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Major distinctions in the antioxidant responses in liver and kidney of Cd^{2+}-treated common carp (*Cyprinus carpio*)

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ABSTRACT

This study is related to the accumulation of Cd^{2+}, its effects on oxidative stress biomarkers and its role in macromolecule damage in liver and kidney of common carp. We present evidence of an increased ratio of reduced to oxidized glutathione (GSH/GSSG) in both organs after 10 mg/L Cd^{2+} exposure, with different underlying biological mechanisms and consequences. In the liver, the expressions and/or activities of superoxide dismutase, catalase, glutathione reductase and glutathione peroxidase increased to cope with the Cd^{2+}-generated toxic effects during the first 48 h of treatment. In contrast, none of these selected antioxidant markers was significantly altered in the kidney, whereas the expression of glutathione synthetase was upregulated. These results suggest that the major defense mechanism provoked by Cd^{2+} exposure involves the regeneration of GSH in the liver, while its *de novo* synthesis predominates in the kidney. High levels of accumulation of Cd^{2+} and peroxynitrite anion ($ONOO^-$) were detected in the kidney; the major consequences of $ONOO^-$ toxicity were enhanced lipid peroxidation and GSH depletion. The accumulation of $ONOO^-$ in the kidney suggests intensive production of NO and the development of nitrosative stress. In the liver the level of hydrogen peroxide was elevated.

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1. Introduction

As a consequence of the chemical loading of the environment, living organisms may be exposed to numerous harmful compounds throughout their lifetime. Cadmium, a highly toxic, widely distributed metal that enters the aquatic environment via natural and anthropogenic sources, is such a potential threat to the environment and human health (Satarug et al., 2003). Aquatic organisms absorb cadmium directly from water in its ionic form (Cd^{2+}) (AMAP, 1998). The toxic effects of Cd^{2+} are generally thought to be caused by "free" Cd^{2+}, i.e. Cd^{2+} not bound to metallothioneins (MTs) or other proteins (Goyer et al., 1989). Cd^{2+} may have a number of adverse effects, including the inactivation of metal-dependent enzymes, and the promotion of oxidative stress by inducing the formation of reactive oxygen species (ROS), among them the superoxide anion ($O_2^{\cdot-}$) and the hydroxyl radical (Wang et al., 2004). Cd^{2+} induces ROS production 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 signaling cascades (Cuypers et al., 2010) and may result in physiological damage to different organs (Nawrot et al., 2008; Järup and Åkesson, 2009).

To protect themselves against oxidative stress, aerobic organisms have evolved complex antioxidant defense systems. A number of antioxidant enzymes, including superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR) and glutathione peroxidase (GPx), have been demonstrated in most organisms, among them teleosts (Basha and Rani, 2003; Cunha Bastos et al., 2007). GSH also plays a critical role in this system, as an antioxidant, enzyme cofactor and major redox buffer (Dringen et al., 2000). GSH synthesis is catalyzed by γ-glutamyl-cysteine synthetase (γ-GCS) and glutathione synthetase (GSS) in ATP-dependent reactions. GSH depletion can result in short-term increases in γ-GCS and GSS activity and GSH synthesis (Rahman et al., 1996). Most or all vertebrate tissues produce GSH, but liver and kidney are the most active sites of GSH synthesis (Shi et al., 1996). GSH is instantly oxidized by ROS to GSSG, which is then recycled into GSH through the action of GR. The antioxidant role of GSH in cells relies on its concentration, rate of turnover and rate of synthesis. Members of this antioxidant defense system in different organisms are useful biomarkers to characterize a polluted environment. They have the advantages of being sensitive, relatively invariable and highly conserved between species (Agrahari et al., 2007).

Cd^{2+} affects many cellular functions and its action has been reported to be cell type-specific (Ranaldi and Gagnon, 2009). Cd^{2+} interferes with antioxidant defense mechanisms, stimulates the production of ROS, and enhances the synthesis of nitric oxide (NO) (Han et al., 2007). The biological actions of NO are controlled at various levels, including mechanisms that regulate NO synthetase localization and activation, and the variable oxidative metabolism of NO, resulting in the generation of reactive nitrogen species (RNS) (Bove and van der Vliet, 2006). The simultaneous generation of NO and $O_2^{\cdot-}$ in sufficiently high concentrations in the same compartment favors the production of a toxic reaction product, peroxynitrite anion ($ONOO^-$) (Radi et al.,

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2001). $ONOO^-$ and other RNS can affect cell functions through the oxidation or nitration of various cellular targets. Although $ONOO^-$ itself is not a free radical, it is a powerful oxidant, whose toxicity is manifested among others in lipid peroxidation (LPO) and GSH depletion (Radi et al., 2001). Thus, the induction of NO synthesis and $O_2^{\cdot-}$ production may contribute to the cytotoxicity of Cd^{2+}, causing a variety of adverse health effects (Bove and van der Vliet, 2006). There is an extensive database of studies of the chronic toxicity of Cd^{2+} in humans. These studies have identified the kidney as one of the sensitive targets of Cd^{2+} toxicity (Brzóska and Moniuszko-Jakoniuk, 2004). Animal studies have confirmed the identification of the kidney and bone as the most sensitive targets of chronic Cd^{2+} toxicity.

The first question raised in the present study, therefore, was whether the increased sensitivity of the kidney is to be detected after even a short-term Cd^{2+} exposure. Accordingly, we made a search for major differences in the activation of both enzymatic and non-enzymatic antioxidant defense systems and macromolecular damage between liver and kidney of common carp from the aspect of Cd^{2+} accumulation and the production of free radicals and oxidants. Besides the gene expression and enzyme activity studies, measurements were made on Cd^{2+}, H_2O_2 and $ONOO^-$ accumulation. As the major consequences of $ONOO^-$ toxicity are LPO and GSH depletion, these parameters were also measured. Additionally, the activity of SOD was assayed in order to assess the level of $O_2^{\cdot-}$.

2. Materials and methods

2.1. Animals and treatments

Carp weighing 800–1000 g, obtained from the Tisza Fish Farm, Szeged, were acclimatized under fasting conditions in well-aerated 400-L water tanks over a 3-week period at 12 °C. For Cd^{2+} treatment, the carp were transferred into 100-L water tanks (2 fish per tank) containing 10 mg/L Cd^{2+} dissolved in the form of cadmium acetate dehydrate, ($Cd(CH_3COO)_2x2H_2O$; Fluka), under static conditions. Cd^{2+} at this concentration is not lethal to common carp at least for 21 days. In all experiments, 3–5 animals were sacrificed at each time point for tissue harvesting. Tissues were frozen immediately in liquid nitrogen and stored at −80 °C.

2.2. GSH measurement

Kidney and liver of each individual fish were homogenized in physiological saline solution using a double glass homogenizer immersed in an ice water bath. The homogenate was centrifuged (Micro star 17R centrifuge, VWR) at 17000 *g* for 15 min at 4 °C to obtain supernatant for measuring GSH, H_2O_2, $ONOO^-$ levels and the activities of antioxidant enzymes. The quantity of protein was determined with Folin reagent, using bovine serum albumin as standard (Lowry et al., 1951). The concentrations of total and reduced GSH in the tissues were measured as described by Sedlak and Lindsay (1968).

2.3. Enzyme activity measurements

GR (EC 1.6.4.2) activity was measured as described by Nagalakshimi and Prasad (2001). One unit of activity (EU) is defined as the amount of enzyme that catalyses the reduction of 1 μmol GSSG in 1 min (37 °C, pH 7.4).

CAT (EC 1.11.1.6) activity was determined by the method of Beers and Sizer (1953), and specific CAT activity was expressed as μmol/min mg protein.

SOD (EC 1.15.1.1) activity was determined on the basis of the inhibition of epinephrine-adrenochrome autoxidation (Misra and Fridovich, 1972). The results were expressed in U/mg protein.

2.4. Determination of H_2O_2 production

For the assay of H_2O_2 0.05 mg/mL horseradish peroxidase and 0.1 mg/mL o-dianisidine in sodium phosphate buffer (100 mM, pH 6.5) were used. H_2O_2 was calculated as nmol/mg protein (Villegas and Gilliland, 1998).

2.5. Determination of $ONOO^-$ production

$ONOO^-$ was assayed by diluting samples into 1.0 M NaOH (60:1) and measuring the increase in absorbance at 302 nm. As a control, samples were added to 100 mM potassium phosphate (pH 7.4) (60:1). The decrease in absorbance was measured at neutral pH as $ONOO^-$ decomposes (Huie and Padmaja, 1993).

2.6. 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 LPO (Nogueira et al., 2003). LPO was estimated by a TBARS assay, as described by Serbinova et al. (1992). Through use of an MDA standard, TBARS were calculated as nmol MDA/mg protein.

2.7. DNA single-strand breaks

In order to detect oxidative DNA damage, DNA samples were prepared from the livers and kidneys of control and treated animals by the salting-out method of Miller et al. (1988). DNA damage was detected by fluorimetric method (Birnboim and Jevcak, 1981).

2.8. Analysis of Cd^{2+} content

Liver and kidney tissues of each individual fish were dried and separately digested in 10 times their weight of concentrated HNO_3 solution at approximately 80 °C for 3 h. The Cd^{2+} 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 Cd^{2+} concentration. The Cd^{2+} contents are reported in μg/g dry mass.

2.9. RNA extraction, reverse transcription and PCR amplification

The whole brain and the whole heart and approximately 50 mg samples of liver, kidney and muscle were homogenized in TRI Reagent (Sigma) 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) to avoid any DNA contamination. For the quantification of *cat*, *gpx1*, *gr* and *gss* mRNAs, RT-PCR was performed. First-strand cDNA was synthesized by using 5 μg total RNA as template, 200 pmol of each dNTP (Thermo Scientific), 200 U Maxima H Minus Reverse Transcriptase, (Thermo Scientific) and 500 pmol random hexamer primers (Sigma) in a final volume of 20 μL, and incubated for 10 min at 37 °C, followed by 1 h at 52 °C. 1 μL reverse transcription product was added to 25 μL of a DreamTaq Green PCR Master Mix 2x (Thermo Scientific). Amplification was performed in a PTC 200 Peltier Thermal Cycler (MJ Research). For the β-actin mRNA, used as internal reference, 25 cycles and for *cat*, *gpx1*, *gr* and *gss* 30 cycles were used. The amplified products were detected on a 2% agarose gel.

2.10. Primers

The following primers were used: in case of *gpx1*: F: tggcttyga gcccaaattcca and R: tcaatgtcgctggtgaggaa; in case of *cat*: F: cgtcatat gaacggatacgg and R: tcagcctgctcaaaggtcat; in case of *gr*: F: attgctgtg caaatggctgg and R: cctgcacgagtggtgttctgga; in case of *gss*: F: gtccatcgg cacattctgaa and R: ggcatgtatccattacggaa. For the normalization of *gpx1*,

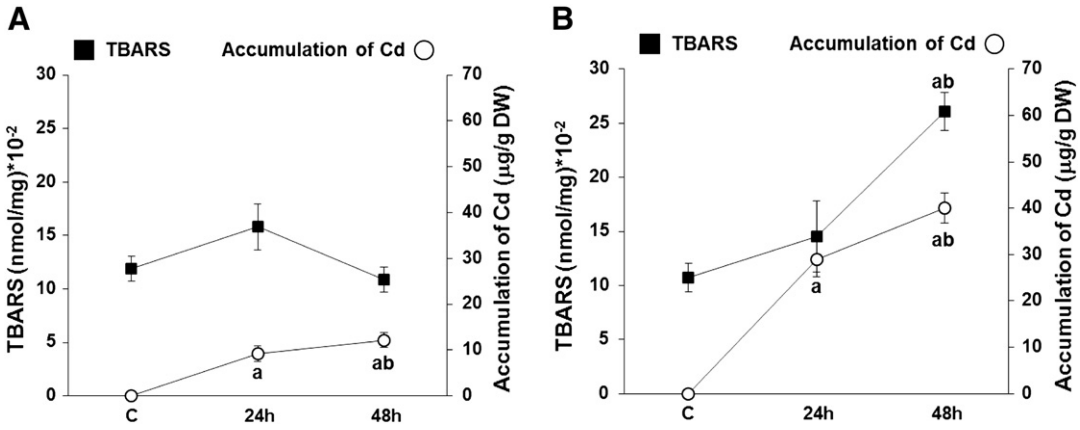

Fig. 1. The levels of lipid peroxidation (■) and accumulation of Cd^{2+} (○) in the liver (A) and in the kidney (B) after treatment with 10 mg/L Cd^{2+}. a indicates significant differences between the control level (c) and that at a given time point; b indicates significant differences between the values at consecutive time points.

cat, *gr* and *gss* mRNA, the level of carp β-actin mRNA was used as internal standard, detected with primer pairs F: caagagaggtatcctgacc, and R: ccctcgtagatgggcacagt (GenBank accession no. **M24113**).

2.11. 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).

2.12. Statistical analysis

For each time point of the experiments, 3–5 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. Significant difference was accepted at $P < 0.05$.

3. Results

3.1. Cd^{2+} accumulation, DNA damage and peroxidation of lipid molecules in the liver and kidney

The levels of Cd^{2+} accumulation in liver and kidney were measured in the first 48 h of Cd^{2+} challenge. The exposure was followed by a clear pattern of tissue-specific accumulation. The amount of Cd^{2+} in the kidney was always about 4–5-fold higher than that in the liver. In both tissues, the accumulation correlated with the duration of exposure (Fig. 1).

The Cd^{2+}-induced modifications of the lipids and DNA macromolecules were followed by DNA single-strand breaks and LPO. In the liver, no damage to selected macromolecules was detected during Cd^{2+} exposure (Fig. 1A and data not shown). In the kidney, there was no measurable DNA degradation, but the level of TBARS underwent a gradual increase: a 2.5-fold elevation was measured after the 48-h exposure (Fig. 1B).

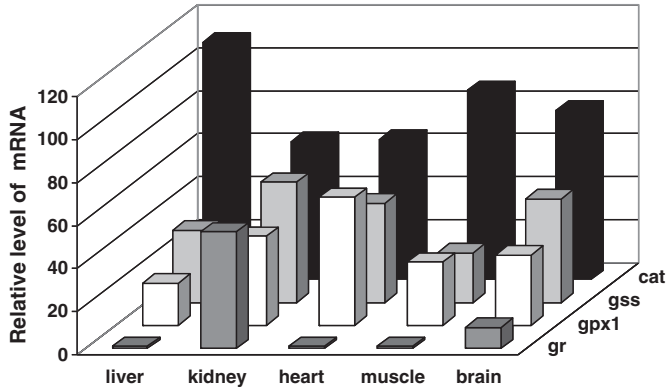

Fig. 2. Transcriptional study of gene expression in untreated carp tissues. For the normalization of *glutathione reductase (gr) glutathione peroxidase (gpx1), glutathione synthetase (gss)* and *catalase (cat)* mRNAs, the level of *β-actin* mRNA was used as internal standard in the PCR reaction.

3.2. Tissue distribution of gr, gss, gpx1 and cat expression

The transcriptional study of gene expression by using PCR amplification acquires sequence information for the design of gene-specific primers. We have identified four partial cDNAs coding for the enzymes GPX1, GR, GSS and CAT involved in the mechanism of defense against oxidative stress. The sequences have been deposited in the GenBank (accession numbers: **GQ376154** for *cat*, **GQ376155** for *gpx1*, **HQ174243** for *gss* and **HQ174244** for *gr*). On the basis of the identified sequences, carp gene-specific primer pairs were designed and used to follow the expression of *gr, gss, cat* and *gpx1* in five different tissues (the liver, kidney, heart, muscle and brain) of untreated animals. For the detection and determination of the transcript levels, semiquantitative-RT-PCR amplification was used. PCR analysis revealed tissue specificity in the expression of all the examined genes, with essential quantitative difference (Fig. 2). The highest level of *gr* mRNA was detected in the kidney; 15–20% of this level was measured in the brain, and in the other examined tissues the content of the *gr* transcript was around the limit of detection. The highest level of *gss* mRNA was also expressed in the kidney, with ~80% of this level detected in the heart and in the brain, and 55% in the liver. The *gss* was least expressed in the muscle. The *cat* gene product was strongly present in all of the examined tissues, the mRNA levels lying in a 2-fold range. The highest level was detected in the liver, and the lowest in the kidney and heart. The pattern of *gpx1* expression was somewhat complementary to that of *cat*. The highest levels of *gpx1* mRNA were detected in the heart and kidney, where the lowest levels of *cat* mRNA were observed.

3.3. Cd^{2+}-induced alterations in gene expressions

In the liver, Cd^{2+} at 10 mg/L induced the expressions of *gpx1* and *cat*. The pattern of induced *gpx1* expression was to a certain extent the opposite of that of *cat*. The peak induction of *cat* expression (2-fold)

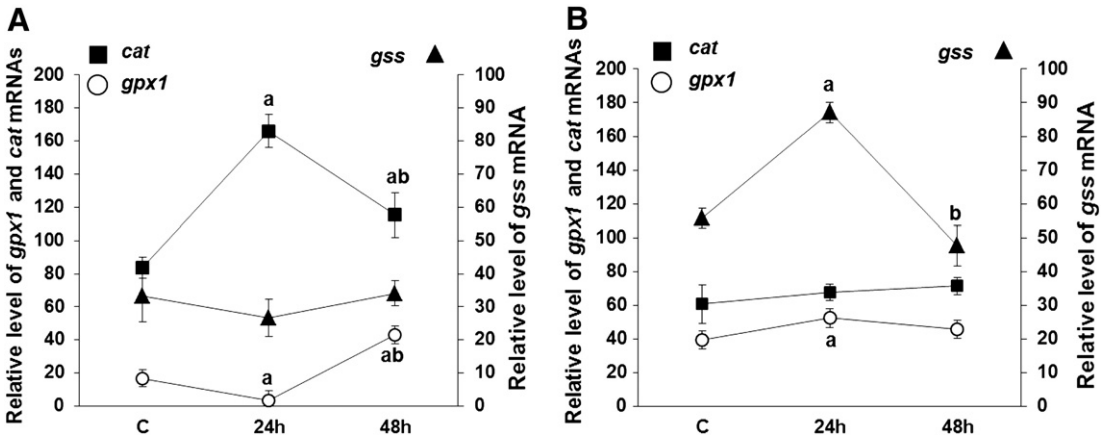

Fig. 3. The expressions of *cat* (■), *gpx1* (○) and *gss* (▲) genes in the liver (A) and in the kidney (B) following treatment with 10 mg/L Cd^{2+}. a indicates significant differences between the control level (c) and that at a given time point; b indicates significant differences between the values at consecutive time points. All data are means ± S.D. of the results of measurements on 3–5 fish at each time point.

was measured after 24 h of Cd^{2+} exposure. At that time point, significantly less *gpx1*-specific mRNA (25% of the control level) was present. After a 48-h exposure, the expression of *cat* mRNA approximated to the control level, while that of *gpx1* was induced (~2.5-fold). The expression of *gss* was not significantly affected by Cd^{2+} loading (Fig. 3A). The level of *gr*-specific mRNA gradually increased during Cd^{2+} treatment: 2.5-fold elevations were detected after a 48-h exposure (data not shown). In the kidney, the Cd^{2+} treatment did not result in any significant changes in the *cat*-, *gpx1*- and *gr*-specific mRNA levels at any time point measured. However, there was a significant increase (1.5–2-fold) in the level of expression of *gss* mRNA (Fig. 3B).

3.4. Cd^{2+}-induced alterations in GR activity, GSH and GSSG content

In the liver, the activity of GR mirrored its mRNA increases: a 2-fold elevated activity was detected after 48 h of Cd^{2+} exposure (Fig. 4A). As a consequence, an increased level of GSH was detected and the ratio GSH/GSSG was also increased significantly, by 25% (Fig. 4A). In the kidney, the Cd^{2+} treatment did not result in a significant alteration in GR activity at any time point. However, there was a notable rise (~2.5-fold) in the ratio GSH/GSSG by 48 h of treatment (Fig. 4B), due to a pronounced loss of GSSG (50%) and a significant increase in the GSH level.

3.5. Cd^{2+}-induced alterations in H_2O_2 and $ONOO^-$ contents and in the activities of SOD and CAT

In the liver, the activities of both SOD and CAT were elevated 1.5–2-fold and the H_2O_2 level was also increased after Cd^{2+} exposure (Fig. 5A and data not shown). As a consequence, there was no significant change in $ONOO^-$ content (Fig. 5A). In the kidney, however, the SOD and CAT activities and hence the level of H_2O_2 production were not changed, whereas a significant elevation in $ONOO^-$ level was detected (~1.8–2-fold) (Fig. 5B).

4. Discussion

Fish and other aquatic animals are particularly subject to environmental stressors, because of their permanent exposure to dissolved substances through their gills, skin, and digestive tract (Mzimela et al., 2003). Cd2+, one of the potentially most deleterious heavy metal ions is toxic for humans, animals and plants, and is one of the widespread trace pollutants with a long biological half-life (Satarug et al., 2003). Cells respond to toxic metals via activation of their natural antioxidant defense systems. In the present study, we compared the efficiencies of these defense systems from the aspect of Cd2+ accumulation and free radical formation in liver and kidney of common carp. Our data revealed that Cd2+ accumulated

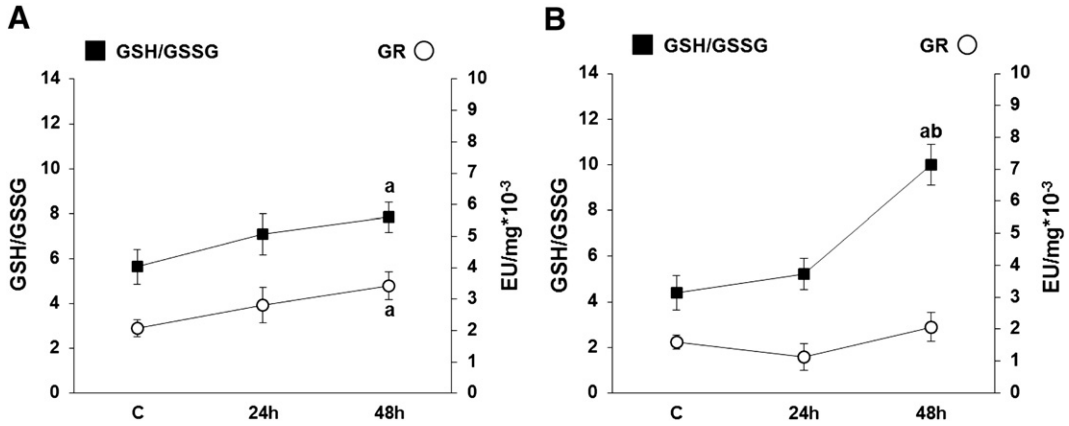

Fig. 4. The ratio GSH/GSSG (■) and the GR activity (○) in the liver (A) and in the kidney (B) after treatment with 10 mg/L Cd^{2+}. a indicates significant differences between the control level (c) and that at a given time point, b indicates significant differences between the values at consecutive time points. All data are means ± S.D. of the results of measurements on 3–5 fish at each time point.

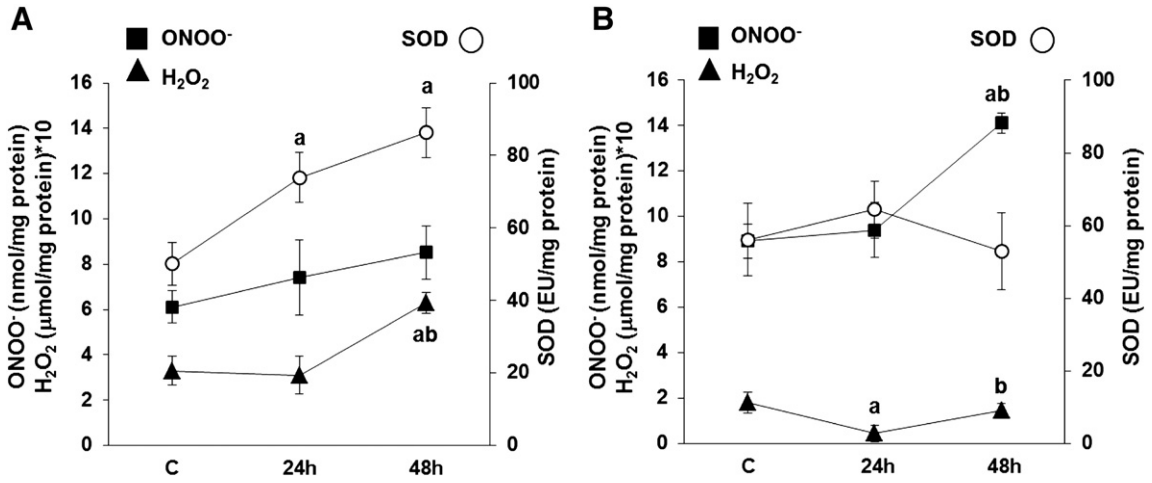

Fig. 5. The $ONOO^-$ level (■) and the SOD activity (○) in the liver (A) and in the kidney (B) after treatment with 10 mg/L Cd^{2+}. a indicates significant differences between the control level (c) and that at a given time point; b indicates significant differences between the values at consecutive time points. All data are means ± S.D. of the results of measurements on 3–5 fish at each time point.

in a tissue-specific manner: the Cd2 + content in the kidney was higher at all time points measured. This finding is in good agreement with the generally accepted view that Cd2 + at low concentration is primarily absorbed by the liver, where it is bound by MTs and transported to the kidney. At higher Cd2 + concentrations, the kidney itself also absorbs Cd2 + directly from the blood (De Conto Cinier et al., 1998).

In the free radical production and antioxidant responses induced by Cd^{2+}, tissue specificity was again involved. $O_2^{\cdot-}$, a quite toxic radical, is neutralized by SOD in co-operation with CAT, and $O_2^{\cdot-}$ and NO can additionally combine spontaneously to form $ONOO^-$ (Radi et al., 2001). In the liver, Cd^{2+} treatment enhanced the activities of SOD and CAT, indirectly indicating an increased level of $O_2^{\cdot-}$ generation. In contrast, Cd^{2+} exposure was not followed by changes in the activities of SOD and CAT in the kidney, but the concentration of $ONOO^-$ was almost doubled. The increase in the level of $ONOO^-$ is indirect evidence of an elevated $O_2^{\cdot-}$ production, which in this case is paralleled by induced NO synthesis. It seems likely from our data that the unaltered SOD activity could be a consequence of the fast depletion of $O_2^{\cdot-}$ through collision with NO and the formation of an increased amount of $ONOO^-$. In the kidney, the increase in $ONOO^-$ level was clearly reflected by a 2.5-fold increase in the level of TBARS.

Our data suggest that the natural antioxidant defense system in the liver is induced to a sufficient extent to cope with the Cd^{2+}-generated toxic effects during the first 48 h of treatment, as depicted in Fig. 6. The genes coding for CAT, GPx1 and GR, i.e. the major enzymes involved in the defense mechanism against oxidative stress, are upregulated, and thiol-containing molecules such as GSH and MTs are present in high amounts on the second day of Cd^{2+} exposure. In the kidney, the highly elevated rate of Cd^{2+} accumulation was not accompanied by significant increases in the levels of most of the selected parameters. Although the *mt* expression is strongly induced, the level of the *mt* transcript is about one-third of that measured in the liver (Hermesz et al., 2001). The upregulation of *gss* expression might reflect an attempt by the organism to rescue the situation via an increase in the level of GSH. In light of the unchanged activities of the antioxidant enzymes, the elevated levels of GSH and *mt* expression do not seem to be capable of effectively warding off the harmful effects of the accumulated Cd^{2+}; the increased level of TBARS is an indicator of the cell damage in the kidney.

In the antioxidant defense, GSH plays a major part 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 and Buettner, 2001). The present study has demonstrated that the ratio GSH/GSSG is significantly increased by Cd^{2+} exposure in both the liver and kidney. These results lead us to conclude that the increase in the ratio GSH/GSSG may be achieved through different underlying biological mechanisms and with different consequences as concerns macromolecule damage. In the liver, the increase in the ratio GSH/GSSG can be accounted for by an elevated extent of regeneration of GSH from GSSG, while in the kidney the *de novo* synthesis of GSH is enhanced. Moreover, there is a dramatic exhaustion of the GSSG pool after a 48-h Cd^{2+} exposure. These results indicate that reliance on the ratio GSH/GSSG alone in attempts to characterize the oxidative stress status might possibly be misleading. The data presented here, in conjunction with our earlier results on the expression of *mt* (Hermesz et al., 2001), may possibly facilitate a deeper understanding of the status of oxidative stress and emphasize the changes in certain parameter in the two main organs involved in detoxification.

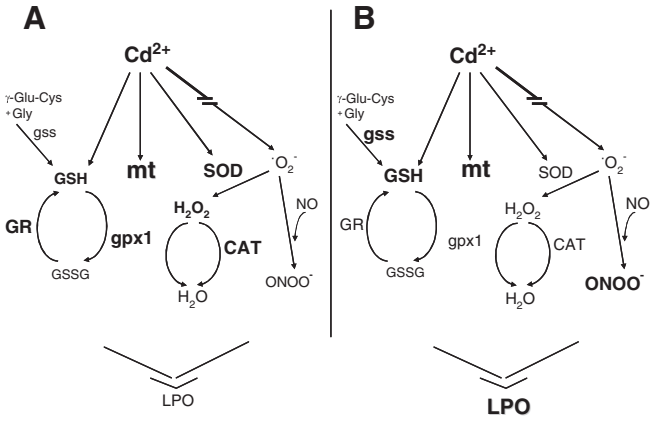

Fig. 6. A schematic illustration of the responses of the biomarkers in the liver (A) and the kidney (B) following treatment with 10 mg/L Cd^{2+} for 48 h. By that time, the levels of accumulated Cd^{2+} in these tissues were about 9 μg/g and 40 μg/g dry weight, respectively. Font sizes are approximately proportional to measured levels. Bold fonts indicate an elevated level relative to the respective controls. *mt*, GR, *gpx1*, *gss*, CAT, LPO, GSH, SOD, $ONOO^-$, H_2O_2.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

Acknowledgments

K.D. and Á.F. contributed equally to this work.

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