *c-jun*, *p*53 and *bax* (Fernandez *et al*. 2003); also two genes known to intervene in response to DNA damage repair: the growth-arrest-DNA-damage (*gadd*) and the *rad51* genes. Due to its constitutive expression, the actine gene was used as a reference gene.

Gene expression levels were investigated by quantitative real-time PCR and normalized according to actine gene expression. MT protein levels were analysed in association with their gene expression in brain, liver and gills. Total Cd concentrations were determined in the four organs after each exposure duration.

Materials and methods

Exposure conditions

Two hundred and fourty adult male fish (average weight: 0.88 ± 0.03 g, wet weight; standard length: 3.63 ± 0.05 cm) were randomly placed in three tanks containing 100 l of chlorine-free, permanently oxygenated water. Female fish were excluded to avoid interference due to reproduction processes. Throughout the experiment, temperature was maintained at 24.5 ± 0.1 °C. Fish from each tank were fed twice a day with the same quantity of artificial food (dried fish meat and cereals) corresponding to 5% of fish wet weight per day. The fish in the first experimental unit were in uncontaminated water and constituted control animals for the genetic analysis, metallothionein content, and Cd bioaccumulation levels. In the second tank (C_1) fish were exposed to water at a low level of contamination (mean over 21 days: 1.9 ± 0.6 $\mu g\ Cd\ l^{-1}$; 17 ± 5 nM). In the remaining unit (C_2), fish were exposed to highly contaminated water (mean over 21 days: 9.6 ± 2.9 $\mu g\ Cd\ l^{-1}$; 86 ± 26 nM). These two exposure conditions were close to field levels observed downstream from industrial sites like zinc ore treatment facilities (Andres *et al*. 2000; Audry *et al*. 2004). Water contamination was based on daily additions of aqueous solutions of Cd (ultrapure $CdCl_2$, Merck). The amounts of Cd added were adapted to compensate for the decrease in metal concentrations over the 24 h cycles, which were measured on a regular basis by analysing water samples (10 ml). One third of the water volume from each tank was changed every two days and tank bottoms were cleaned to eliminate fish faeces and the remains of food. Forty fish per condition were removed after 7 and 21 days and were killed within seconds by immersion in melting ice (T = 0 °C). From each *D. rerio*, brain, liver, gills and skeletal muscle were independently harvested. The fish were dissected on ice. Brain, gills, and liver

Table 1. Accession numbers and specific primer pairs for the 15 *D. rerio genes used in our study*.

Gene name	Accession number	Primer (5′–3′)
mt1	X97278	CTGCGAATGTGCCAAGACT[a] GCTGGAGCCACAGGAAT[b]
mt2	AY305851	TGCGAATGCGCCAAGAC[a] GCCCTTACACACGCAGC[b]
tap	AL672164	GCAAGATAGCGATGGTGGG[a] CGTTGGCTTTGCTTGCAG[b]
coxI	NC_002333	GGAATACCACGACGGTACTCT[a] AGGGCAGCCGTGTAAT[b]
hsp70	AB062116	CATCGACGCCAACGGG[a] CCAGGGAGTTTTTAGCAGAAATCTT[b]
pyc	NM_131550	GTCCGGTGGACTGCCT[a] CCTTGACCAAGACTGAACGC[b]
sod(*Cu*/*Zn*)	BC055516	TGAGACACGTCGGAGACC[a] TGCCGATCACTCCACAGG[b]
sod(*Mn*)	CB923500	TTCAGGGCTCAGGCTGG[a] ATGGCTTTAACATAGTCCGGT[b]
actine	NM_131031	AAGTGCGACGTGGACA[a] GTTTAGGTTGGTCGTTCGTTTGA[b]
c-jun	BC065976	TGGATACAACCACAAGGCTCT[a] GTCACGTTCTTGGGACACAG[b]
p53	AF365873	GGGCAATCAGCGAGCAAA[a] ACTGACCTTCCTGAGTCTCCA[b]
bax	AF231015	GGCTATTTCAACCAGGGTTCC[a] TGCGAATCACCAATGCTGT[b]
gadd	BC059472	GCTTGTTCGTGTCTTCTGTGG[a] CTTCCCGCATTCAGCGAT[b]
rad51	BC062849	TGCTGCGTCTCGCTGA[a] GCCTCGGCCTCTGGTAA[b]
cyt	NM_152952	CTTCCCATCGGCGAAGC[a] CACCTTGTGGCGCAGC[b]

Abbreviations: *mt* – metallothionein; *coxI* – cytochrome C oxidase subunit I; *pyc* – pyruvate carboxylase; *sod*(*Cu*/*Zn*) – superoxide dismutase; *sod*(*Mn*) – mitochondrial superoxide dismutase; *gadd* – growth-arrest-DNA-damage; *cyt* – cytoglobine. [a]Upstream primer. [b]Forward primer.

were collected whole. The two skeletal muscles of each fish were taken in the region located between the head and the tail. Next, for each organ, three replicates were prepared by pooling four fish. Each replicate was divided into two for genetic analysis and Cd concentration determinations. MT protein levels were determined from three distinct replicates (pools of four fish). The remaining fish were conserved at −80 °C in case of analysis failure of any of the pooled organs and constituted a pool of samples that could be used for further investigations.

Cd analysis

Dissected organs were thawed, dried on absorbent paper and weighed (wet weight). Samples were digested by nitric acid (3 ml of pure HNO_3) in a pressurized medium (borosilicate glass tubes) at 100 °C for 3 h. After dilution of the digestates to 20 ml with ultrapure water (MilliQ plus), cadmium concentrations were measured by atomic absorption spectrophotometry with Zeeman correction, using a graphite tube atomizer (AAS, Thermoptec M6 Solaar). In order to avoid interference, analyses were carried out in a tube atomizer with a blend of Pd and $Mg(NO_3)_2$. The detection limit was 0.1 μg Cd l^{-1} (3 × SD of the reagent blanks). The analytical method was simultaneously validated for each sample series by analysing standard biological reference materials (TORT-2, Lobster hepatopancreas; DOLT-2, Dogfish liver; NCR/CRNC, Ottawa, Canada). Values were in

agreement with the certified ranges (data not shown). Recovery of metal was 98 ± 2% for Cd. The results are expressed as average metal concentrations accumulated in organs (three replicates per organ per exposure condition) in nmol Cd g^{-1} ± SE (Standard Error), wet weight.

Metallothionein quantification

The levels of total MT proteins in gills, liver, and brain were determined by mercury-saturation assay as previously described, using cold inorganic mercury (Dutton *et al.* 1993; Baudrimont *et al.* 2003). MT analysis was conducted on three replicates (four pooled organs from four different fish) per exposure condition, the saturation assay being repeated twice per sample. This technique is based on the quantification of Hg bound to the saturated MTs. The denaturation of non-MT proteins was performed with trichloroacetic acid and excess Hg not bound to the MTs was removed by scavenging with lyophilised beef hemoglobin (Sigma) prepared in 30 mM Tris–HCl buffer (pH 8.2 at 20 °C). The final supernatant was then quantitatively recovered and used for Hg determination by flameless atomic absorption spectrometry (AMA 254, Altec, Prague, Czech Republic). The detection limit was estimated at 1 ng Hg. Owing to the fact that the exact quantity of Hg binding sites per MT molecule is unknown for this species, MT concentrations cannot be directly expressed in nmol MT g^{-1} (wet weight), but in nmol Hg g^{-1} (wet weight).

Total RNA purification

Total RNAs were extracted from 40 mg of fresh tissue using the Absolutely RNA RT-PCR Miniprep kit (Stratagene), according to the manufacturer's instructions. The quality of all RNAs produced was evaluated by electrophoresis on a 1% agarose-formaldehyde gel, and their concentration determined by spectrophotometry. For each exposure condition, samples were carried out in triplicate.

Reverse transcription of RNA

First-strand cDNA was synthesized from 5 μg total RNA using the Stratascript First-Strand Synthesis System (Stratagene). RNA was adjusted to 38 μl with DEPC-treated water. After the addition of 2 μl of oligo(dT) and 1 μl of random primers, the reaction was incubated at 65 °C for 5 min. Next, 40 units of Rnase Inhibitor, 2 μl of 100 mM dNTPs, 5 μl of 10× first-strand buffer and 50 units of Stratascript reverse transcriptase were added. The reaction was incubated for 1 h at 42 °C in an Eppendorf Mastercycler. The cDNA mixture was conserved at −20 °C until it was used in a real-time PCR reaction.

Primer design

The accession numbers of the 15 genes used in our study are reported in Table 1. For each gene specific primer pairs were determined using the LightCycler probe design software (version 1.0, Roche). All theses primer pairs are reported in Table 1.

Real-time PCR

Real-time PCR reactions were performed in a LightCycler (Roche) following the manufacturer's instructions (one cycle at 95 °C for 10 min, and 50 amplification cycles at 95 °C for 5 s, 60 °C for 5 s and 72 °C for 20 s). Each 20 μl reaction contained 2 μl of reverse transcribed product template, 1 μl of master mix including the SyberGreen I fluorescent dye (Roche), enabling the monitoring of the PCR amplification, and the gene-specific primer pair at a final concentration of 300 nM for each primer.

Reaction specificity was determined for each reaction from the dissociation curve of the PCR product. This dissociation curve was obtained by following the SyberGreen fluorescence level during a gradual heating of the PCR products from 60 to 95 °C. Relative quantification of each gene expression level was normalized according to the actine gene expression.

Results

Cd bioaccumulation in fish organs

Cd concentrations determined in gills, liver, skeletal muscle and brain after the two exposure durations

are shown in Table 2. The average Cd concentrations in organs from control fish throughout the experiment (C_0) were in decreasing order: liver (1 ± 0.1 nmol Cd g^{-1}), gills (0.9 ± 0.3 nmol Cd g^{-1}), brain (0.45 ± 0.1 nmol Cd g^{-1}) and muscles (0.2 ± 0.04 nmol Cd g^{-1}). At 1.9 μg Cd l^{-1} in the water (C_1 tank), the highest bioaccumulation after 21 days of Cd exposure was observed in gills (15 ± 2.2 nmol Cd g^{-1}) and in liver (10.8 ± 0.4 nmol Cd g^{-1}), followed by brain (3.9 ± 1.5 nmol Cd g^{-1}) and a weak metal load in skeletal muscle (1.6 ± 0.4 nmol Cd g^{-1}). At 9.6 μg Cd l^{-1} in the water (C_2 tank), the bioaccumulation ranking order between organs after 21 days of Cd exposure was: liver (24.9 ± 5.2 nmol Cd g^{-1}), gills (12.8 ± 1.1 nmol Cd g^{-1}), brain (10.6 ± 0.4 nmol Cd g^{-1}) and skeletal muscle (1.2 ± 0.4 nmol Cd g^{-1}).

Accumulation in gills in water contaminated at 9.6 μg Cd l^{-1} showed a significant decrease during the second exposure period (Table 2): the highest Cd concentration was observed in this organ after the first 7 days (17.4 ± 1.8 nmol Cd g^{-1}), which was more than twice that in liver. Between 7 and 21 days, however, Cd accumulation decreased to reach a level similar to that observed in water contaminated at 1.9 μg Cd l^{-1}. In the skeletal muscle, a low level of bioaccumulation was observed. After 7 days of exposure the highest values were obtained in C_2 (0.9 ± 0.2 nmol Cd g^{-1}). Only a small increase was evidenced between 7 and 21 days in C_2 and also in C_1. At the end of the experiment, skeletal muscles from the two exposure conditions presented nearly the same values of Cd bioaccumulation.

Gene expression levels

The genetic analysis evidenced a tissue-specific basal expression rate. For instance, the *mt2* gene was more expressed at the basal level in liver than in brain, gills or skeletal muscles where it was 16, 32 and 64 times higher, respectively (Table 3). These increased basal expression levels in liver as compared to the other organs were observed for many of the genes investigated: *sod*(*Cu*/*Zn*), *pyc*, *mt1*, *mt2*, *sod*(*Mn*), *p*53 and *bax* genes. In the skeletal muscles, several genes showed the highest basal expression levels: *coxI*, *hsp70*, *tap* and *gadd*. In contrast, basal expression levels in brain were most often lower than in the other organs.

Besides these basal gene expressions, there were marked differences in the genetic response to Cd exposure conditions between organs (Table 4).

In the brain, no differential expression was observed after 7 days, whatever the contamination pressure. Only at 21 days were up-regulated *c-jun*, *mt1* and *mt2* genes, an expected hallmark of Cd contamination, but with no differences between the two exposure conditions. However, the oxidative stress responsive genes, *sod*(*Cu*/*Zn*), *sod*(*Mn*) and *hsp70*, were not influenced by Cd within brain cells.

In liver, no significant variations were determined for most genes after 7 days of exposure to Cd, except for the *c-jun* gene expression level which decreased three fold in the C_2 condition. Although the liver was the organ that accumulated the highest concentrations of Cd, along with the gills, only *mt1* and *c-jun* genes were up-regulated after 21 days.

In sharp contrast and rather surprisingly, many genes were found to be up-regulated in skeletal muscles, the tissue that accumulated by far the weakest Cd concentrations. At day 7, for instance, muscles accumulated 7 and 9 times less Cd than liver in the C_1 and C_2 tanks respectively, and yet DNA repair genes *gadd* and *rad51* were up-regulated along with the pro-apoptotic *bax* gene, *mt1*, and the cytoglobin gene *cyt*. At day 21, muscles accumulated 21 times less Cd than liver, but as in liver, *mt1* and *c-jun* were stimulated. However, three additional genes were induced in muscles at the end of the experiment: *cyt*, the pyruvate

Table 2. Cd accumulation (nmol g^{-1}, wet weight) in *D. rerio* organs after 7 and 21 days of contamination (mean ± SE; $n = 3$).

Tanks	Brain		Gills		Liver		Skeletal muscles	
	7	21	7	21	7	21	7	21
C_0	0.6 ± 0.2	0.3 ± 0.04	0.9 ± 0.2	0.9 ± 0.3	1.1 ± 0.2	0.9 ± 0.02	0.2 ± 0.03	0.2 ± 0.05
C_1	1.2 ± 0.4	3.9 ± 1.5	5.8 ± 0.9	15 ± 2.2	2.7 ± 0.1	10.8 ± 0.4	0.4 ± 0.1	1.6 ± 0.4
C_2	1.8 ± 0.6	10.6 ± 0.4	17.4 ± 1.8	12.8 ± 1.1	8.3 ± 2.1	24.9 ± 5.2	0.9 ± 0.2	1.2 ± 0.4

Table 3. Comparative basal expression for the selected genes observed in brain, gills, liver and skeletal muscles from control fish.

Function	Genes	Brain	Liver	Muscle	Gills
Mitochondrial metabolism	*coxI*	4	16	64	32
	pyc	0.00003	0.002	0.0001	0.00003
	cyt	0.002	0.008	0.004	0.0005
Oxidative stress	*sod*(*Cu*/*Zn*)	1	4	0.5	0.5
	sod(*Mn*)	0.125	0.5	0.5	0.125
	hsp70	0.002	0.002	0.125	0.004
Detoxification process	*mt2*	0.25	4	0.06	0.125
	mt1	0.002	0.008	0.00025	0.008
	tap	0.0005	0.002	0.015	0.004
DNA repair	*gadd*	0.03	0.125	0.25	0.03
	rad51	0.008	0.004	0.004	0.008
Apoptosis	*p*53	0.008	0.015	0.008	0.015
	bax	0.008	0.015	0.015	0.008
	c-jun	0.015	0.015	0.06	0.008

carboxylase *pyc* gene, and the ABC transporter *tap* gene.

Gills are the organ at the interface with the contaminated water and are thus the tissue from which the metal loading of the organism will proceed. It is therefore not surprising that we observe the induction of expression of 9 out of 14 selected genes. At day 7 and at the highest contamination level (C_2) where gills accumulated twice as much Cd as liver, only expressions of *mt1*, *mt2*, *sod*(*Cu*/*Zn*), *c-jun* and *p*53 genes were not up-regulated. Worth noting, impressive induction factors were observed for *cyt* (×64), *pyc* (×128), *hsp70* (×64), *coxI* (×32), *rad51* (×12), and *tap* (×100) genes, meaning that mitochondrial metabolism was markedly influenced and that responses to DNA damage and oxidative stress were triggered. At the highest Cd contamination and after 21 days, Cd concentration in gills showed a significant decrease in comparison to the average level determined after 7 days' exposure and remained quite similar to that observed after 21 days for the lowest Cd contamination level (Table 2). Meanwhile, of the nine genes up-regulated at day 7 for the C_2 condition, only *bax* was still induced. No clear relationship appeared between Cd tissue loading and gene expression, since at day 21 around 14 nmol Cd g^{-1} accumulated in gill tissues, whatever the Cd concentration in the medium and however the largest genetic response was obtained in fish in the C_1 tank. Indeed, *cyt*, *pyc*, *mt1* and *mt2* genes were up-regulated in the fish in this tank, whereas only *bax* and *mt1* did so for the C_2 tank fish. Even *mt1* was more up-regulated in fish from C_1 tank than from C_2 (×10 against ×4, respectively).

Metallothionein gene expressions and protein levels

With the exception of the *mt1* gene induction at day 7 in muscles (×16), all of the organs featured an *mt1* gene up-regulation at day 21 (Table 4). Nevertheless, *mt1* gene expression was observed in muscles of C_1 tank fish at day 7 but not at day 21 by which time Cd concentration was 4 times higher. In gills, *mt1* gene expression was increased (×4) at day 21 in C_2 tank fish, although Cd concentration in this tissue was fairly similar to day 7. *mt2* was up-regulated in gills at day 21 (×3) but only in C_1 tank fish, which nevertheless did not accumulate more Cd than C_2 tank fish. In the brain, the same induction factor was obtained at day 21 in both C_1 and C_2 fish for *mt1* (×4) and *mt2* (×2) genes although Cd concentration was three times higher in brains of C_2 fish than in C_1 fish.

MT protein levels were quantified by mercury saturation in brain, gills and liver (Table 5). According to gene expression, differential MT concentrations were observed in the three organs at the basal level. The highest protein levels were measured in the liver (47.9 ± 6.9 nmol Hg g^{-1}, wet weight), where *mt2* is notably over-expressed, followed by the brain and the gills (12.3 ± 2.9 and 1.5 ± 0.5 nmol Hg g^{-1}, respectively). After Cd-contamination, MT protein levels remained constant in brain and liver whatever the contamination pressure and the time duration (around

Table 4. Differential gene expression observed in brain, gills, liver and skeletal muscles of zebrafish after direct contamination with $CdCl_2$ for 7 and 21 days.

Organs and functions	Genes[a]	Contaminated tanks			
		C_1 (17 ± 5 nM)		C_2 (86 ± 26 nM)	
		7	21	7	21
Brain					
Pro-apoptotic gene	*c-jun*	/	2	/	2
Detoxification	*mt1*	/	4	/	4
	mt2	/	2	/	2
Liver					
Pro-apoptotic gene	*c-jun*	/	16	1/3	4
Detoxification	*mt1*	/	64	/	4
Skeletal muscles					
Mitochondrial metabolism	*cyt*	8	4	/	4
	pyc	/	/	/	4
Detoxification	*mt1*	16	/	/	6
	tap	/	/	/	3
Pro-apoptotic genes	*bax*	/	/	2	/
	c-jun	/	8	/	8
DNA repair	*gadd*	4	/	55	/
	rad51	/	/	32	/
Gills					
Mitochondrial metabolism	*coxI*	4	/	32	/
	cyt	2	4	64	/
	pyc	/	4	128	/
Detoxification	*mt1*	/	10	/	4
	mt2	/	3	/	/
	tap	/	/	100	/
Oxidative stress	*hsp70*	8	/	64	/
	sod(*Mn*)	/	/	4	/
Pro-apoptotic genes	*bax*	1/2	/	16	2
	c-jun	1/14	/	1/32	/
DNA repair	*gadd*	/	/	3	/
	rad51	/	/	12	/

[a]Results are given as induction (> 1) or repression (< 1) factors as compared to control zebrafish. /: identical to control levels.

20 nmol Hg g^{-1} and 50 nmol Hg g^{-1}, respectively). In gills, a two and five fold increase in MT proteins was observed at 7 days for fish from C_1 and C_2, respectively, and at 21 days' exposure, a two fold increase in C_2 fish.

Discussion

Numerous field studies have shown that skeletal muscles are not considered to be storage compartments for Cd in fish and similarly in other

Table 5. MT concentration (nmol Hg g^{-1}, wet weight) in *D. rerio* brain, gills and liver after 7 and 21 days of contamination (mean ± SE; $n = 3$).

Tanks	Brain		Gills		Liver	
	7	21	7	21	7	21
C_0	12.3 ± 2.9	24.6 ± 1.5	1.5 ± 0.5	4.4 ± 0.9	47.9 ± 6.9	53.1 ± 5.1
C_1	19.7 ± 2.5	22.3 ± 3	4.1 ± 1.3	4.9 ± 1	40.8 ± 7.5	43.9 ± 10.7
C_2	20.5 ± 1.2	23.1 ± 3.2	7.9 ± 1.5	9.4 ± 1.8	58.9 ± 6.4	69.5 ± 3.8

Table 6. Expression ratios *Mt2*/*Mt1 in D. rerio* organs after 7 and 21 days of contamination.

Tanks	Brain		Gills		Liver		Muscles	
	7	21	7	21	7	21	7	21
C_0	128	128	16	16	512	512	256	256
C_1	128	64	16	5	512	8	16	256
C_2	128	64	16	4	512	128	256	43

aquatic species (Andres *et al*. 2000). However, the low levels of Cd burdens accumulated in the zebrafish muscles trigger a large genetic response, much more potent, for example, than that in liver, which is one of the main Cd accumulator organs, suggesting that there is no direct relationship between Cd accumulation and gene expression levels. In fact, Cd loading seems to be necessary but not sufficient to obtain a genetic response.

It should be stressed that in most published studies dealing with various animal models and cell cultures, authors are using extremely high amounts of Cd, which bear no relation to environmental pollution. For instance, zebrafish embryos were incubated for 23 h in culture media containing 1–1,000 μM $CdCl_2$ and deformities such as head, eye, heart and tail malformations (Cheng *et al*. 2000) were induced. When zebrafish embryos were given a 100 μM $CdCl_2$ treatment for 20 h, this of course led to Cd-induced apoptosis in the neural tube and defects in axon growth (Chan and Cheng 2003; Chow and Cheng 2003). The discrepancy between results obtained at low doses versus acute high doses can be illustrated by a study of zebrafish embryo-derived fibroblasts. These cells were transfected with a construct allowing expression of the firefly luciferase gene under the control of a synthetic MT gene promoter consisting of a pentamer of MRE. After an overnight treatment, a maximal induction (four fold) was obtained with 30 μM $CdCl_2$. However, at 3 μM no induction of the reporter gene could be produced (Carvan III *et al*. 2000). In our laboratory conditions, a 64 fold induction of *mt1* gene was observed in muscles after 21 days of exposure to 17 nM.

From our results, MT gene expression appeared to be a late hour biomarker. This delay is probably explained by the low Cd concentrations used in this study. In contrast, when zebrafish are contaminated with huge amounts of Cd, a quick MT gene expression can be seen: the induction of MT genes has been reported in liver and gills of zebrafish treated for 4 h with 50 μM $CdCl_2$ (Airaksinen *et al*. 2003). One might say that this expression pattern is governed by the necessity of reaching high Cd bioaccumulation levels within tissues before the onset of MT gene expression. However, this is not always verified and examples in this study are not compatible with this belief.

At the basal level, *mt1* and *mt2* genes were not expressed at the same levels. *mt2* was by far the more expressed in various organs of control fish, like the liver and muscles, for example, where the *mt2* to *mt1* ratios reached 512 and 256, respectively (Table 6). However, *mt1* represented 6% of the total transcripts in gills and its influence was emphasised under Cd contamination where it accounted for 20 and 25% of the total MT transcripts at day 21 in C_1 and C_2 tank fish, respectively. This result suggests that *mt1* could have an important physiological role within gills during contamination by a low Cd concentration in water.

Discrepancies were observed between MT protein levels and up-regulation of MT gene expressions. This could be explained by a time delay between gene expression and protein accumulation, suggesting that MT synthesis in *D. rerio* is regulated at the transcriptional level as well as the translational level. Such a delay has also been reported for MT from rats (Vasconcelos *et al*. 2002) and mussels (Lemoine and Laulier 2003). This observation showed that MT gene expression levels and MT protein concentrations give complementary results and could both be investigated during Cd contamination studies.

Up-regulation of *c-jun* in brain, liver, and muscles after 21 days of contamination suggests an induction of AP1 transcription factor, a heterodimer made of *c-jun*, *c-fos*, and ATF2. AP1 is known to be involved in cell cycle regulation, apoptosis, DNA repair and metal detoxification since many MT promoters contain AP1-binding

Table 7. Comparison of differentially expressed genes from the zebrafish between direct cadmium and trophic methylmercury contamination.

Categories	Genes[a]	
	$CdCl_2$	MeHg
Specific to MeHg		*p53*[l], *sod*(*Cu*/*Zn*)[l,m]
Specific to Cd(II)	*hsp70*[g], *mt1*[b,g,l,m], *pyc*[g,m]	
Specific to both Cd(II) and Hg(II)	*gadd*[g,m], *mt2*[b,g], *rad51*[g,m], *tap*[g,m]	*gadd*[l], *mt2*[l], *rad51*[l], *tap*[l]
Specific to both Cd(II) and MeHg	*bax*[g,m], *coxI*[g], *c-jun*[b,l,m], *sod*(*Mn*)[g]	*bax*[l,m], *coxI*[l,m], *c-jun*[l,m], *sod*(*Mn*)[l,m]

[a]Tissues found to express the listed genes are indicated by upper case letters: b, brain; g, gills; l, liver; m, skeletal muscles.

sites (Lee *et al*. 1987). *c-jun* is activated by c-Jun NH_2-terminal kinase (JNK), a mitogen-activated protein kinase whose phosphorylation is dependent on cadmium-induced hydrogen peroxide generation. Reactive oxygen species (ROS) production is itself dependent on cadmium-stimulated calcium release from storage compartments (Kim and Sharma 2004). Hydrogen peroxide has been reported to enhance the AP1 binding activity of the nuclear extract from Jurkat cells to the *gadd153* AP1 binding site (Oh-Hashi *et al*. 2004). In agreement with this finding, *gadd* induction in zebrafish gills is correlated with up-regulation of the ROS-reactive *hsp70* and *sod*(*Mn*) genes. Induction of the cytoglobin gene might also be considered as a ROS reaction since the brain globin counterpart, neuroglobin, has been reported to have a role in the detoxification of ROS and NO (Herold *et al*. 2004).

Gills, liver and brain are peculiarly sensitive to Cd toxicity. This may be due to the high Cd concentrations within these organs, whereas muscles appear to be much less sensitive to Cd because they accumulate very low doses of this metal. But why do muscles accumulate such small quantities of Cd? It is often suggested that Cd entry routes into muscles are not efficient (Andres *et al*. 2000). We prefer to suggest that protection and detoxification devices such as Cd ion pumping are much more efficient in muscles than in liver and brain. First, basal levels of the DNA repair *gadd* gene are 2 and 8 times lower in liver and brain, respectively, than in muscles. The same holds true for the ROS and Cd responsive *hsp70* and *sod*(*Mn*) genes: basal level of *hsp70* is 64 times lower in brain and liver than in muscles, and that of *sod*(*Mn*) is 4 times lower in brain than in muscles. Second, the ABC transporter *tap* gene is much more expressed in muscles than in the other tissues: 4, 8 and 32 times more compared with gills, liver and brain, respectively. And *tap* is induced in gills and in muscles by Cd contamination but not in liver and brain. Third, the 100 fold increase in *tap* expression observed in C_2 tank fish gills at day 7 is correlated with a small decrease in Cd loading between days 7 and 21. A similar pattern occurred in C_2 tank fish muscles where a three fold increase in *tap* expression at day 21 paralleled a stagnation of Cd loading between days 7 and 21. This stagnation of Cd loading suggested that between 7 and 21 days gills, and also muscle, were dispatching Cd into the whole organism or that efficient Cd detoxification and scavenging devices were in action. However, we could not exclude the fact that this release of Cd into the organism reflected the occurrence of the apoptotic phenomenon. Indeed, the expression levels of apoptotic genes were up-regulated, notably in gills.

We thus propose that the stagnation of Cd loading in gills and muscle might involve Cd extrusion by *tap* ATPase-catalysed pumping of a glutathione–Cd complex or a complex associating Cd to cysteine-rich polypeptides. This would explain the low Cd burden in the muscles. What is the experimental evidence to support such a hypothesis? (1) *tap* belongs to the ABC transporter superfamily which also contains, besides MDR1, the multidrug resistance-associated protein (MRP1), another molecular device involved in the resistance of tumor cells to chemotherapy, the *Leishmania* LtpgpA transporter and the yeast cadmium resistance factor 1 (*Ycf1*). These last three proteins, which are genuine xenobiotic pumps, are also involved in metal detoxification. *LtpgpA* pumps out arsenite and antimony (Dey *et al*. 1996). The *Ycf1* transporter catalyses the pumping of bis(glutathionato)cadmium (Li *et al*. 1997) and the human *MRP1* protein functionally

complements the *Ycf1* factor (Tommasini *et al.* 1996). This means that a xenobiotic pump can also be a metal pump, provided that the metal ion forms a complex with glutathione. (2) *MRP1* is overexpressed in human metal-selected tumor cells (Vernhet *et al.* 1999). (3) The induction of the human *MDR1* gene has been observed in human cells exposed to sodium arsenite or cadmium chloride (Chin *et al.* 1990). (4) An *MDR* homologue is induced by various metals including Cd, zinc, copper and mercury in the Asiatic clam *Corbicula fluminea* (Achard *et al.* 2004). (5) Rat *mdr1* gene overexpression protects kidney cells against cadmium-mediated apoptosis (Thévenod *et al.* 2000). (6) The nematode *Caenorhabditis elegans* was hypersensitive to metals when both the *MRP* homologue and a member of the *MDR1* gene family were deleted (Broeks *et al.* 1996). (7) The only known member of the Tap/MDR family in zebrafish is the *tap* gene. (8) Recently, hMDR1 and bacterial homologues have been shown to protect cell against Cd through Cd pumping (Achard-Jorris *et al.* 2005)

In a prior study, using the same set of selected genes, the effects of dietary methylmercury (MeHg) were analysed on gene expression in three organs of the zebrafish (liver, skeletal muscles and brain). Adult fish were fed for 7 and 21 days with three different trophic exposure conditions: a control diet and two diets (C_1 and C_2) contaminated by MeHg at 25 and 67 nmol Hg g^{-1} (Gonzalez *et al.* 2005). When comparing the gene expression patterns between MeHg and Cd contamination, some categories of genes are highlighted (Table 7). One set of genes appears to be specific to Cd contamination: *mt1*, *pyc*, and *hsp70*. Pyc is an anaplerotic enzyme allowing replenishment of the Krebs' cycle pool of metabolites, thus indicating a possible toxic effect of Cd on mitochondrial metabolism. *pyc* was reported to be expressed in response to Cd in *C. elegans* (Liao and Freedman 1998). A second set of genes including *sod*(*Cu*/*Zn*) and *p*53 are specific to MeHg exposure. A third set of genes is common to both types of contamination and includes *c-jun*, *bax*, *sod*(*Mn*) and *cox1*, indicating pro-apoptotic response and mitochondrial damage. A last set of genes is said to be common to Cd(II) and Hg(II) contamination and includes *mt2*, *gadd*, *tap* and *rad51*. Indeed, these genes were found to be up-regulated under MeHg contamination only late in time in liver when the demethylation process leading to Hg(II) reached 28% of the total Hg burden in this organ. In contrast, in muscles or brain, where demethylation does not operate, these genes were either down-regulated or unresponsive.

Our results open the way to future experiments, which could investigate the whole genomic expression pattern in *D. rerio* using the SAGE (Serial Analysis of Gene Expression) approach (Velculescu *et al.* 1995), after environmentally relevant contamination with Cd by the direct exposure route. These investigations would be of great interest in defining the molecular mechanisms developed by fish in response to metal contamination and to better understand the toxicological effects at organ level. These investigations would also furnish new and more specific molecular biomarkers for use in determining the structural and functional impacts of metals on fish in the natural environment.

Acknowledgements

The authors are grateful to Bruno Etcheverria, Aurélie Godrant, Régine Maury-Brachet and Véronique Roques-Duflo for technical support during the exposure conditions and the harvesting of organs from *D. rerio*. We are grateful to Jean-Charles Massabuau for comments on the manuscript and his helpful competence in fish physiology.

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