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Metallothionein mRNA induction is correlated with the decrease of DNA strand breaks in cadmium exposed zebra mussels

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Abstract:

We have previously shown that cadmium (Cd) and Benzo[a]pyrene (BaP) induced early DNA damages in zebra mussels, and that the level of DNA strand breaks (SB) returned to a basal level after 3 days of exposure to Cd. The aim of the present study was to go further in the mechanisms of Cd and BaP detoxification. For that purpose, expression of genes encoding for metallothionein (MT), Aryl Hydrocarbon Receptor (AHR), P-gP, catalase, glutathione S-transferase and Heat shock protein 70 (HSP70) proteins have been measured using RT-qPCR. Data reported here show that Cd is a strong inducer of MT and HSP70 genes, and that BaP is a strong inducer of P-gP and AHR genes. Exposure to Cd and BaP resulted in moderate changes in antioxidant enzymes mRNA. Since the increase of MT mRNA occured when the DNA SB level returned to its basal level, we can suggest that MT is implicated in cadmium detoxification.

Highlights

► cadmium (Cd) and Benzo[a]pyrene (BaP) induced DNA damages in zebra mussels. ► Cd is a strong inducer of Metallothionein (MT) and HSP70 genes. ► Metallothionein might be implicated in cadmium detoxification. ► BaP is a strong inducer of P-gP and AHR genes. ► Cd and BaP induced in moderate changes in antioxidant enzymes mRNA.

Keywords : Benzo[a]pyrene ; cadmium–metallothionein ; Aryl Hydrocarbon Receptor ; antioxidant enzymes ; RT ; qPCR

1. Introduction

The zebra mussel is an organism of choice for the monitoring of metallic and organic contaminants in freshwater ecosystems [1-4]. The sensitivity of zebra mussels to genotoxic contaminants has been demonstrated through the induction of micronuclei, DNA strand-breaks and DNA adducts [5-9]. Similarly, field studies revealed that DNA damages were higher in mussels from polluted sites compared with reference site [10, 11]. However, little is know about the regulation of proteins implicated in detoxification following DNA strand breaks.

Recently, gene expression profiles of proteins implicated in detoxification and stress were analyzed in zebra mussels exposed to cadmium and xenobiotics (metoprolol and levonorgestrel). These first observations improved our knowledge in the regulation of these genes [12-14]. These genes encode for proteins that are reliable biomarkers in field and laboratory exposures to contaminants, including metals and organic pollutants. MT has been suggested to be key elements causing the retention of Cd in mussels [15]. High level of MT mRNA was observed in many organisms exposed to cadmium, including zebra mussel [16]. Heat shock proteins are widespread in plants, bacteria and animals and belong to chaperone proteins, which are important for protein folding, protein transport and cell stabilisation [17, 18]). Superoxide dismutase (SOD) and Catalase (CAT) are antioxidant enzymes and reliable biomarkers of reactive oxygen species [19], SOD is responsible for the reduction of the superoxide radical into hydrogen peroxide, and CAT for catalysing hydrogen peroxide to water. Glutathione S-transferase gene (GST) encodes a phase II metabolizing enzyme known to catalyze the conjugation of glutathione with various electrophilic substances and plays a role preventing oxidative damages [20]. The P-glycoprotein, P-gp, is a member of the multidrug transporter proteins that are ATP dependent proteins which efflux a variety of moderately hydrophobic compounds out of cells [21-23]. The HSP70, which is involved in the processing of misfolded proteins due to different kinds of stress, is used as a very

general ecotoxicological endpoint for protein damage and subsequent protective mechanisms [24].

The data presented here are the second part of a article published earlier in which we first shown that zebra mussels exposed to environmentaly relevant concentrations of Cd and BaP displayed DNA damages [25]. Cadmium, as well as Benzo[a]pyrene (BaP), are model environmental contaminants, classified as a human carcinogen by the IARC [26]. Cd shows a co-genotoxic effects in combination with other mutagenic agents such as UV light, alkylating agents and B[*a*]P in mammalian cells. Cadmium is assumed to be a weak genotoxicant that amplifies the genotoxic effect of B[*a*]P [27, 28]. Therefore, based on these studies, we wanted to determine whether Cd could have the same effect in mussels. We have shown that Cd induced early DNA damages (DNA strand break and DNA oxidation), and that BaP induced DNA damages only on the third day of exposure. The most surprising is that the level of DNA returned to the basal level despite the continued presence of cadmium.

The aim of the present study was to go further in the mechanisms of Cd and BaP detoxification to determine why the level of DNA stand breaks return to its basal level. For that purpose, we measured gene expression of several proteins described to be involved in detoxification, such as metallothionein, AHR, P-gP, catalase, SOD and glutathione S-transferase. HSP70 gene expression was also measured as a general indicator of stress. Samples analyzed here are frozen samples of the first published study.

2. Materials and Methods

2.1 Chemical reagents

$CdCl_2$, BaP, DMSO, agarose, trypan-blue and PBS were purchased from Sigma (France).

2.2 Mussel sampling and maintenance conditions

Adult specimens of the zebra mussel *Dreissena polymorpha* (shell length 25±2 mm) were collected in the East channel (Commercy, France), which is a reference site. Cd concentration in mussels is in good agreement with those usually found in bivalves from clean waters [29]; BaP was not detected [11, 30]. Mussels were detached from the rock by cutting their byssus threads and carried to the laboratory in their original water. The mussels were randomly placed in a 20-L aerated tank with about 100 specimens each, and acclimatized to Valvert mineral water and temperature (15°C), a day/night lighting system was applied. The mussels were fed every 3 days with algae (*Pseudokirchneriella subcapitata)* and the water was changed every 2 days. The animals that had not become attached to the tank were removed. The mussels were maintained in the above conditions for 8 days before the start of treatment since it was demonstrated that this time is needed to reach a baseline level of DNA damage [31].

2.3 In vivo exposure of zebra mussels

We chose two genotoxicants with different modes of genotoxic action: benzo[a]pyrene, which prevalent mechanism of action relies on DNA adducts, and cadmium for its pro-oxidant properties and its role in the inhibition of DNA repair. In order to work with more environmentally realistic concentrations, the Cd and BaP concentrations used here were low-level concentrations equal to the lowest genotoxic concentration published for zebra mussels or marine mussels [6, 32]. A stock solution of $CdCl_2$ and BaP was prepared in water or pure DMSO, respectively. Preliminary experiments showed that DMSO in water (0.001%) did not induce genotoxic effects.

Following overnight equilibrium of tanks with chemical compounds to avoid adsorption to the wall of the tank, mussels were added after renewing the water. Ten mussels were exposed to BaP at 10 µg /L, Cd at 10 µg /L and to a combination of Cd (10 µg/L) and BaP (1 µg/L) dissolved in 8 L of water for 11 days at 15°C. In order to maintain the chemical concentration constant, water was renewed every 2 days and then contaminated again with chemicals. For each parameters analyzed, four mussels were sacrificed after 0, 12 h, 24 h and 3, 5 and 11 days of exposure for further analysis (bioaccumulation, DNA damages and gene expression). Cadmium bioaccumulation, DNA damages and nuclear abnormalities data were already published [25]. Gills of 4 mussels were pooled and frozen for gene expression analysis.

2.4 RNA extraction, RT-PCR and qRT-PCR analysis

Total RNA was isolated from frozen gills of *Dreissena polymorpha* using the phenolic reagent TRIZOL (Invitrogen, France). RNA concentration and purity was measured by spectrophotometric absorption (260/280 and 230/280 ratio), RNA quality was checked with denaturing gel electrophoresis. First strand cDNA synthesis was carried out on 1 µg of total RNA extract with oligo-dT primers according to Improm II Reverse Transcriptase kit (Promega, France).

Real-time PCR assays for Ribosomal protein S3 (S3), Catalase (CAT), superoxide dismutase (SOD), Glutathion S-transferase (GST), Metallothionein (MT), Heat-shock protein 70 (HSP70), Aryl Hydrocarbon receptor (AHR), and P-gp were run in a LightCycler 480 Real Time PCR System (Biorad) using SYBR Green Power Master Mix (Invitrogen, France). PCR reactions and primers pairs were those published by [12] except for Ribosomal protein S3 [14]. Determination of the transcript abundances in individual sample was conducted with the comparative C_T method ($\Delta\Delta C_T$) in consideration of a calibrator sample (control sample). Samples were normalized to Ribosomal S3 gene due to its constant expression [14]. At the end of each PCR reaction a melting curve analysis was carried out to proof assay specificity. PCR efficiency values for reference and tested genes were calculated as described [33], and assumed to be close to 100% from these calculations.

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160 ***2.5 Statistic analysis***

161 RT-qPCR results are given as mean values ± S.D. of 3 values (4 mussels per condition
162 pooled and 3 repetitions of each test). The calculated values were compared among different
163 groups (model agents' concentration) using an Analysis of Variance (ANOVA) followed by a
164 Tukey post hoc test. Three levels were considered significant: $p<0.05$ (*), $p<0.01$ (**) and
165 $p<0.001$ (***). All statistical analysis was performed with R software.

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3. Results

The data published here are the second part of a study published earlier [25] in which we observed an increase of DNA strand breaks in gill cells of mussel exposed to environnementaly relevant concentrations of Cd and BaP (see Table 1, Supporting information). The renewal of the water every 2 days ensured a constant cadmium concentration over the 11-day experiment: 8.27 ± 0.18 and 8.23 ± 0.34 µg/L in $CdCl_2$ and $CdCl_2$ + B[*a*]P media, respectively. Bioaccumulation of cadmium in soft tissues of mussels exposed to $CdCl_2$ (10 µg/L) or $CdCl_2$ + B[*a*]P (10 µg/L + 1 µg/L) increased over the 11 days of exposure from 9 µg/g dw after 24 hr of exposure to 45 µg/g dw at the end on day 11 [25].

Strongest changes in the expression of detoxification genes were found for MT, HSP70 and P-gp genes. A significant increased was noticed, up to 6.5, 10 and 17 fold, respectively after 5 days of exposure to BaP for P-gp ($p<0.001$), after 3 days of exposure to Cd and Cd+BaP for MT ($p<0.001$), and after 11 days of exposure to Cd for HSP70 ($p<0.001$).

It is important to note that DNA strand breaks induced by Cd return to the basal level when MT gene expression started to increase. Indeed, MT mRNA expression increased gradually from day 3 until day 11. The same trend was observed when mussels were exposed to Cd+BaP. The presence of BaP in the mixture does not inhibit the effect of Cd on MT gene induction.

Also, as expected, BaP is as well a strong inducer of AHR gene in zebra mussel, as we observed an early and transient increase of the AHR mRNA expression as soon as 12 hr after the beginning of the exposure to BaP alone ($p<0.01$). and to the mixture Cd + BaP ($p<0.05$) AHR mRNA expression was slightly decreased at day 1 and day 5, respectively in BaP ($p<0.05$), Cd+BaP ($p<0.001$) and in Cd exposed mussels ($p<0.01$).

P-gp was slightly up-regulated by Cd and BaP during the 11 days of exposure, with the strongest induction at day 5 in Cd exposed mussels.

HSP70 was slightly up-regulated during the first days of exposure to Cd, and strongly up-regulated at day 11 as already mentioned, indicating protein damage.

In contrast, the effect of Cd an BaP was less important on SOD, GST and CAT mRNA levels. More precisely, CAT mRNA was two times decreased after 12 hr of exposure in BaP exposed mussels ($p<0.01$) and after 24 hr ($p<0.001$) in Cd and Cd+BaP exposed mussels compared to control. On the contrary, CAT gene was up-regulated, two times, after 3 and 5 days of exposure, respectively in Cd+BaP and Cd exposed mussels.

GST mRNA was 1.5 time increased after 12 h of exposure to Cd+BaP, and significantly decrease after 3 days and 11 days of exposure to Cd, BaP and Cd+BaP.

SOD mRNA was about two times decreased in Cd exposed mussels from the first day until the eleventh day but this was significant only on days 3 and 5. SOD mRNA was 1.5 times increased after 12 hr, 5 and 11 days of exposure to Cd+BaP ($p<0.001$ and $p<0.05$) and after 3 days and 11 days of exposure to BaP ($p<0.001$).

4. Discussion

The present study is the second part of a study in which we reported early genotoxic effects of Cd and BaP in gill cells of zebra mussels. We wanted to determine whether stress-related genes and detoxification genes are regulated at the transcriptional level in the hours following genotoxic damage in gills.

Tissue-specific differences in gene expression following exposure to metals (Cd, Cu, Hg) and BaP have been published previously [14] [34], hence we focused here on gills in the hours following genotoxic damage. Indeed similar patterns of mRNA abundance were observed between gills an digestive gland in Cd exposed mussels, while in BaP exposed mussels, we noticed an early induction of *HSP70*, P*g*P, *AHR* and *SOD* mRNA levels in the gills compared to the digestive glands. Therefore, it appears from these studies that gill tissue represents an interesting model to investigate the molecular mechanisms of detoxification [34]**.**

We show here that, at environmentally relevant concentrations, Cd is a strong inducer of MT and HSP genes, and that BaP is a strong inducer of PgP and AHR genes. Exposure to Cd and BaP resulted in moderate changes in antioxidant enzymes mRNA. The mRNA level of metallothionein increases when the level of DNA strand breaks returned to its basal level, which is probably the most interesting result of this study

We confirmed that Cd (10 µg/L) is a strong inducer of MT gene, 8 times more than control. Indeed, zebra mussels accumulate Cd at water concentrations as low as 9 µg/liter, excluding the possibility of a homeostatic control [35], and as a consequence, MT protein is induced [36]. Therefore it appears that MT mRNA induction and MT protein induction are early increase in zebra mussel as Lecoeur et al. [37] observed an early increase of total MT biosynthesis after exposure to Cd (2-20 µg/L). Only one isoform of MT gene has been reported for zebra mussel, the Dp MT [38]. Engelken and Hildebrandt [38] first shown that cadmium was an inducer of MT gene but this was for elevated concentration of cadmium; afterwards, an increase of this gene expression has also been observed for lower Cd concentrations [14, 38-40] [16]. Cadmium

increases the synthesis rate of metallothionein messenger-RNA via transcriptional activation of metal-responsive factors located in the upstream region of MT genes [41]. We used here cadmium at environmentally relevant concentrations, measured in rivers strongly impacted by mining activities [40].

Our data provide evidence on the exposure time required for the induction of MT gene by cadmium: MT mRNA level gradually increases from the third day until the eleventh day of exposure suggesting that during the first three days, the physiological concentration of MT protein was not high enough to adsorb the Cd. A time-dependent increase was also reported but for higher concentration of cadmium (20 µg/L) [14].

Interestingly, the increase of MT mRNA occurs as the DNA strand break level return to its basal level, suggesting that DNA repair was efficient. However as a constant concentration of cadmium in the water and an increase of bioaccumulated cadmium were measured, these data also suggest a decrease in Cd bioavailability. Indeed, the MT protein concentration is probably higher on the third day, compared to the first day; a higher level of MT protein can lead to a decreased bioavailability of Cd and, as a consequence, Cd induced DNA damage could be decreased too. Similar observations were recently reported by Qu & Waalkes [42]; these authors demonstrated that MT-competent cells activate MT in response to Cd, while MT-deficient cells adapt to Cd primarily by turning on oxidant response systems.

Cd is known to induce reactive oxygen species (ROS) which in turn lead to DNA strand breaks and Oxidative DNA damage [43], an effect that we also observed with zebra mussels [25]. MT has two major functions in Cd toxicity inhibition: (i) MT detoxicates the metal by direct binding and (ii) the cysteines in MT appear able to react directly with ROS, thus MT may also act as an antioxidant independently of metal sequestration [45, 46]. Our data highlight the role of MT in the inhibition of Cd genotoxicity.

One of the known protective mechanisms that aquatic animals have developed in response to stresses is the induction of HSPs. We observed that Cd is a strong inducer of HSP70 gene, on the contrary to Navaro et al. [14] who reported HSP70 gene induction only in the digestive gland. Interestingly the strongest up-regulation of HSP mRNA, seventeen

267 times more than control, was observed after 11 days of constant exposure to Cd. HSP70 are
268 protein chaperones that are induced by various environmental stressors including organic
269 pollutants [47] and metals such as Hg, Cu, Cd [14]. HSP acts to prevent protein aggregation
270 and to maintain functional conformations. This transcriptional regulation of HSP70 is
271 probably necessary to enhance the tolerance to cadmium, as elevated levels of HSPs have
272 been proven to protect against the negative impact of metals on protein integrity [48, 49].
273 We confirmed that BaP (4.4 nM) is an inducer of HSP70 as we observed previously [34]. The
274 regulation of HSP70 gene in response to BaP remains unclear. For example, high
275 concentration of BaP (10 µM) suppress the transcription of HSP70 gene in human
276 endothelial cell [50], and BaP (1 µM) up-regulate HPS70 mRNA in bronchial cells, suggesting
277 a potential role of HSP70 in the NER DNA repair [51].
278 We observed that Cd up-regulated both MT and HSP70 genes. It is well known that
279 HSP70 and MT promoters possess anti-oxidant response element (ARE) which may provide
280 a mechanistic explanation to this correlated response [52, 53]. Cd has been described to
281 induce HSP70 and MT proteins in mammals and oysters [54, 55].
282 In Mammals, Cytochrome P450 enzymes are important in the metabolization of
283 xenobiotics, such as PAHs [56]). Induction of CYP1 is mediated mainly through a specific
284 cytosolic receptor, the aryl hydrocarbon receptor (AHR). Activated AHR is also a transcription
285 factor of other genes that encode phase I and II xenobiotic metabolizing enzymes [57]. Our
286 data confirmed that BaP is a strong inducer of AHR gene as we recently observed [34]. AHR
287 mRNA induction was lower in Cd+BaP exposed mussels, suggesting an interaction between
288 Cd and BaP, as observed for fish and Human hepatocytes. Indeed, it has been shown that
289 Cd decreases the induction of AHR by BaP in that biological models [58, 59]. AHR seems to
290 be implicated in the detoxification of BaP in zebra mussels; as the AHR serves as a
291 transcription factor for enzymes of the CYP450 family, our data suggest that BaP is
292 metabolised by CYP450, as it was recently proposed for pharmaceutical compounds [12].
293 We observed that exposure to Cd and BaP resulted in moderate changes in
294 antioxidant enzymes, characterized by a slight decrease of mRNA levels of SOD, GST and

CAT genes, which suggest a moderate regulation of these genes at the transcriptional level. It is known that Cd induced a depletion of cellular GSH, which could explain the slight decrease of anti-oxidant enzymes mRNA level. We previously observed that when BaP was added in aquaria containing zebra mussels two times a day, animals presented an increase in GST, CAT and SOD mRNA levels after 12h of exposure. In the present study, the same concentration of BaP (renewed every two days) did not induce significant increase of mRNA levels of those genes. As CAT, SOD and GST are the first enzymes induced after animal exposure to xenobiotics, we can suggest that their mRNA levels probably increased before the twelfth hourof exposure which would explain that we have not been able to detect their increase. At the protein levels, data of the literature also appeared to be contradictory, hence it is suggested that catalase activity was dependent on the animal tested, the nature of chemicals and the intensity of exposure [60], which is confirmed in the present study. Hoarau *et al.* [61] observed an inhibition of GST gene expression after marine mussel exposure to BaP whereas mussels collected in a site highly contaminated with PAH exhibited an induction of its expression.

The increase of P-gp expression observed in the present study is not surprising as this protein is implicated in non specific excretion of xenobiotics, metabolites as well as waste products derived from cell damage [62, 63]. The induction of P-gp by BaP was first reported in Caco-2 cells [64, 65]. The inducilbility of P-gp by BaP was also observed in blue mussels at the protein level [65] and at the mRNA level [66]; in zebra mussel, we recently shown an increase at the transcriptional level [34].

In conclusion, gill tissue represents an interesting model to investigate the expression of genes encoding for stress related proteins. Cd and BaP induced a regulation at the transcriptional level of genes implicated in either metabolism or detoxification such as AH-R, HSP70, P-gp and MT. Cd seems to decrease the induction of AHR by BaP as already described for fish and human hepatocytes. In Cd exposed mussels, the up-regulation of MT mRNA is correlated with the restoration of the DNA SB basal level suggesting that MT is

implicated in cadmium detoxification. Future studies need to be done to clarify the interaction of metals and PAHs on AHR gene expression.

Conflict of interest:

The authors declare that there are no conflicts of interest.

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Figure captions

Figure 1: Relative mRNA expression genes

mRNA expression of genes in gills of zebra mussels exposed to Cd (10 µg/L), BaP (10µg/L) and Cd+BaP (10 µg/L, 1 µg/L) for 12 hr, 24 hr, 3, 5 and 11 days. Determination of the transcript abundances in individual sample was conducted with the comparative C_T method ($\Delta\Delta C_T$) in consideration of a calibrator sample (control sample). Samples were normalized to Ribosomal S3 gene due to its constant expression. Results (mean values ± S.D.) were compared among different groups using an ANOVA followed by a Tukey post hoc test. Three levels were considered significant: $p<0.05$ (*), $p<0.01$ (**) and $p<0.001$ (***).

A: metallothionein (MT), P-glycoproteion (P-gp), Aryl hydrocarbon receptor (AHR) and Heat shock protein 70 (HSP70)

B: superoxide dismutase (SOD), Glutathion S transferase (GST) and catalase (CAT)

Supporting information :

Table 1:Tail DNA (%) in gill cell comets of *Dreissena polymorpha*

DNA strand breaks were measured with the comet assay and expressed as Tail DNA (%). Control and various treatment groups of mussels were exposed to constant concentration of dissolved $CdCl_2$ (10 µg/l), BaP (10 µg/l) and Cd+BaP (Cd=10 µg/L, BaP=1µg/l) for various duration. Tail DNA % were reported as mean ± standard error; *p<0.05; **p<0.01; ***p<0.001.

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	Control	Cadmium	Cd+BaP	BaP
12 hr	15.42±0.8	24.23±1.22	13±0.61	15.56±0.85
Day 1	10.95±0.54	22.12±1.37***	16.92±0.72***	14.12±0.73**
Day 3	14.55±0.88	24.95±1.17***	19.13±0.99***	32.39±1.47***
Day 5	11.66±0.63	11.83±0.61	13.27±0.77	14.98±0.84
Day 11	12.84±0.68	14.95±0.88*	12.84±0.73	15.51±0.95

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Highlights

- cadmium (Cd) and Benzo[a]pyrene (BaP) induced DNA damages in zebra mussels
- Cd is a strong inducer of Metallothionein (MT) and HSP70 genes
- Metallothionein might be implicated in cadmium detoxification
- BaP is a strong inducer of P-gP and AHR genes
- Cd and BaP induced in moderate changes in antioxidant enzymes mRNA