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Impact of acute arsenic and cadmium exposure on the expression of two haeme oxygenase genes and other antioxidant markers in common carp (*Cyprinus carpio*)

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ABSTRACT: The aim was to study the effects of cadmium (Cd) and arsenic (As) on haeme oxygenases (HOs) and other oxidative stress biomarkers, and their roles in macromolecule damage in liver and kidney of common carp (*Cyprinus carpio* L.). HOs play a critical role in the defence system against oxidative damage, producing biliverdin and carbon monoxide with important free radical scavenging properties. However, increased HO activity in haeme degradation may also lead to a pro-oxidant effect through the liberation of Fe-modifying Cd and As toxicity. The response of an organism to exposure to toxic metals is in many cases brought about by changes at the level of gene expression. In this study, the genes *ho-1* and *ho-2* of the common carp were identified, and the changes in gene expressions were analysed from the aspect of Cd and As accumulation. Both *ho-1* and *ho-2* are transcriptionally induced by Cd and As, but their inductions differ in time course, dose response and tissue specificity. The expression of *ho1* was mostly affected by As, primarily in the liver (45-fold), whereas it was enhanced with higher efficacy by Cd in the kidney (25-fold). The cellular redox status and the damage of lipid molecules were monitored via the ratio of reduced to oxidized glutathione, the levels of H_2O_2 and lipid peroxidation, and the activities of superoxide dismutase (SOD) and catalase (CAT). Copyright © 2014 John Wiley & Sons, Ltd.

Keywords: heavy metals; kidney; liver; lipid peroxidation; oxidative stress

Introduction

A variety of contaminants including toxic heavy metals (Cd, Cu, Hg, Pb, Zn and As), which are ubiquitously present in rivers, lakes and reservoirs, are disadvantageous for aquatic organisms (Olsson, 1998; Bhattacharya *et al.*, 2002). In general, they do not undergo biodegradation and therefore accumulate in fish, oysters, mussels, sediments and other components of aquatic ecosystems (Abdullah *et al.*, 2007). Through the disturbed integrity of these ecosystems, human health is challenged through the food chain. Exposure to heavy metals increases the formation of reactive oxygen species (ROS), and promotes oxidative stress by inducing loss of the cellular pro-oxidant–antioxidant balance (Halliwell & Gutteridge, 1984; Sies, 1991). The disturbed redox balance leads to enhanced formation of ROS, such as the superoxide anion ($O_2^{\bullet-}$), the hydroxyl radical (•OH), hydrogen peroxide (H_2O_2) and singlet oxygen and peroxynitrite. The pathway of increased ROS generation in response to heavy metal exposure is metal dependent (Valko *et al.*, 2005). For example, cadmium (Cd), a highly toxic, widely distributed bivalent cation, induces ROS production and oxidative stress via indirect pathways, such as the induction of NADPH oxidases, binding to thiol groups and replacing Fenton metals from their active sites. The disturbed redox balance influences both damaging and repair processes, through the activation of several signalling cascades (Cuypers *et al.*, 2010), and the toxic manifestations of Cd may lead to the enhancement of cellular damage, including the peroxidation of membrane lipids and the disturbance of DNA integrity (Halliwell & Whiteman, 2004). Arsenic (As), a ubiquitous, potentially toxic trace element present in both inorganic and organic forms and various oxidation states that is released into freshwater and marine environments (Welch *et al.*, 1988), is able to generate ROS directly (Flora, 2009).

Under stress conditions, genes coding for molecules involved in the biological defence and cellular repair are markedly upregulated, and the changes in gene expression can be more characteristic, sensitive and measurable endpoints than the toxicity itself (Nuwaysir *et al.*, 1999). Members of this antioxidant defence system in different organisms are useful biomarkers to characterize a polluted environment. The pathways of haeme biosynthesis and degradation affect the cellular oxidant metabolism because both are closely linked with Fe cycling. Heme oxygenases (HOs) play roles in haeme degradation, yielding equimolar quantities of biliverdin, and CO with important free radical-scavenging properties and free Fe ions. HO-1 has emerged as a central component of the mammalian stress response, as the corresponding gene can be induced by multiple chemical and physical stimuli (Maines, 1988; Ryter & Tyrrell, 1997). Three isozymes, HO-1, HO-2 and HO-3, have been identified. HO-1 is

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ubiquitously expressed in animal cells, and has a tissue-specific gene expression pattern. *ho-1* expression is highly inducible by its substrate haeme and by numerous stress stimuli, such as ultraviolet irradiation, heat shock, lipopolysaccharide, (pro-)inflammatory cytokines (IL-6 and TNF-α), heavy metals, hypoxia and hyperoxia. *ho-2* has a relatively constant expression, with an important regulatory role in haeme homeostasis (Maines, 1999).

To minimize oxidative damage, antioxidants may decrease the cellular level of free radicals, either by inhibiting the activities or expressions of free radical-generating enzymes or by enhancing the activities and expressions of antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) (Halliwell, 2006). SOD can convert two $O_2^{\bullet-}$ into H_2O_2 and O_2 (Fridovich, 1989), CAT and GPx then take part in the elimination of H_2O_2 before the Fenton reaction can create a •OH (Lü *et al.*, 2010). GSH also plays a critical role in this system as an antioxidant, enzyme cofactor and major redox buffer (Wang & Ballatori, 1998). Most vertebrate tissues produce GSH, but the liver and kidney are the most active sites of GSH synthesis (DeLeve & Kaplowitz, 1991).

In this paper, we report the identification of two *ho* genes in common carp (*Cyprinus carpio* L.), and their basal expression patterns in different tissues and organs. We also followed the changes in their expressions after exposure to heavy metals (Cd and As) in liver and kidney, the organs most strongly involved in the processing and excretion of toxic agents. As the activity of HO in haeme degradation, besides producing biliverdin, and CO with antioxidant capacity, may lead to a pro-oxidant effect by releasing Fe, we also measured parameters affecting cellular stress-response and macromolecule damage (H_2O_2, SOD, CAT and GSH/GSSG, and lipid peroxidation levels) from the aspects of the expression of *ho*s and metal accumulation.

Materials and Methods

Animals and Treatments

Common carp weighing 800–1000 g, obtained from the Tisza Fish Farm, Szeged, were acclimatized in well-aerated 400-l water tanks at 16 °C. Water was kept oxygen-saturated by aeration and was changed twice a week. Fish were transferred into 100-l water tanks (2 fish per tank) at the end of the acclimatization period. For metal treatment, the carp were exposed to 1 mg l^{-1} or 10 mg l^{-1} Cd^{2+} [$Cd(CH_3COO)_2 \times 2H_2O$; Fluka Buchs, Switzerland] or 1 and 10 mg l^{-1} As (Na_2HAsO_4; Fluka Buchs, Switzerland) respectively, for up to 72 h, under static conditions. In all experiments, 4–6 Cd- or As-treated and three untreated animals were sacrificed at each time point for tissue harvesting. Organs and tissues were frozen immediately in liquid nitrogen and stored at −80 °C. Experiments were conducted in accordance with national and institutional guidelines for animal welfare.

RNA Extraction, Reverse Transcription and PCR Amplification

Approximately 100 mg of frozen tissues/organs (liver, kidney, skin, gill, spleen, blood and muscle) or whole heart and brain was homogenized in RNA Bee reagent (Tel-Test, Inc. Friendswood, TX, USA) and total RNA was prepared according to the procedure suggested by the manufacturer. Total RNA was routinely treated with 100 U RNAse-free DNAseI (Thermo Scientific Baltic, Vilnius, Lithuania) to avoid any DNA contamination.

For the identification of common carp *ho* genes, first-strand cDNA was synthesized using 5 μg total RNA as a template, prepared either from the liver of untreated and Cd-exposed animals. First-strand cDNA was synthesized using 5 μg total RNA as a template, 200 pmol of each dNTP (Thermo Scientific Baltic, Vilnius, Lithuania), 200 U Maxima H Minus Reverse Transcriptase, (Thermo Scientific Baltic, Vilnius, Lithuania) and 500 pmol random hexamer primers (Sigma St. Louis, MO, USA) in a final volume of 20 μl, and incubated for 10 min at 37 °C, followed by 1 h at 52 °C. Next, 1 μl reverse transcription product was added to 25 μl DreamTaq Green PCR Master Mix 2× (Thermo Scientific Baltic, Vilnius, Lithuania). Amplification was performed in a PTC 200 Peltier Thermal Cycler (MJ Research Waltham, MA, USA) using 40 cycles of 95 °C for 30 s, 55 °C for 30 s and 72 °C for 120 s. The amplified products were electrophoresed on 0.8% agarose gel (SeaKem LE, Lonza Rockland, ME, USA) and isolated from it using Ultrafree-MC Centrifugal Filter Units (Millipore Bedford, MA, USA) and the sequences of the PCR population were determined.

For the quantification of *ho* mRNAs, reverse transcription-PCR (RT-PCR) amplification was performed. For the β-actin mRNA, used as an internal reference (Hansen *et al.*, 2006), 25 and for *ho*, and 30 cycles, respectively, were used. The amplified products were detected on a 2% agarose gel. The relative levels of *ho* mRNAs are expressed as ratios (*ho*/β-*actin* × 100).

Primers

To amplify common carp *ho*-specific cDNA(s), oligonucleotides (HO1F (cgctgctcatcccgcagttca), HO2F (caaggttcatcctgatctggc), HOR (cgggtgtaagcgtgggccac), HO1F2 (atggaatccacgaaaagcaaa) HO1R (actcccatgccaaccgtdgc), HO2R (tcacataaggtaccaagcag), based on the zebrafish *Danio rerio* Hamilton 1822 (NM_001127516; NM_001103139), goldfish *Carassius auratus* L. 1758 (Wang *et al.*, 2008) and nile tilapia *Oreochromis niloticus* L. 1758 (XM_003449966) *ho* sequences were used. For the normalization of *ho* mRNAs, the level of common carp β-actin mRNA was used as internal standard, detected with the primers β-actin-3: gcaagagaggtatcctgacc, and β-actin-4: ccctcgtagatgggcacagt (GenBank accession no. M24113).

Phylogenetic Analysis

HO phylogenetic analysis was conducted with known vertebrate HO amino acid sequences, using the Philogeny software package (Dereeper *et al.*, 2008, 2010). The sequences compared with common carp Ho-1 and Ho-2 were as follows: Homo sapiens NP_002124.1 and NP_002125.3; Macaca mulatta XP_001113241.2 and NP_001252998.1; Tursiops truncatus XP_004315933.1 and XP_004310399.1; Mus musculus NP_034572.1 and NP_001129538.1; Rattus_norvegicus NP_036712.1 and NP_077363.1; Gallus gallus NP_990675.1 and XP_004945440.1; Bos taurus NP_001014912.1 and XP_005224478.1; Xenopus tropicalis XP_002934766.1 and NP_001072640.1; Anolis carolinensis XP_003220978.1; Crotalus horridus JAA96881.1; Latimeria chalumnae XP_006006976.1 and XP_006001510.1; Alligator sinensis XP_006026050.1 and XP_006034310.1; Chrysemys picta bellii XP_005300880.1 and XP_005312205.1; Danio rerio NP_001120988.1 and NP_001096609.1; Xiphophorus maculatus XP_005797578.1 and XP_005806580.1; Takifugu rubripes XP_003972631.1 and XP_003964694.1; Oryzias latipes XP_004065741.1 and

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XP_004066399.1; Oreochromis niloticus XP_003443134.1 and XP_005454959.1; Carassius auratus (Wang *et al*., 2008).

Densitometry

Images of the ethidium bromide-stained agarose gels were digitized with a GDS 7500 Gel Documentation System and analyzed with the GelBase/GelBlot™ Pro Gel Analysis Software (UVP Inc., SanGabriel, CA, USA).

GSH Measurement

For the measurement of GSH and H_2O_2 levels, lipid peroxidation and the activities of antioxidant enzymes, 1 g samples of the liver and the kidney of each individual fish were homogenized in 4 volume of ice-cold physiological saline solution, using a double glass homogenizer immersed in an ice-water bath. The homogenate was centrifuged at 17 000 *g* for 15 min at 4 °C to obtain supernatant. The quantity of protein was determined with Folin reagent, using bovine serum albumin as a standard (Lowry *et al*., 1951). Spectrophotometric measurements were carried out with Biomate 5 Double-Beam UV–VIS (Thermo Spectronic) and GENESYS 10S UV–vis (Thermo Scientific Madison, WI, USA) spectrophotometers. The concentrations of total and reduced GSH in the tissues were measured as described by Sedlak and Lindsay (1968).

Lipid Peroxidation Estimation Assay

In biochemical evaluations of metal toxicity, the level of thiobarbituric acid-reactive substances (TBARS) is regarded as an appropriate indicator of the extent of lipid peroxidation (LPO) (Nogueira *et al*., 2003). LPO was estimated by a TBARS assay, as described by Serbinova *et al*. (1992). The malondialdehyde (MDA) reaction with 2-thiobarbituric acid (TBA) was measured at 532 nm against a blank that contained all the reagents except the tissue homogenate. Through the use of a MDA standard, TBARS were calculated as nmol MDA mg^{-1} protein.

Determination of H_2O_2 Production

For the assay of H_2O_2, 0.05 mg ml^{-1} horseradish peroxidase and 0.1 mg ml^{-1} *o*-dianisidine in sodium phosphate buffer (100 mM, pH 6.5) was used. The H_2O_2 concentration was determined spectrophotometrically at 400 nm and was calculated as nmol mg^{-1} protein (Villegas & Gilliland, 1998).

Enzyme Activity Measurement

CAT (EC 1.11.1.6) activity was determined spectrophotometrically at 240 nm by the method of Beers and Sizer (1953) and specific CAT activity was expressed as μmol min^{-1} mg^{-1} protein.

SOD (EC 1.15.1.1) activity was determined on the basis of the inhibition of the epinephrine–adrenochrome autoxidation (Misra & Fridovich, 1972). Spectrophotometric measurement was carried out at 480 nm. The results were expressed in U mg^{-1} protein.

Analysis of Cd and As Contents

Next, 3 g of liver and kidney tissues of each individual fish were dried and separately digested in 10 w/v of concentrated HNO_3 solution at 80 °C for 3 h. The Cd and As contents of the homogenates were determined with a Hitachi Z8200 Zeeman polarized atomic absorption spectrophotometer. Flame or graphite furnace atomization was used, depending on the metal concentration. The Cd and As contents are reported in μg g^{-1} dry weight.

Statistical Analysis

For each time point of the experiments, four to six fish were used. RT-PCR reactions were performed in triplicate to increase the reliability of the measurements. Statistical differences were calculated with one-way analysis of variance (ANOVA) (MedCalc Statistical Software version 9.4.2.0; Broekstraat, Belgium) with a Student–Newman–Keuls follow-up test. A significant difference was accepted at $P < 0.05$.

Results

Identification of Common Carp *ho* Genes

To identify common carp *ho*-specific transcripts, RT-PCR was performed on total RNAs extracted from the liver of untreated and Cd-treated animals, to ensure that both the inducible and the constitutively expressed *ho* mRNAs were represented. In order to design PCR primers, database entries were searched for *ho* genes of fish species (*Danio rerio*, *Carassius auratus*, *Oreochromis niloticus* and *Takifugu rubripes*), the sequences were aligned, and the regions with the highest divergences between the *ho-1* and *ho-2* sequences were sought. On the basis of the sequence differences, two gene-specific primer pairs (HO1F2/HO1R and HO2F/HO2R) were designed, located at the 5′ and 3′ ends of the known coding regions of fish *hos* (Fig. 1). The HO1F2/HO1R primer pair had the potential to amplify an 819-bp PCR product corresponding to 100% of the zebrafish *ho-1* coding region. Amplification with the primer pair HO2F/HO2R was expected to result in a 679-bp DNA fragment carrying ~ 70% of the *ho-2* coding region of the zebrafish. Both reactions yielded the product with the expected size; these fragments were gel-purified and sequenced directly. As expected, the sequence analysis yielded two cDNA species, carp *ho-1* and *ho-2*, with the highest homology to their orthologous forms of fish species. The longer carp DNA sequence (819 bp) had the highest homology to goldfish *ho-1* (92%), whereas the closest relative of the shorter sequence (658 bp) was zebrafish *ho-2* (81%). The similarity levels were much lower when the carp *ho* paralogues were compared (48%). An open reading frame spanned the entire length of both cDNAs. The deduced amino acid sequence of carp HO-1 was 92% identical to goldfish HO-1. Multiple alignments revealed that,

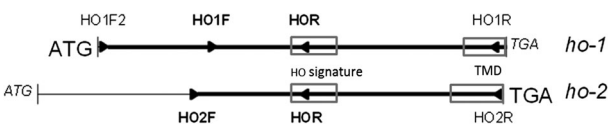

Figure 1. Schematic drawings of the *ho-1* and *ho-2* coding regions. The straight lines indicate the coding regions of the two *ho* genes with the start and stop codons. Heavy lines highlight the newly identified sequences deposited in the GenBank. Grey boxes indicate the positions of sequences coding for the haeme oxygenase domain (HO signature) and the transmembrane domain (TMD). Arrows show the locations and orientations of the primers. The HO1F2/HO1R (1-20/795-817 bp) and HO2F/HO2R (288-309/909-929) primer pairs were used to amplify gene-specific cDNAs. The HO1F/HOR (221-241/404-424 bp) and HO2F/HOR (288-309/474-491) primer pairs were used for the determination of ho mRNA levels.

like mammalian HO-1 proteins, carp HO-1 carries a putative transmembrane segment (from aa 249 to 271), a HO domain (from aa 14 to 218) and a HO signature motif (from aa 129 to 152). Overall, HO-1 is 45–92% identical to orthologous proteins from birds, mammals and fish, with 53–88% identity over the HO domain and 83–96% identity at the level of the HO signature motif. The deduced primary structure of carp HO-2 showed the highest similarity to its *Takifugu rubripes* orthologue (73%). Multiple alignments revealed that the carp HO-2 protein also carries the HO signature motif. The overall identity of the deduced amino acid sequences of the common carp paralogues was 31%. As concerns the most highly conserved HO signature motif, the homology was 87%.

The phylogenetic analysis of known HO sequences revealed that HO-1 and HO-2 comprise two definite groups, with separate clusters of fish, amphibia, birds and reptiles, and mammals (Fig. 2).

Basal Expressions of Two *ho* Genes

The basal levels of the two *ho* transcripts relative to *β-actin* mRNA were determined in the liver, kidney, spleen, brain, heart, skin, blood, gill and muscle. For PCR amplification, carp-specific primers were designed: the common reverse primer complementary to the highly conserved HO motif (HOR), and the gene-specific forward primers (HO1F and HO2F) complementary to the substantially divergent regions between the *ho* sequences. *ho-2* was highly expressed in all of the examined tissues, with the mRNA levels lying in a four-fold range. The highest level was detected in the skin, and the lowest in the kidney (Fig. 3). The basal levels of the *ho-1* transcripts were at the limit of detectability in all the examined tissues, with three exceptions. The highest *ho-1* mRNA level was found in the spleen, the function of which is related to the immune response and blood supply. The ratio *ho*-2/*ho-1* in this organ was ~2.5. *ho-1* was also detected in the blood and skin, with ratios *ho*-2/*ho-1* of 13 and 8.5, respectively (Fig. 3).

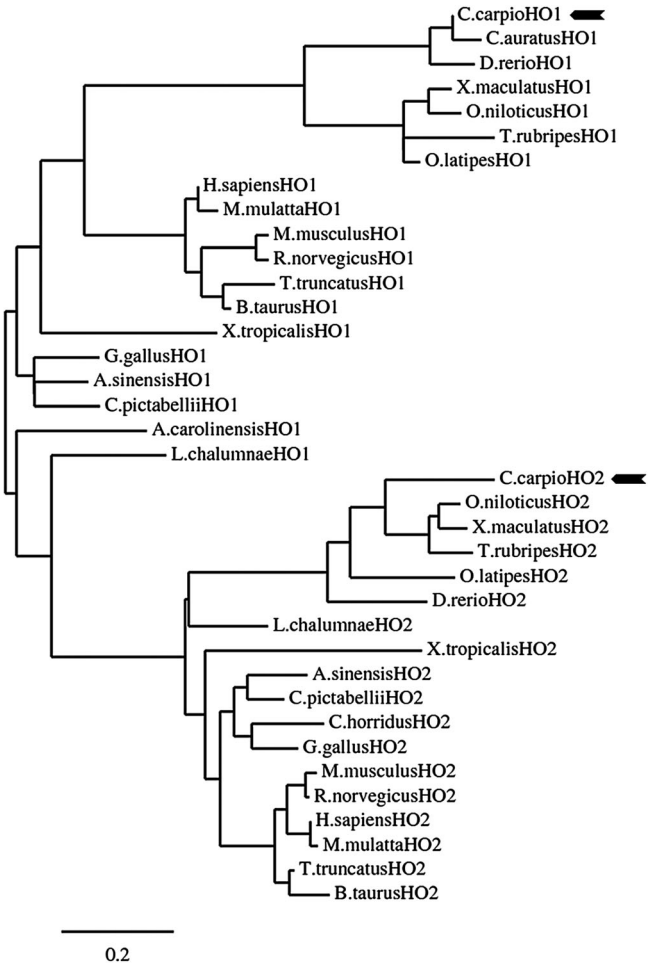

Figure 2. The phylogenetic tree, showing the relationship of carp haeme oxygenases (HOs) to known vertebrate HO-1 and HO-2. The scale bar indicates evolutionary distances between groups. Common carp are indicated by arrows. Full-length HO sequences of known vertebrates were retrieved from GenBank and alignment was made by the Phylogeny.fr program (Dereeper *et al*., 2008, 2010).

Effects of Heavy Metal Exposure on The Expressions of The *hos*

Impact of Cd Exposure on ho Expression. Cd at 1 mg l^{-1} had no effect on the *ho-1* expression in the liver; its mRNA remained undetectable or at the threshold of detectability at all the time points examined. *ho-2* was induced to 2.5-fold in this organ. In the kidney, *ho-2* was induced to four-fold at 24 h, whereas the *ho-1* expression was increased about 13-fold, with the peak at 48 h.

Cd at 10 mg l^{-1} transiently upregulated the expressions of both *ho* genes. The highest levels of *ho-1* were 15- and 25-fold the basal expression in the liver and kidney, respectively. In the liver, a more or less steady elevated level was observed between 6 and 72 h, whereas in the kidney the peak expression was measured at around 24 h of exposure. This was the only time point at which the *ho-1* level was higher than that of *ho-2*. Cd at this concentration induced the expression of *ho-2* in two phases: the 2.5- to 3.5-fold peak expression was detected at around 6 h, and a second increase in mRNA level was observed at around 72 h in both organs (Fig. 4).

Impact of As Exposure on ho Expression. The time courses for *ho-1* and *ho-2* gene expression in the liver and kidney after acute exposure to 1 or 10 mg l^{-1} As are shown in Fig. 5. In the liver, 1 mg l^{-1} As induced *ho-1* expression in two phases: the first peak at 24 h (~30-fold) was followed by a mild, but significant decrease before the second increase in expression at 72 h (~45-fold). In the kidney, the two-phase pattern was detected at 6 and 48 h (~25-fold), with a complete absence of the *ho-1* transcript at 24 h. As concerns the *ho-2* expression, a moderate induction (1.5-2.5-fold) was observed in both the liver and the kidney. Surprisingly, at 10 mg l^{-1} As had less impact than at 1 mg l^{-1} on the expression of *hos* in the kidney: the expression of *ho-2* was not affected at all, whereas the *ho-1* mRNA reached a steady level (Fig. 5). In the liver, the levels of both *ho-1*and *ho-2* mRNA were significantly increased after exposure for 48 h (25- and 1.8-fold, respectively), but had returned to the basal level by 72 h of treatment (Fig. 5).

Metal Accumulation in The Liver and Kidney. The levels of Cd and As accumulated in the liver and kidney were measured during the first 72 h of metal exposure. In the liver, at 1 mg l^{-1} an increased metal uptake was observed between 24 and 48 h, followed by a second stage between 48 and 72 h, when the change in metal content was close to zero. At 10 mg l^{-1}, this two-phase intake was less expressed. In the kidney, Cd exposure at both doses was characterized by a time-dependent intake (Fig. 4).

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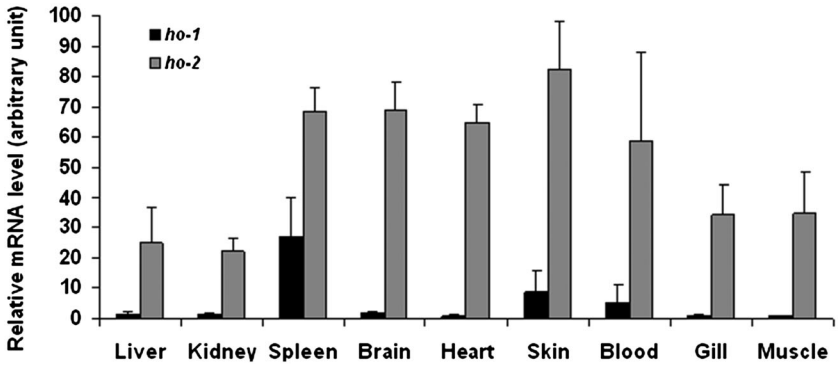

Figure 3. The relative levels of *ho* transcripts in untreated carp tissues. The level of β-actin mRNA was used as an internal standard in the PCR reaction. All data are means ± SD from measurements on 4–6 fish for each tissue.

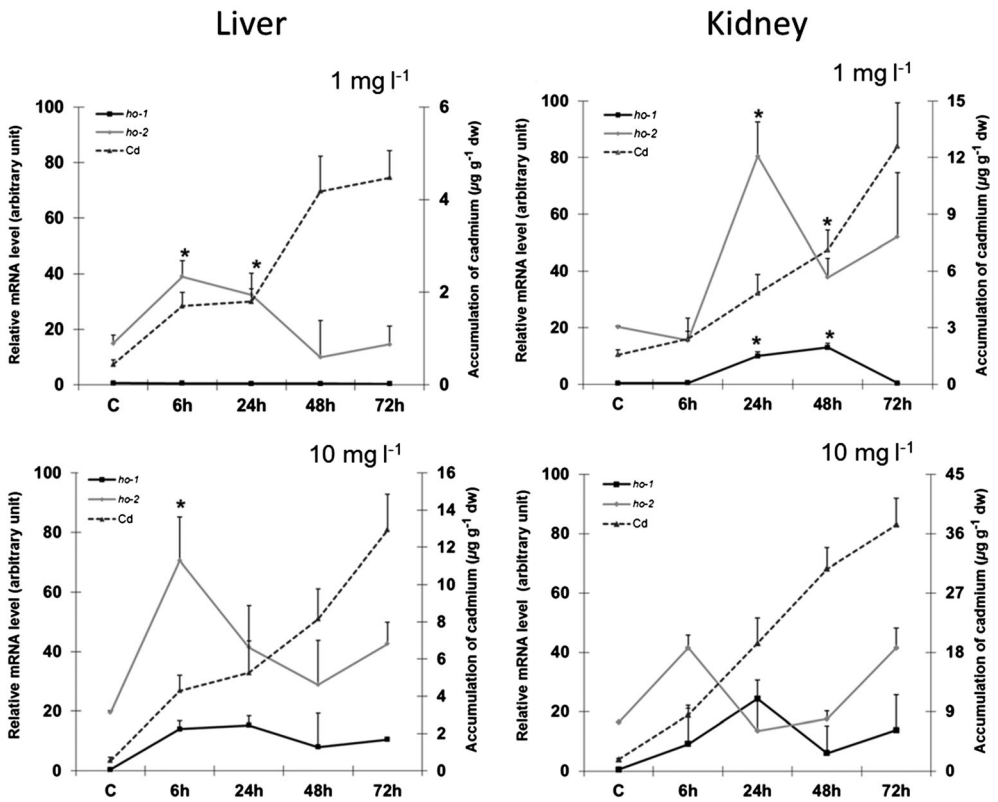

Figure 4. Time courses of induction of the genes *ho-1* and *ho-2* and the accumulation of cadmium in the liver and in the kidney after treatment with 1 and 10 mg l^{-1} cadmium (Cd). All data are means ± SD of the results of measurements on three to five fish at each time point. Statistical differences were calculated with one-way analysis of variance (ANOVA) with a Student-Newman-Keuls follow-up test. Significant differences at the $P < 0.05$ level: *, between the control (C) and the value at a given time point.

The highest rate of As accumulation took place during the first 6 and 24 h of treatment in the liver and kidney, respectively, followed by a slower increase in metal content. The only exception was the liver at 10 mg l^{-1}, where there was no further increase after 6 h; instead, a significant fluctuation was detected (Fig. 5).

GSH and GSSG Contents and Lipid Peroxidation in The Liver and Kidney. The ratio GSH/GSSG is frequently used as an established indicator of the cellular redox status. In the liver, the low-dose Cd treatment led to a 50% decrease in GSH/GSSG after 24 h of exposure, whereas in the kidney there was no significant change. Exposure to the high dose of Cd resulted in increased levels of GSH/GSSG in both organs: elevations of 25% in the liver and ~2.5-fold in the kidney. In the kidney, As decreased GSH/GSSG in both doses (~ 40% and ~70%, respectively), whereas there was no detectable effect in the liver (Table 1).

Exposure to As at the low dose induced the peroxidation of lipids in both the liver and the kidney, whereas the high dose of As had no effect on the membrane lipids in the two examined

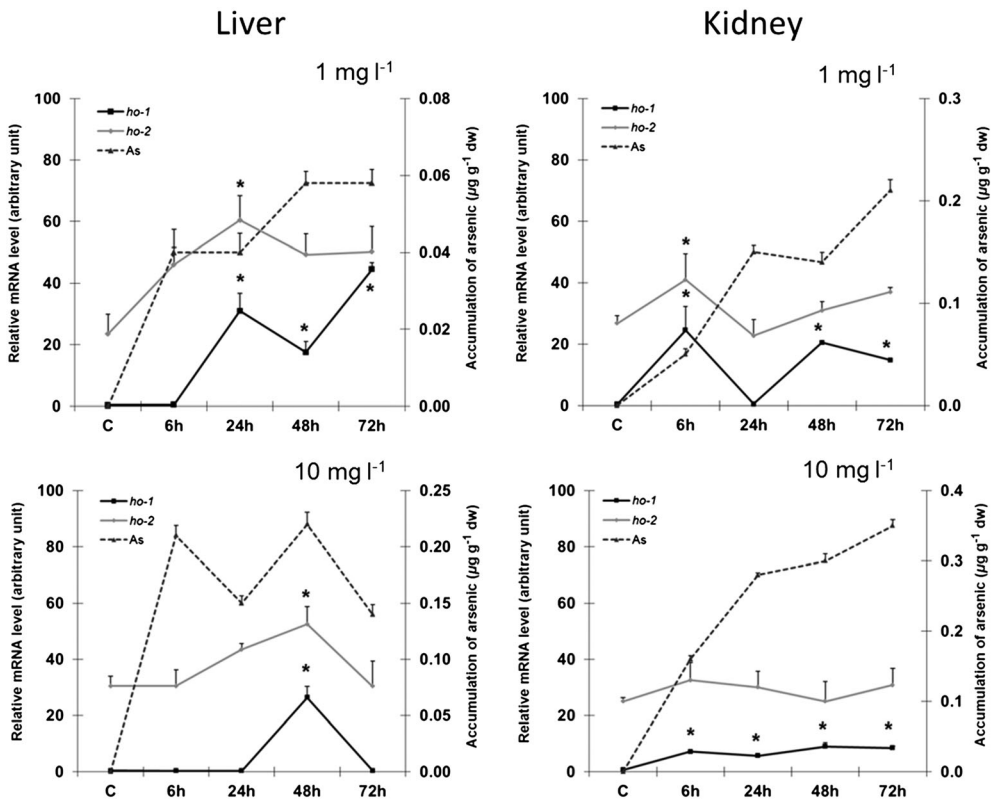

Figure 5. Effects of arsenic treatment on the expression of the genes *ho-1* and *ho-2* and the accumulation of As in the liver and in the kidney after treatment with 1 and 10 mg l^{-1} arsenic (As). All data are means ± SD of the results of measurements on three to five fish at each time point. Statistical differences were calculated with one-way analysis of variance (ANOVA) with a Student-Newman-Keuls follow-up test. Significant differences at the *P* 0.05 level: *, between the control (C) and the value at a given time point.

tissues. Cd affected the membrane lipids only in the high dose and only in the kidney (Table 1).

Metal-induced Alterations in The Activities of SOD and CAT and in H_2O_2 Content. SOD in cooperation with CAT catalyses the conversion of $O_2^{\bullet-}$ to H_2O. In the liver, the low dose of Cd decreased the activity of CAT and the level of H_2O_2, whereas the SOD activity was unaltered. Exposure to the high dose of Cd increased SOD activity, and as a consequence the H_2O_2 content was also elevated besides an unaltered CAT activity. Cd had no effect on the above markers in the kidney at either applied concentration: the SOD and CAT activities, and hence the level of H_2O_2 production, were not changed (Table 1).

In the liver, As in both doses decreased the SOD and CAT activities, with no alteration in the H_2O_2 content. In the kidney, As in both concentrations increased the SOD activity and the

Table 1. Maximum fold of induction of the *ho* genes and changes in the level of antioxidant markers during heavy metal exposure

			Fold of induction						
			ho-1	***ho-2***	GSH/GSSG	LPO	SOD	Catalase	H_2O_2
Cadmium	Liver	L	1×	2.5×	↓	—	—	↓**	↓*
		H	15×	3.5×	—	—	↑*	—	↑*
	Kidney	L	13×	4×	—	—	↓	—	—
		H	25×	2.5×	↑*	↑*	—	—	—
Arsenic	Liver	L	45×	2.5×	—	↑*	—	↓*	—
		H	25×	1.7×	—	—	↓**	↓***	—
	Kidney	L	25×	1.5×	↓**	↑*	↑**	—	↑**
		H	10×	1.3×	↓**	—	↑**	↑**	↑*

The arrow indicates the direction of changes. Analysis of variance (ANOVA):
* $P < 0.05$;
** $P < 0.01$;
*** $P < 0.001$. Data are mean ± SD. L = 1 mg l^{-1}; H = 10 mg l^{-1}.

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content of H_2O_2 to about the same extent. CAT activity, however, was induced only by the high-dose As treatment (Table 1).

Discussion

Heavy metals and other pollutants that accumulate in the environment can be hazardous for ecosystems, acting on specific enzymes, proteins and genes. Fish in particular are subject to environmental stressors, because of their permanent exposure to dissolved substances through their gills and skin, and to the incorporation of harmful substances via their food (Mzimela *et al*., 2003). Thus, the use of fish species to monitor heavy metal contamination is highly accepted and recommended and studies based on the responses to aquatic metal ions may promote a better understanding of the potential toxicological mechanism (Zhou *et al*., 2007). Cellular adaptation to the adverse environmental conditions, a multifactorial process, occurs in association with increases in the expression of distinct stress protein genes. Cd and As are well-known effectors of the expression of these genes.

In the present study, we investigated the efficiency of antioxidant defence mechanisms from the aspects of the expression of *ho* genes, the accumulation of two toxic metals and free radical formation in liver and kidney of common carp. The importance of HO-1 in the antioxidant defence lies in the fact that the products of HO-1-derived haeme degradation, CO and bilirubin, may provide additional protective effects against oxidative stresses through the binding of CO to residual haeme-containing oxygenases to inhibit any further production of ROS and through the bilirubin-dependent scavenging of peroxyl and hydroxyl radicals (Stocker *et al*., 1987).

Fish HOs have been less extensively studied than those of other taxonomic groups. Only two *ho* paralogues, the *ho-1* and *ho-2* genes, have been described in higher vertebrates and our GenBank search of the lower vertebrate genomes also indicated the existence of single copies of *ho*-1 and *ho*-2. Common carp is considered to be a tetraploid animal, mainly on the basis of its chromosome numbers (2n = 100–104) and high DNA content (Ohno *et al*., 1967). The tetraploidization of carp has been suggested to have taken place about 50 million years ago (David *et al*., 2003), although recent studies on duplicated genes suggested a more recent divergence time, about 16 million years ago (Zhang *et al*., 2008). Tetraploidity might pose a problem in the course of the identification of genes and investigations of their expressions. During our study we have seen no indication of the expression of multiple ho genes: the amplification of the cDNAs resulted in single, well-defined products for both *ho-1* and *ho-2*, and sequencing the PCR product populations *en mase* indicated no sequence variations in the regions investigated. We therefore conclude that the *ho* genes identified by our approach are the only ones expressed in *C. carpio*. Zebrafish is closely related to common carp. A blast search against the draft zebrafish genome indicated single copies of *ho-1* and *ho-2* genes in this species. The results of the analysis of the phylogenetic tree appropriately mirror our established view of phylogeny: fish, amphibia, birds and reptile, and mammals form well-separated groups. Within this system, the members of *ho-2* seem to be less divergent, indicating a possible evolutionarily later separation from the *ho-1* genes.

We also report the analysis of the expression patterns of the two *ho* paralogues after exposure to heavy metals. The basal *ho-1* level was below the threshold of detection in all the organs examined but not the spleen, skin and blood. The expression of *ho-2* was demonstrable in all the tissues examined, although with quantitative differences. Whereas the brain is usually considered not to play a significant role in haeme degradation, we observed a notably high expression of *ho-2* in the brain. It was reported earlier that *ho-2* mRNA and protein are abundant in the brain of rat, and the CO produced by HO has been postulated to function as a neurotransmitter, in much the same way as proposed for NO (Yamanaka *et al*., 1989; Marks *et al*., 1991; Ewing & Maines, 1992; Verma *et al*., 1993). Another potential explanation is that the brain may require a greater degree of antioxidant protection owing to its critical role in regulating the organ functions (Maines, 1997).

Our results under physiological conditions indicated that both *ho*s were expressed most strongly in the spleen, where senescent erythrocytes are sequestered and Fe is recirculated. One of the lowest levels of expression was detected in the liver. A rational explanation could be that HO-2 limits the intrahepatic turnover of the haeme enzymes (Goda *et al*., 1998), as the haeme molecules in these enzymes are known to be metabolized exclusively by the HO reaction (Maines, 1988). The present study also addressed the accumulation of these two metals in liver and kidney. The levels of incorporated Cd appeared to increase steadily throughout the exposure period, without reaching a peak; this held true at both doses applied, regardless of the tissues examined. The uptake of As proved to be more variable; it was both tissue- and dose-dependent. It is interesting that the rates of metal accumulation in liver and kidney in the first 6 h of As exposure was more or less similar, regardless of the dose. From 24 h of treatment, the kidney was characterized by a higher metal content than that in the liver, and exposure to the lower dose always resulted in greater differences between the two organs. In the liver, As in the high dose exhibited two-phase accumulation, with peaks at 6 and 48 h, whereas in the kidney a more or less continuous increase was observed, with a high accumulation rate in the early period. How the Cd and As contents influence the expression of the *ho* genes in the kidney and the liver was also addressed in the present study. The effects of acute exposure to the two metals on the two *ho* genes were quite different. From the maximum increase in the level of induction, As appeared to be a more potent inductor of *ho-1* than Cd, and the influence was more pronounced in the liver, in spite of the higher As content in the kidney. It is worth mentioning that As had the higher impact on the expression of *ho-1* in the lower dose, regardless of the tissue. *ho-1* also displayed differential responses during Cd exposure; the highest increase in *ho-1* expression was measured in the kidney, where the Cd content was also the highest.

In the antioxidant defence, GSH plays an important role in countering oxidative damage, and the intracellular ratio GSH/GSSG is often used as a measure of cellular toxicity; a decrease in the value of this ratio is an indication of an oxidative impairment (Schafer & Buettner, 2001). The present study has demonstrated that the ratio GSH/GSSG decreased significantly only in the kidney, because of an enhanced GSH depletion after As treatment at both doses. Besides the lowered GSH level, significant increases in the activity of SOD and the H_2O_2 content may reflect As-induced oxidative stress in the kidney in both doses. In spite of the similar patterns of changes in the above parameters, the peroxidation of lipid molecules was detected only when As was applied in the low dose. One of the mechanisms by which As produces toxic effects is through its interaction with cellular sulfhydryl groups in proteins or elsewhere (Aposhian, 1997). It

has been shown that As reacts with GSH to form As–GSH complexes in the liver of mammals (Scott *et al*., 1993). In this organ, membrane damage was observed only on exposure to the low dose of As. However, lipid peroxidation was not accompanied by changes in the levels of GSH and H_2O_2. The observed variations could possibly be attributed to an alternative mechanism for the toxicity of As, a secondary generation of a molecular stressor. Arsenic depletes ATP in rabbit and human erythrocytes, inducing cell death within a few hours after exposure (Winski & Carter, 1998). This mechanism could be connected with the As-induced increase in ho-1 expression. Intracellular Fe cycling plays a critical role in how cells cope with the generation of ROS. The haeme molecule itself is a potentially harmful Fe chelate, and the release of Fe during haeme degradation before its sequestration by ferritin may make Fe available for the catalysis of harmful oxidation reactions. Our experimental results therefore might support the hypothesis that increased HO-1 activity in haeme degradation may lead to both pro- and antioxidant effects (Kvam *et al*., 2000). In addition to Fe release by haeme degradation, exposure to arsenic species induced a high rate of ferritin-Fe release in horse spleen (Ahmad *et al*., 2000). It has also been observed that Cd promotes Fe release from biological membranes (Casalino *et al*., 1997). The pro-oxidative effects of both metals are therefore indirect as they are mediated by Fe. Cd potently induced the expression of *ho-1* in the kidney when it was applied in the high dose (~25-fold). At this point a mild increase in GSH level was detected, coupled with a pronounced depletion of GSSG. This combination resulted in an increased GSH/GSSG ratio, but in spite of an increased level of lipid peroxidation, the toxic effects of Cd were detected. Cd has been shown to induce *ho* expression, in mouse hepatoma cells and in Chinese hamster ovary cells (Alam *et al*., 1989; Taketani *et al*., 1989). As Cd is able to bind free sulfhydryl groups and nucleic acids, it may simply mimic or modify a physiological mediator involved in a signal transduction system leading to activation of the *ho* genes (Vallee & Ulmer, 1972; Gong *et al*., 2001).

Altered patterns in gene expressions and enzyme activities, and the damage suffered by macromolecules may serve as fingerprints specific for the various tissue targets, and the toxicity of examined stressors. In this study, we followed the cellular stress responses relating to exposure to Cd and As from the aspect of the expression of *ho* genes. These responses proved to be metal specific, and the results suggest that secondarily generated stressors such as the Fe released by HO during the degradation of haeme might be involved in the toxicity of the examined metals.

Conflict of Interest

The Authors did not report any conflict of interest.

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