Identification and expression of the genes of Metal-Responsive Transcription Factor-1 and glutathione peroxidases of common carp

Summery of Ph.D. Thesis

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1. Introduction

Environmental pollution, including the contamination of rivers and lakes, is still a persistent and significant problem. In some cases, it may be increased by disasters, such as the pollution of the River Tisza with cyanide and heavy metals in 2000. Fish are in great danger, because they accumulate harmful substances in their bodies via their food, and they are permanently exposed to dissolved substances through their gills and skins.

Almost all physiological processes are influenced by heavy metals. The proteins, nucleic acids are damaged and the formations of reactive oxygen species (ROS) are induced by metals, which may cause intensified cell and tissue damage. The rapid response to environmental stress is a general characteristic of organisms, which (from bacteria to mammals) have a high degree of homology in the response of the cells.

One of main pathways of stress responses is based on the metallothioneins (MTs) and their primary transcription regulator, metal-responsive transcription factor (MTF-1). Another pathway is associated with reduced glutathione (GSH) and certain enzymes, such as glutathione peroxidase (GPx), glutathione-S-transferase (GST), glutathione reductase (GR) and glutathione synthetase (GSS). It is clear, therefore, that the *mtf-1*, *gpx1* and *gpx4* genes are key members in the antioxidant defence system. Study of the expression patterns of these genes contributes to a better understanding of the stress response.

Because of the high metal-binding capacities of MTs, they play important roles in the homeostasis of essential metal ions (Zn^{2+} and Cu^{2+}) and in the elimination of toxic heavy metals (Cd^{2+}, As^{3+} and Pb^{2+}). The expression of MTs is primarily transcriptionally regulated and the Zn^{2+}-sensitive MTF-1 is an essential participant in this process.

GSH is an antioxidant molecule, consisting only three amino acids (γ-Glu-Cys-Gly). In the event of oxidative stress, GSH is readily oxidized, because of the free SH group; when exposed to heavy metals, GSH binds metal ions. GSH also participates in the regeneration of ascorbic acid and vitamin E. GSH is a cofactor for antioxidant enzymes reducing H_2O_2 and lipid peroxides and neutralizing xenobiotics.

The GPx enzymes protect against the harmful effects of ROS, because they reduce H_2O_2, some organic lipid peroxides and cholesterol esters, during the oxidation of GSH. The phospholipid hydroperoxide GPx (GPx4) binds directly to the membranes, and prevents the initiation of radical chain reactions. Eight different GPx families have been identified in mammals; in fish, only the classical GPx1 and GPx4 have been studied.

2. Aims

The main aims of our group are the identification and functional characterization of stress-response gene families in fish. At the beginning of these experiments, our primary aim was to identify gene(s) parts of defense system against heavy metal toxicity in the common carp, for example *mtf-1*, *gpx1* and *gpx4*.

Other aims were to study the expressions of *mtf-1* and *gpx* genes in the liver and the kidney, the organs most involved in the processing and excretion of toxic agents, and also in the heart, the muscle and three isolated brain regions (olfactory lobe, midbrain region and cerebellum) of untreated fish, and to follow the expressions of these genes in the liver and brain of stressed fish. The liver is one of the most important organs of detoxification, while the brain has high oxygen content, and is therefore the primary target of the harmful effects of ROS. The effects of treatment with a divalent heavy metal (Cd^{2+}), a variable valence heavy metal (As^{3+}; As^{5+}) and physical stress (rapid temperature change) was investigated.

In the third part of the experiments, our aims were to examine the parameters of cell damage, such as lipid peroxidation, the changes in the amounts of reduced and oxidized GSH, the ratio of single and double-strand DNA, and the enzyme activities of GPx and GR after Cd^{2+} treatment.

3. Materials and methods

Animals and treatment conditions

Common carp (*Cyprinus carpio* L.), obtained from the Tisza Fish Farm, Szeged, were acclimatized under fasting conditions in well-aerated 400-l water tanks over a 3-week period**.** In cold shock experiments, the fish were transferred from 12 ^{o}C to 5 ^{o}C for 1 or 5 h and tissues were taken after a 1-h recovery period, following cold treatment at the acclimatization temperature. For metal treatment, the carp were transferred into 100-l water tanks containing 10 mg/l Cd^{2+} ($Cd(CH_3COO)_2x2H_2O$) or 10 mg/l As^{3+} ($Na_2HAsO_4x7H_2O$), under static conditions. Samples were obtained directly after the cold shock, or after the 1-h recovery period. At each time point of the treatments, 3 or 4 animals were used for the measurements.

Applied Methods

Preparation of nucleic acid (genomic DNA, mRNA, plasmid DNA)

In vitro recombinant DNA techniques: ligation, transformation

Reverse transcription polymerase chain reaction

Determination of GSH / GSSG levels

Measurement of lipid peroxidation

Measurement of GPx and GR activity

Fluorimetric method for determination of the ratio of DNA breaks

Phylogenetic analysis

4. Results

In the common carp, as in most fish, sequence analysis indicated a single *gpx1* gene, in contrast with the zebrafish (another member of the *Cyprinidae*), where two *gpx1* genes were found. In higher vertebrates, *gpx4* has been identified as a single copy gene with multiple transcription and translation start sites, but in the common carp, as in other fish species (zebrafish, goldfish and salmon), two *gpx4*, *gpx4a* and *gpx4b*, have been isolated. More than 95% of the protein coding regions has been determined in all three *gpx* genes of the carp.

Two different *mtf-1* genes (*mtf-1.1* and *mtf-1.2*) and the splice-variant of *mtf-1.1* (*mtf-1.1a*) have been identified in the common carp. The overall identity of the deduced amino acid sequences of MTF-1.1 and MTF-1.2 was also 98%. The two sequences could be aligned with 1 gap and 9 amino acid differences, located in the C-terminal transcriptional activation domains, mostly in the proline-rich and serine/threonine-rich domains. The two MTF-1 proteins of the carp therefore probably interact with different partners. The common carp, like the other members of the *Cyprinidae*, demonstrates many genome and gene duplications; the common carp is a tetraploid species. In the case of the isoform, MTF-1.1a, the amino acid sequence of the C-terminal region of the original MTF-1.1 is altered by a frame shift. The DNA-binding domains of MTF-1.1a are identical to those of the MTF-1.1 protein, but the acidic transactivation domain is changed and the proline- and serine/threonine-rich domains are missing. MTF-1 without the transcriptional activation domains retains its translocating and DNA-binding capacity, but is presumably unable to activate its target genes.

The mRNA levels of *gpx4a* were expressed in all examined tissues of the carp under unstressed conditions; the highest level was found in the liver. A high expression of *gpx4b* was determined in the olfactory lobe, but in the kidney and cerebellum it was virtually undetected. The expressions of *gpx1* were known in the liver, kidney and brain of the goldfish and silver carp, but the expression of the *gpx1* gene of the carp differs significantly from those of other fish. The carp *gpx1* was detected with the highest level in the heart, kidney and midbrain regions. In other fish, the *gpx1* expression was liver-specific, and in the goldfish brain it was undetectable.

The tissue-specific expression of the *mtf-1* gene was undetectable by Northern hybridization in untreated fish and mammal species. All three *mtf-1* mRNAs of the common carp were detected in significant quantities in the brain. In the liver, the level of *mtf-1.1* mRNA was low and the level of *mtf-1.1a* mRNA was at the limit of detection. The *mtf-1.2* mRNA was virtually undetected in any organ but the brain. The *mtf-1.1*:*mtf-1.2* ratio in the brain was 3:2. In all examined tissues, the expression of *mtf-1.1* was the determining factor.

The fish were treated with 10 mg / l Cd^{2+} and As^{3+} for 24-96 hours. The expressions of the *gpx1*, *gpx4a* and *mtf-1.1* genes were slightly increased after Cd^{2+}treatment in the liver, but Cd^{2+} did not influence the levels of *mtf-1.2* and *mtf-1.1a* mRNA. The level of *gpx4b* mRNA was virtually undetected as early as after 24 h. In the brain regions, time-dependent decreases were measured in the expressions of all examined genes. The As^{3+} treatment caused lower changes, but with similar tendencies in both tissues, except in the case of *mtf-1.1a*. After 48 h of As^{3+} exposure, the mRNA levels of the spliced variant were increased in both the liver and the brain. There was a significant difference, in that the expression of the *gpx1* gene was undetected after Cd^{2+}treatment in these tissues of the goldfish.

Rapid changes in water temperature were chosen as physical stress, because fish in a freshwater environment are subject to temperature fluctuations. Relatively rapid changes in water temperature can influence the gene expressions and induce an oxidative stress-like status in the tissues. The fish were exposed to a 1- or 5-h 7 °C temperature drop and some fish were then allowed a 1-h recovery period to the temperature of incubation, which is basically a heat shock for them. The expressions of the *mtf-1* genes showed induction after the 5-h treatment, while those of the *gpx1* genes were increased after the 1-h treatment. The *gpx4a* mRNA level was decreased in both tissues. The changes in the *mtf-1*, *gpx1* and *gpx4a* genes were not influenced by the 1-h recovery. In the case of the expressions of the *gpx4b* gene, which were decreased by metal exposure, the direct exposure to low temperature increased

the transcription by ~ 2.5-fold, but it had returned to the control level by the end of the 1-h recovery period.

In addition to the expressions of the stress genes following high-dose Cd^{2+} treatment, other parameters of cell damage and the activities of some key antioxidant enzymes were investigated. The lipid peroxidation, the ratio of single- and double-stranded DNA and the changes in the reduced and oxidized GSH levels are the most frequently studied parameters of cell damage. In the early stages, damage to the cells was indicated by the changes in three parameters, but the repair systems were activated effectively after the 48-hour treatment. The activation of two enzymes of GSH homeostasis, the GPx and GR, was induced.

The identification and expression analyses of several genes were continued in our current work, which will promote a better understanding of the stress reactions of fish after exposure to environmental effects.

Publications

Publications directly used in and related to the thesis:

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