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DIFFERENTIAL REGULATION OF THE TWO METALLOTHIONEIN GENES IN COMMON CARP

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The expression of two metallothionein (MT) genes was followed in carp *(Cyprinus carpio) in vivo* during exposure to As and Cu. Changes in the levels of MT-1 and MT-2 mRNA in the liver and kidney were detected by semi-quantitative RT-PCR. The inducibility of the two MT isoforms was tissue- and metal-specific. Regardless of whether As or Cu was applied, the liver was more responsive than the kidney. Copper influenced the expression of both isoforms: the MT-1 and MT-2 mRNA levels increased in both the liver and the kidney in a time- and dose-dependent manner. Arsenic affected mostly the MT-2 expression, while the level of the MT-1 transcript did not change significantly in either organ.

Keywords: Carp – gene expression – metallothionein mRNAs – metal treatment – RT-PCR

INTRODUCTION

Copper is a trace element that plays a fundamental role in the biochemistry of all organisms. It is essential in cellular respiration, free radical defense, neurotransmitter function and cellular iron metabolism. Nevertheless, the chemical properties that make Cu biologically useful are also potentially highly toxic. Excess Cu accumulation always results in toxicosis [4, 20]. Arsenic is a relatively abundant non-essential element. Acute exposure to As results in toxicity: liver enlargement, cell degeneration, necrosis and fibrosis in human and rat [6, 16]. It displays strong affinity for the sulfhydryl groups of amino acids, and it is thought to exert its toxicity by inhibiting thiol-dependent enzymes [23].

To protect against the damage induced, cells respond to toxic metals via activation of the metallothionein (MT) gene expression. The MTs are low molecular mass, cysteine-rich, inducible intracellular proteins that bind metal ions with high affinity. The MTs, first reported as Cd-binding proteins in horse kidney [7, 14], are ubiquitous in both prokaryotic and eukaryotic organisms. Although the precise biological functions of the MTs remain obscure, it has been suggested that they are involved in the

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detoxification of certain heavy metals, the homeostasis of essential trace elements such as Zn^{2+} and Cu^{2+}, the scavenging of free radicals, and protection against alkylating agents [1, 8, 10, 13, 19].

Despite the large literature on fish MTs, few data have been published regarding the gene expression *in vivo*. Most studies on the expression of MTs have focused on the changes in protein level after treatment with various inducers [10–12]. Others have followed the MT mRNA levels in different tissue cultures or in developing embryos, where the regulation of stress genes might differ substantially from that in fully developed animals [3, 15]. In previous work, we isolated two MT cDNA species (MT-1 and MT-2) from the common carp, and designed isoform-specific primers which enabled us to directly monitor changes in the transcription of the two MT genes in fully developed, adult animals [5]. We addressed the effects of two types of stressors (Cd and cold exposure) and found that the regulations of the two MT genes differ substantially; tissue dependence is also involved, which suggests distinct physiological roles. In the present work, we have investigated the inducibility of the two carp MT genes by the essential metal Cu and by As, a known inducer of stress and heat shock proteins in mouse [9, 18, 22].

MATERIALS AND METHODS

Animals and treatments

Carp *(Cyprinus carpio)* 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 in January and February. During the acclimatization, the water was changed twice a week. For metal treatment, the carp were transferred into 100-l water tanks containing 1 and 10 mg/l As (Na_2HAsO_4 – Fluka), or Cu ($CuSO_4$ – Reanal) respectively. In all experiments, 3 or 4 animals were sacrificed at each time point for organ harvesting, and samples were taken from the liver and kidney. The gall-bladders were removed to avoid bile contamination of the liver. The tissues were frozen immediately in liquid nitrogen and stored at –80 °C.

RNA extraction, reverse transcription and PCR amplification

Approximately 100 mg of frozen tissue was homogenized in RNAzol B reagent (Tel-Test, Inc.) and the total RNA was prepared according to the procedure suggested by the manufacturer. The total RNA was routinely treated with 100 U RNAse-free DNAseI (Boehringer-Mannheim) to avoid any DNA contamination.

To detect carp MT-specific mRNAs, an RT-PCR-based strategy was employed. First-strand cDNA was synthesized using, as template, 5 µg total RNA prepared from the kidney and the liver. The RNA was denatured at 90 °C, mixed with 200 pmol of each dNTP (Boehringer-Mannheim), 200 U M-MuLV reverse transcriptase

(Sigma) and 500 pmol random hexamer primer in 20 µl final volume, and incubated for 10 min at 37 °C, followed by 1 h at 42 °C. The reaction was stopped by heating at 65 °C for 5 min. One microlitre reverse transcription product was added to a 49 µl PCR reaction mixture containing 250 µM of each dNTP, 1x Sigma PCR buffer/$MgCl_2$, 5 U Taq polymerase (Sigma) and 50 pmol MT primers. Amplification was performed in a PTC 150-16 MiniCycler (MJ Research), using 30 cycles of 95 °C for 40 sec, 60 °C for 40 sec, and 72 °C for 40 sec. The amplified products were electrophoresed on 2% agarose (Sigma) gel.

Primers

For amplification of carp MT mRNAs, primers CMT6 and CMT7, specific to MT-1 and MT-2, respectively, were used in pairs with CMT3 [5]. The sequences of primers β-actin3 and -4 were derived from GeneBank entry M24113 and used to amplify β-actin mRNA for internal standard.

CMT3 forward (104-123) 5'GCAAGTGCACTAATTGCCAG3',

CMT6 reverse (283-263) 5'CGAACAGGTTCACATAGGTGA3',

CMT7 reverse (221-202) 5'ACAAGTTCACATTGCTGTAG3',

β-actin3: 5'GCAAGAGAGGTATCCTGACC3',

β-actin4: 5'CCCTCGTAGATGGGCACAGT3'.

Densitometry and statistical analysis

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). The results were submitted to Student's *t*-test analysis, with a probability $P < 0.05$ taken as the limit of significance.

RESULTS

Isoform-specific induction of the MT genes by a high concentration of As

Arsenic at a concentration of 10 mg/l proved to be an efficient inducer of the MT-2 gene in both the liver and the kidney of carp. We observed a 2–2.5-fold increase in the hepatic MT-2 mRNA content after 24 h. It reached the maximum (4.5–5-fold) at around 48 h. The induction was transient: by 72 h, the MT-2 mRNA level had decreased somewhat, though it was still elevated (3-fold) (Fig. 1A, B). There was no significant change in the amount of the MT-1 transcript during the treatment. Since the ratio of the basal MT-1/MT-2 mRNA levels in the liver is high (3 : 1), the amount

of the MT-2 transcript (relative to that of β-actin) at the peak of induction was not much higher than that of the MT-1 mRNA. In the kidney, the kinetics of the induction was very similar to that in the liver, but this organ was less responsive (Fig. 1C). There was at most a 1.5- and 3-fold increase in the level of the MT-1 and MT-2 transcript, respectively.

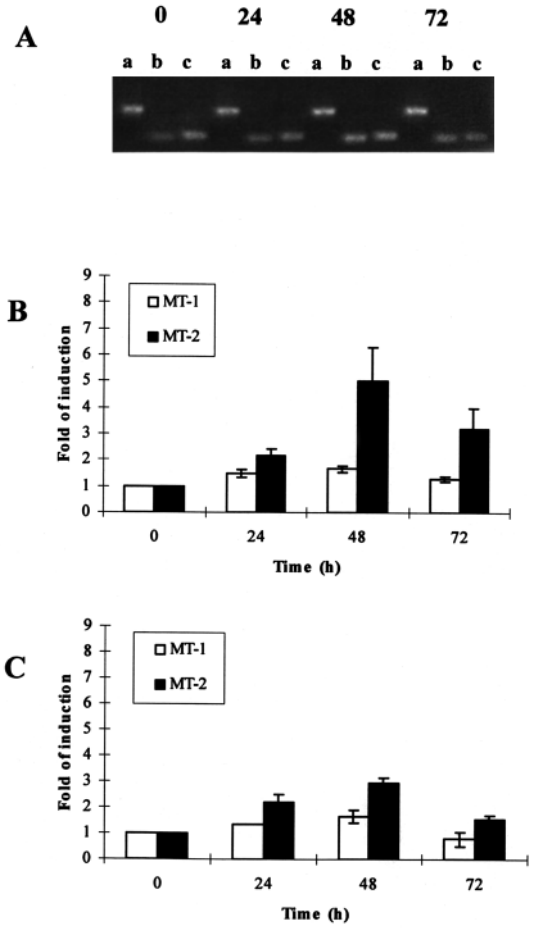

Fig. 1. (A) A representative result of RT-PCR amplification. In parallel with the MT isoform-specific transcripts, β-actin mRNA was amplified and used as an internal control to determine the relative levels of the two MT transcripts. Template RNAs were prepared at the time points indicated from the liver of carp that were either untreated or exposed to 10 mg/l As. PCR amplifications were performed in triplicate. Time course of induction of the MT-1 and MT-2 genes by 10 mg/l As in the liver (B) and the kidney (C). The data are normalized to time 0 (control), and are means ± SD from 9–12 measurements on 3–4 fish at each time point. The mRNA levels at all time points are significantly different from 0 time. a: β-actin; b: MT-2, c: MT-1

We did not detect a significant effect of As at a concentration of 1 mg/l on the expression of the two carp MT genes, in either of the tissues examined (data not shown).

Induction of the genes coding for MT isoforms in the liver and kidney by Cu

To examine the effect of Cu on the MT mRNA levels, carp were kept in water containing 10 mg/l Cu for up to 72 h. This treatment did not affect the survival rate of the animals during the span of the experiment. Regardless of whether liver or kidney were examined, Cu treatment resulted in a transient induction of both genes, with a broad maximum at 6–48 h (Fig. 2). We observed a drastic rise in the hepatic MT-2 content after 6 h (6–7-fold) and this high level of the transcript persisted for 24 h. The

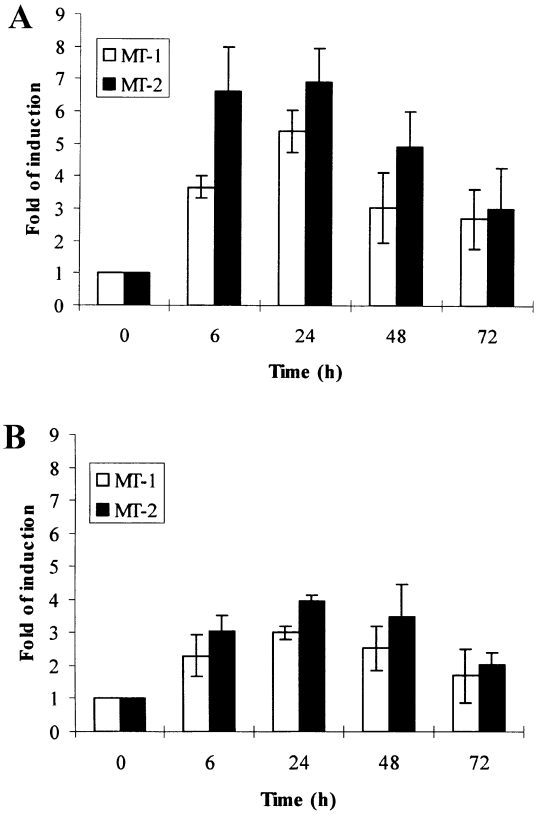

Fig. 2. Time course of the accumulation of the two MT mRNAs induced by 10 mg/l Cu in the liver (A) and the kidney (B). The data are normalized to time 0, and are means ± SD from 9–12 measurements on 3–4 fish at each time point. The mRNA levels at all time points are significantly different from 0 time.

amount of this mRNA was still high one day later (5-fold), but it had decreased substantially by 72 h (3-fold). Cu exposure, unlike As exposure, dramatically induced the level of the other MT isoform. By 6 h of treatment, there was a maximum 3.5-fold increase in MT-1 mRNA; this level was dynamically elevated further, the peak (5–5.5-fold) being reached by 24 h. One and 2 days later, the MT-1 content was still high (3–3.5-fold). In spite of the high induction of the MT-2 gene, the level of the MT-1 transcript was significantly higher than that of the MT-2 transcript at each time point of this experiment.

In the kidney, the two MT genes responded in a similar fashion to the high dose of Cu, but the efficiency of the treatment was about 2 times less, with a maximum of 3- and 4-fold for MT-1 and MT-2, respectively.

Copper at a concentration of 1 mg/l resulted in at most a 1.5-fold enhancement of the induction in the liver at 48 h of treatment, while in the kidney there were no detectable changes in the MT mRNA levels (data not shown).

DISCUSSION

Little information is available concerning the transcriptional regulation of MT genes in fish. Even less data have been published on the isoform-specific expression of these genes under stress conditions [16]. We earlier identified two MT isoforms in the common carp and showed that their inducibility is stress-specific [5]. The present work extends that study, addressing the effects of two metals (As and Cu) on the expression of the carp MT-1 and MT-2 genes. The effects on MT transcription of exposure to As or Cu have previously been studied in juvenile winter flounder [3] and rainbow trout [2], but the distribution of the elevated mRNA between the different isoforms was not the subject of those works.

We have found that regardless of whether Cu or As was applied, or whether liver or kidney was investigated, the treatment resulted in transient rises in the levels of the MT transcripts. The transient nature of the induction suggests that the phenomenon observed is part of the physiological regulation of the two MT genes, and not an experimental artifact generated by the high concentration of the metal. Besides this similarity, significant metal-, tissue- and isoform-dependent differences were found. Copper proved to be a more potent inducer of both isoforms than As in two respects. 1. The Cu-induced levels of both MT transcripts (at their peaks in the same organ) were always higher than the As-induced levels. 2. The kinetics of induction by the two metals were also markedly different: Cu treatment resulted in a faster response in both tissues, with a peak at around 24 h, while the highest level of induction by As was observed at about 48 h.

The present study additionally demonstrates that the inducibility of the MT-1 gene is metal-specific. Treatment with As did not significantly affect the MT-1 transcription in any dose we applied, whereas Cu at high concentration caused a dramatic rise in the level of MT-1 mRNA. The level of induction achieved in this experiment was comparable to that reported for Cd treatment (5–6-fold vs. 6–7-fold in the liver, and

3-fold vs. 3–4-fold in the kidney) [5]. Cu is generally considered to be a weaker MT inducer than Cd. The carp MT-2 gene was, indeed, about 2-fold less responsive to this metal than to Cd (i.e. 6–7-fold vs. 14–15-fold in the liver, at 10 mg/l). However the induction of the MT-1 gene to similar levels by Cd and Cu indicates that a particular metal- and isoform-specific response does not necessarily fit into a simplified, general picture. The good inducibility of MT-1 by Cu suggests that it plays a distinctive role in protecting against a high dose of Cu. One such function of MT-1 could be the scavenging of free radicals, since Cu is a known catalyst in the formation of reactive oxygen species [4, 20]. An example for differential regulation of the MT-1 and MT-2 expression in response to Cu treatment was reported earlier in rat [21]. After Cu treatment, the accumulation of MT-1 protein run parallel with that of the MT-1 mRNA, whereas a translational control of MT-2 mRNA was observed.

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