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EXPRESSIONS OF HEAT SHOCK AND METALLOTHIONEIN GENES IN THE HEART OF COMMON CARP *(CYPRINUS CARPIO)*: EFFECTS OF TEMPERATURE SHOCK AND HEAVY METAL EXPOSURE

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Heat shock proteins (HSPs) and metallothioneins (MTs) play important roles in protection against environmental stressors. The present study analyzes and compares the regulation of heat shock (*hsp70*, *hsc70-1* and *hsp90α*) and metallothionein (MT-1 and MT-2) genes in the heart of common carp, in response to elevated temperature, cold shock and exposure to several heavy metal ions (As^{3+}, Cd^{2+} and Cu^{2+}), in whole-animal experiments. Among these metals, arsenate proved to be the most potent inducer of the examined stress genes; the *hsp90α* and MT-1 mRNA levels were elevated 11- and 10-fold, respectively, after a 24-h exposure. In contrast, Cd^{2+} at 10 mg/L had no impact on the expression of *hsp90α*, and the MT genes also proved to be rather insensitive to Cd^{2+} treatment in the heart: only a 2–2.5-fold induction was observed in response to 10 mg/L Cd^{2+}. Heat shock resulted in a transient induction of *hsp70* (19-fold) and *hsp90α* (15-fold), while elevated temperature had no effect on the expression of the MTs. Direct cold shock induced *hsp70* expression (14-fold), while the *hsp90α* (26-fold) and MT-2 (2-fold) expressions peaked after the recovery period following a direct cold shock. The five stress genes examined in this study exhibited a unique, tissue-specific basal expression pattern and a characteristic sensitivity to metal treatments and temperature shocks.

Keywords: Carp – heart – heat shock – heavy metal – metallothionein

INTRODUCTION

Diverse physiological and environmental stresses produce multiple intracellular changes that ultimately affect protein structures and function. Multiple endogenous pathways are engaged in restoring cellular homeostasis.

One of the best-characterized mechanisms assisting protein folding involves the family of heat shock proteins (HSPs). HSPs play a key role in cell protection by preventing protein aggregation, facilitating folding of newly synthesized proteins, stabilizing and refolding damaged proteins, and targeting misfolded proteins to specific degradative pathways. Some HSP family members (*bona fide* HSPs) are not or only weakly expressed under normal conditions, but they are up-regulated in cells

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exposed to a wide variety of stressors, including both abiotic (e.g. heat shock, osmotic stress and heavy metal exposure) and biotic (e.g. bacteria and viruses) factors. Others (HSP cognates, HSCs), expressed constitutively, are at best slightly inducible, and play essential roles in the protein metabolism. Depending on their apparent molecular weights and degrees of homology, the HSPs are classified into several families. The major classes of mammalian HSPs are the HSP90s, the HSP70s, the HSP60s and the small HSPs (25–28 kDa) [6, 11, 19]. The families of HSP90 and HSP70 have been characterized in a wide range of organisms from *Escherichia coli* to human. Two closely related *hsp90* genes, *hsp90*α and *hsp90*β, have been identified in vertebrates, and it has been shown that the *hsp90*α and *hsp90*β genes exhibit different patterns of expression during embryonic development and cell differentiation, and also in response to environmental stress [4]. HSP90α plays a specific role in the myogenesis in zebrafish and chicken embryos and its expression is induced strongly under stress conditions. Vertebrates also appear to contain more than one isoform for HSP70s and HSC70s. Among lower vertebrates however, only two fish, *Danio rerio* [23] and common carp [1] are known to express two or more closely related HSC70s. In common carp, two *hsc70* genes have been identified *(hsc70-1* and *hsc70-2).* In the brain and heart, both *hsc70*-specific mRNAs have been detected and in the kidney, liver and muscle the *hsc70-1* and *hsc70-2* genes are expressed in a "complementary" manner [1].

HSPs play cardioprotective roles against stressful stimuli. Stimuli which result in HSP induction in the intact heart can produce a protective effect against subsequent exposure of the heart to ischemia/reperfusion either on a perfusion apparatus or within the intact animal [6, 20, 27].

Health hazards caused by heavy metals have become a great concern to the population. Their toxic manifestations are caused primarily by the imbalance between pro-oxidant and antioxidant homeostasis.

An antioxidant role for mammalian metallothioneins (MTs) is well documented [24]. The MTs are cysteine-rich, intracellular proteins that bind metal ions with high affinity [15, 21]. The induction of MTs under radical-generating circumstances suggests a free radical-scavenging capacity of these molecules. It has been shown *in vitro* that MTs function in cardioprotection by acting against oxidative damage [8, 16, 17].

In fish, the expressions and roles of MTs have mostly been studied in organs that play a central part in metal uptake and accumulation (i.e. the liver, kidney and gills) [10, 18], or in developing embryos, where the regulation of stress genes might differ substantially from that in fully developed animals [7]. Different fish species exhibit differences in metal accumulation and MT level, and different organs accumulate metals to different extents due to the differences in physiological and biochemical functions [5, 25].

The aim of this study was, in the first step, to learn the consequences of temperature shock and heavy metal loading on the expressions of different stress genes in the heart; and in the second step, to compare the regulation of these stress genes under different stress conditions in the heart and in other organs, mostly involved in detox-

ification. We followed the expression patterns of genes coding for members of the HSP (*hsc70-1, hsp70* and *hsp90*α) and MT (MT-1 and MT-2) families in response to elevated temperature, cold shock and metal loading (As^{3+}, Cu^{2+} and Cd^{2+}), in whole-animal experiments.

MATERIALS AND METHODS

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. During the acclimatization, the water was changed twice a week. In heat shock treatments, the fish were transferred from 12 °C to 26 °C for up to 3 h, and in cold shock experiments from 12 °C to 5 °C for 1 to 5 h. Samples were taken from the tissues either immediately after the heat or cold treatment, or after a 1-h recovery period at the acclimatization temperature. For metal treatment, the carp were transferred into 100-L water tanks (2 fish/tank) containing 1 mg/L or 10 mg/L Cd^{2+} ($Cd(CH_3COO)_2 \times 2H_2O$, Fluka), 10 mg/L Cu^{2+} ($CuSO_4$, Reanal) or 10 mg/L As^{3+} (Na_2HAsO_4, Fluka) under static conditions. In other experiments, carp were injected intraperitoneally with 10 mg/kg Cd^{2+}. In all experiments, 4 animals were sacrificed at each time point for organ harvesting, frozen immediately in liquid nitrogen and stored at –80 °C.

RNA extraction, reverse transcription and PCR amplification

Frozen, intact hearts were homogenized in RNAzol B reagent (Tel-Test, Inc., Friendswood, Texas, USA) and the total RNA was prepared according to the procedure suggested by the manufacturer. To detect carp MT- and *hsp*-specific mRNAs, an RT-PCR-based strategy was employed. First-strand cDNA synthesis, PCR amplification and primers, specific to MT-1 and MT-2, *hsp70*, *hsc70-1*, *hsp90*α and β-actin genes of carp were used as described earlier [1, 12, 13]. Amplification was performed in a PTC 200 Peltier Thermal Cycler (MJ Research). The number of amplification cycles during which PCR product formation was limited by the template concentration was determined in pilot experiments: for *hsp*s and MTs 30 cycles were used. The amplified products were electrophoresed on 2% agarose (Sigma) gel.

Measurements and statistical analysis

At each experimental time point, 3–4 fish were used to prepare RNA. RT-PCR reactions for each animal were performed in triplicate in order to increase the reliability of the measurements. For normalization of the amount of *hsp* or MT mRNAs, the

carp β-actin mRNA level was used as internal standard. Images of ethidium bromide-stained agarose gels were digitized with a GDS 7500 Gel Documentation System and analyzed with GelBase/GelBlot™ Pro Gel Analysis Software (UVP). The relative levels of *hsp* and MT mRNAs are expressed as ratios [*hsp* or MT/β-actin]. 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 $P < 0.05$.

Analysis of cadmium content

Tissues were dried and digested with HNO_3/H_2O_2. The metal 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 concentration. Metal contents are reported in μg/g dry weight (DW).

RESULTS

Basal levels of expression

The expression of *hsp90α* and *hsp70* in the heart of the unstressed animals were very low. The specific mRNA varied between undetected (17 animals) and around 5% of that of the β-actin transcript (9 animals).

The highest basal expression of the heat shock genes examined in this study was found for *hsc70-1*. The level measured in the different experiments varied between 20–30% of that of the β-actin.

Both MT-1 and MT-2-specific mRNAs were readily detectable in the heart of the control animals. The amount of the MT-1 transcript was always less than that of the MT-2 transcript. For the actual basal expression levels, see the control columns of Figs 1–4.

Induction of the stress-genes by As^{3+}, Cu^{2+} and Cd^{2+} treatments

hsp90α

Of the three metal ions examined As^{3+} influenced the expression of *hsp90α* most. After 24 h of treatment, we detected an 11-fold induction, with the specific mRNA level reaching 53% of that of the β-actin. A somewhat lower level was detected at 48 h (44%), and the basal level was reached by 96 h (Fig. 1A). The gene was also transiently induced in the heart of the Cu^{2+}-treated animals; Cu^{2+} resulted in a 4–5-fold induction by 24 h, but only a modestly elevated (2-fold) and the basal level

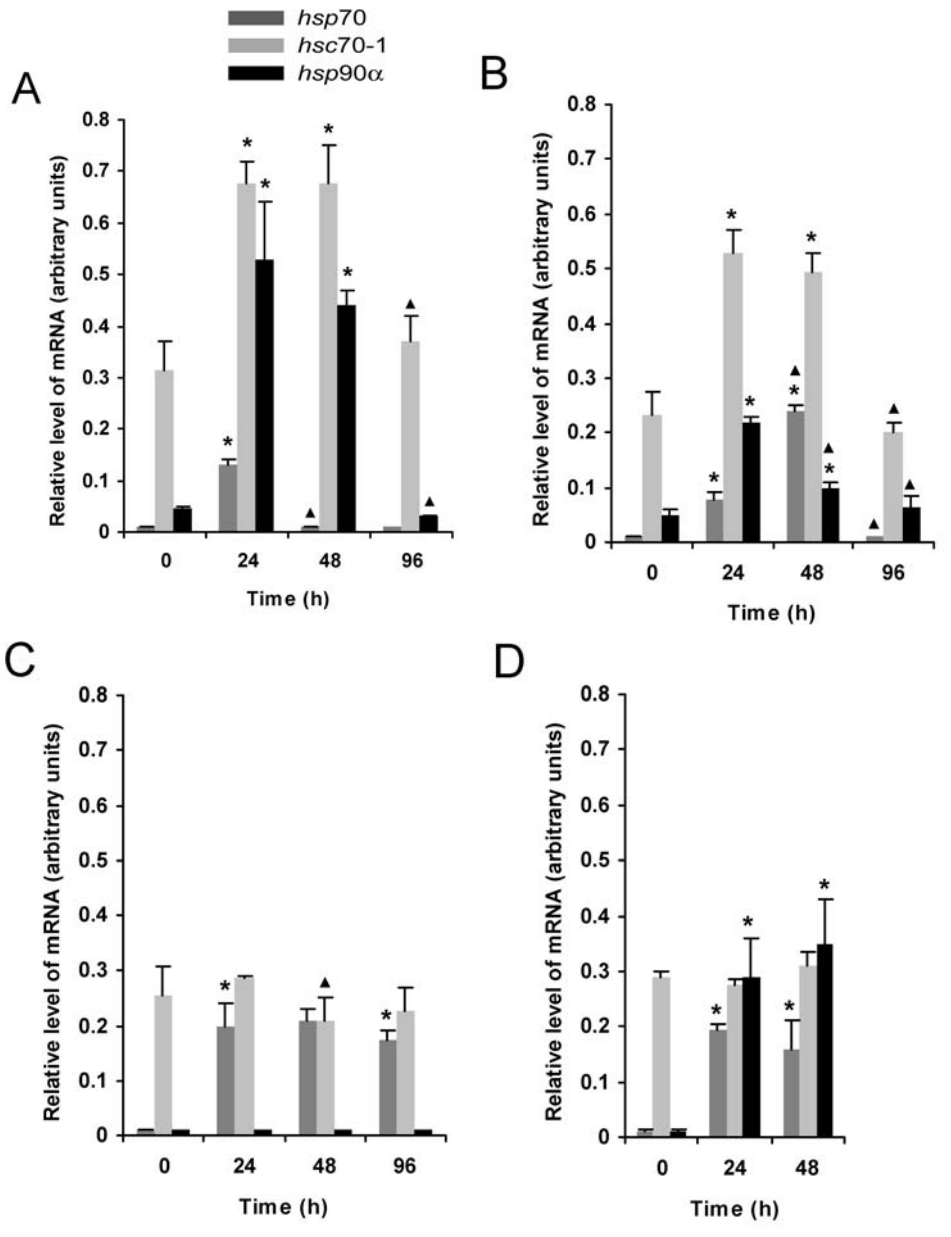

Fig. 1. Time course study of the effects of 10 mg/L metal treatments: A: As^{3+}; B: Cu^{2+}; C: Cd^{2+}; and D: Cd^{2+} at 10 mg/kg, on the expressions of *hsp70, hsc70-1* and *hsp90*α genes in carp heart. Significant difference: *, between the control (0) and the value at a given time point; ▲, between the values at consecutive time points of treatments (24, 48 and 96)

expression were measured at 48 h and at 96 h, respectively (Fig. 1B). We did not observe any detectable effect of Cd^{2+} on the *hsp90α* expression at 1 mg/L (data not shown) and 10 mg/L (Fig. 1C); only the very drastic treatment in which 10 mg/kg was administered intraperitoneally resulted in a significant induction (Fig. 1D). However, the 30–35% relative level of the specific mRNA detected at 24 h and at 48 h was still significantly lower than the 53% measured after As^{3+} treatment at the same time points.

hsc70-1

As^{3+} and Cu^{2+} affected the *hsc70-1* expression to similar extents. Both treatments resulted in a 2-fold induction by 24 h. The elevated specific mRNA levels were not significantly different at 48 h, but the basal level was restored by 96 h in both cases (Fig. 1A and B). Cd^{2+} treatment did not change the expression of this gene significantly, either at 1 mg/L (data not shown) and 10 mg/L or at 10 mg/kg (Fig. 1C and D).

hsp70

The best inducers of this gene were Cu^{2+} and Cd^{2+}. Cu^{2+} treatment resulted in an 8-fold induction by 24 h. The expression was further increased at 48 h, reaching the 24-fold maximum, but had returned to its basal level by 96 h (Fig. 1B). In contrast, the induction triggered by 10 mg/L or 10 mg/kg Cd^{2+} was not transient; the maximal amount of specific mRNA (20% relative to β-actin) was already reached by 24 h, and remained at this level for the duration of the experiments (Fig. 1C and D). *Hsp70* was least affected in the heart of the animals exposed to As^{3+}. Induction (13-fold) was detected only at 24 h; the basal expression level was observed at all other time points (Fig. 1A).

Metallothioneins

Cu^{2+} and As^{3+} induced MT expression transiently, in very similar manners. The MT-2 mRNA level increased 3-fold in the first 24 h of the treatment, where it remained up to 48 h. By 96 h, however, a significant decrease was detected, though the measured values were still 1.5-times higher than the controls. The expression of MT-1 displayed a more drastic increase; after 24 h or 48 h of Cu^{2+} challenge, the induced transcript level was 9-fold and 11-fold, respectively, higher than the control (Fig. 2B). As^{3+} proved to be a somewhat more potent inducer of MT-1; mRNA level was elevated 12-fold after 24 h of treatment and remained there up to 48 h. Due to the difference in the induction rates, the amount of MT-1 mRNA reached that of MT-2. By 96 h, the expressions of both genes were reduced sharply; only 1.5–2 times more mRNA was detected than in the heart of the control animals (Fig. 2A).

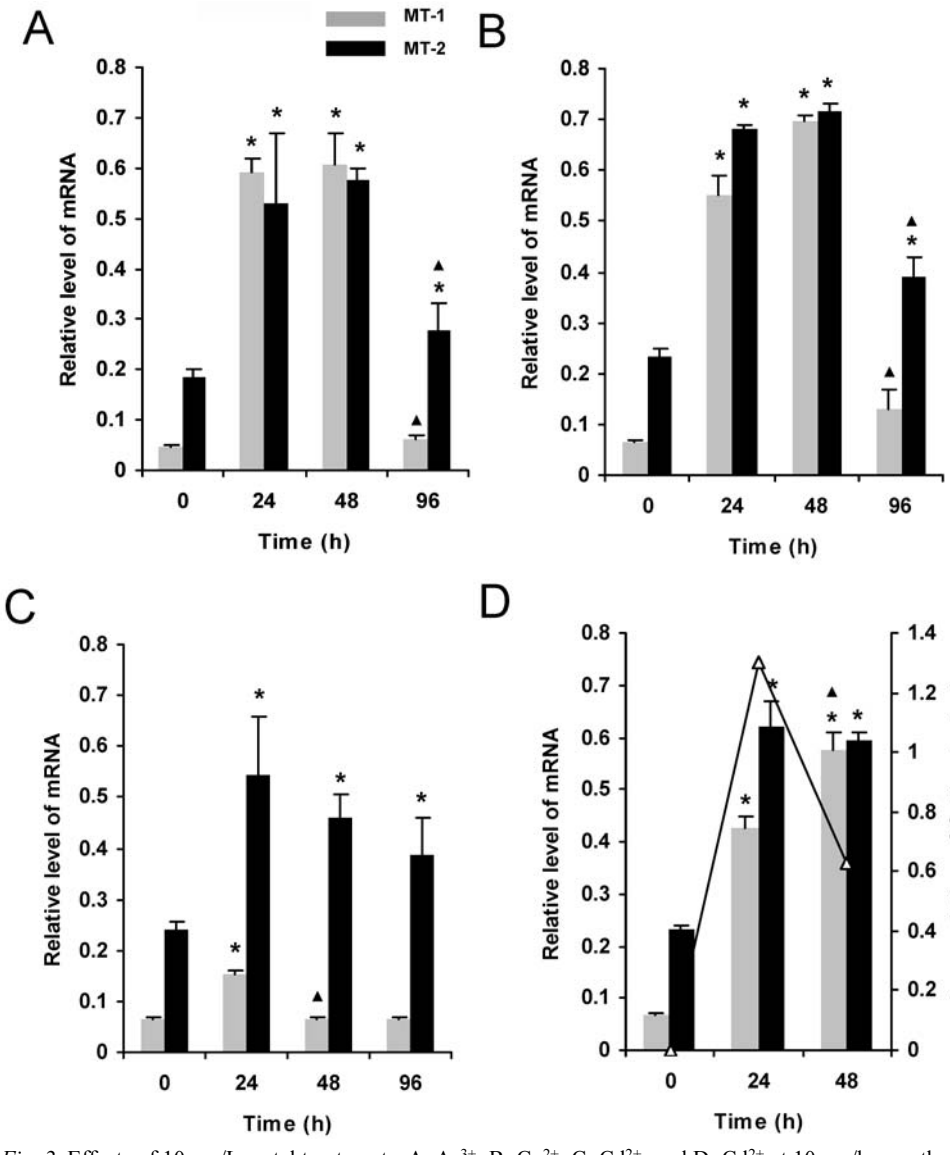

Fig. 2. Effects of 10 mg/L metal treatments: A: As^{3+}; B: Cu^{2+}; C: Cd^{2+}; and D: Cd^{2+} at 10 mg/kg, on the expressions of MT-1 and MT-2 in carp heart. D: Cadmium accumulation in the heart during Cd challenge. Significant difference: *, between the control (0) and the value at a given time point; ▲, between the values at consecutive time points of treatments (24, 48 and 96)

The expressions of the MTs were not induced by 1 mg/L Cd^{2+} (data not shown). The 10 mg/L dose of the metal transiently induced both MT genes. Maximal induction was observed at 24 h of treatment, when the MT-1 and MT-2 mRNA levels were 2–2.5-fold higher than the control. The level of MT-2 mRNA subsequently gradually decreased to about 150% of the control by 96 h, while the MT-1 transcript level already approximated to the control value by 48 h (Fig. 2C).

Ten mg/kg Cd^{2+} caused a 2.5-fold increase in MT-2 transcription 24 h post-injection, which was not significantly different from this at 48 h. These values did not differ greatly from those measured after the 10 mg/L treatment. However, Cd^{2+} at this high dose proved to be a more efficient inducer of MT-1; about a 6-fold elevated transcription was detected at 24 h, which increased further, reaching 830% of the control level by 48 h.

Cadmium accumulation

Cadmium content was followed in the heart in parallel with the MT and *hsp* mRNA levels. It was not detected in the heart of the fish exposed to 10 mg/L Cd^{2+}. However, a detectable amount of cadmium was present when the metal was applied at 10 mg/kg. In the liver, the cadmium accumulation correlated with the duration of the exposure, whereas in the heart, the cadmium content peaked at 24 h (1.3 μg/g dry weight) and was decreased by 48 h of treatment (0.63 μg/g dry weight) (Fig. 2D).

Effect of temperature shock on stress gene expression

Hyperthermia

The concentrations of *hsp70* and *hsp90*α mRNA in the heart of the unstressed animals was around the limit of detectability. The sensitivities of these two genes to elevated temperature were about the same, but the kinetics of upregulation of the gene expressions revealed marked differences. Incubation for 30 min at the elevated temperature resulted in a 7-fold increase in the transcription of *hsp70.* Its peak expression was about 19 times the basal level detected after 3 h of direct heat shock. A similarly high value was measured after the 1-h recovery period following the 30-min heat shock. This high level of expression did not persist during the recovery following the 3-h heat shock; only a 4-fold elevated transcription was measured at this time point. *Hsp90*α was also substantially induced by heat shock; the maximal induction was observed after incubation for 30 min at the elevated temperature, reaching a value 15 times the control. After 3 h of direct heat treatment, a 7-fold induction was observed, and this elevated transcription persisted during both recovery periods (Fig. 3).

A high level of the *hsc70-1* transcript was detected in the heart of the unstressed animals; the amount of specific mRNA was about 25% of the β-actin mRNA level.

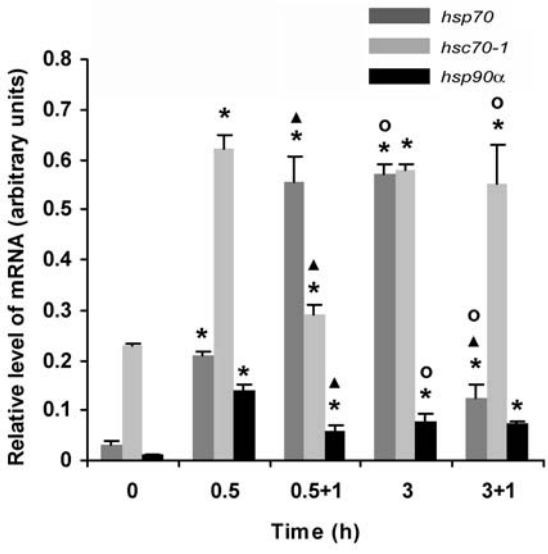

Fig. 3. Effects of hyperthermia on the expression of *hsp* genes in carp heart. The 0.5 and 3 indicate the duration of heat treatment. The 0.5 + 1 and 3 + 1 indicate the same treatments followed by a 1-h recovery period at the acclimatization temperature. Significant difference: *, between the control (0) and the value at a given time point; ▲, between the values directly after the heat shock and after the corresponding recovery period (0.5, 0.5 + 1 and 3, 3 + 1); ○, between the values at consecutive time points directly after heat treatment (0.5 and 3) or after the recovery period (0.5 + 1 and 3 + 1), respectively

Heat shock for 30 min resulted in a 2.5–3-fold elevation in mRNA level, which was not increased further even after 3 h of hyperthermia. This high level of the *hsc70-1* transcript also persisted during the 1-h recovery period following 3 h of heat stress. However, the mRNA level decreased rapidly during the recovery period after the 30-min incubation at the high temperature (Fig. 3).

In the heart, the elevated temperature had no measurable effect on the expressions of the MT genes either immediately after the heat stress or after the recovery period.

Hypothermia

The *hsp70* and *hsp90α* expressions demonstrated time-dependent induction both directly after a cold shock and after a cold shock followed by a recovery period. The *hsp70* mRNA level was increased 14-fold, 12-fold and 5-fold directly after the 1-h, 2-h and 5-h cold treatment, respectively. A cold shock had similar effects on the expression of *hsp90α*: a 10-fold, 8.5-fold and 5-fold increase was detected after 1 h, 2 h and 5 h of incubation, respectively. However, the 1-h return to the adaptation temperature affected the expression patterns of the two genes quite differently. While the *hsp70* mRNA level gradually decreased, the *hsp90α* expression was further induced during the recovery period. Following the 1+1-h incubation, the *hsp70* mRNA level

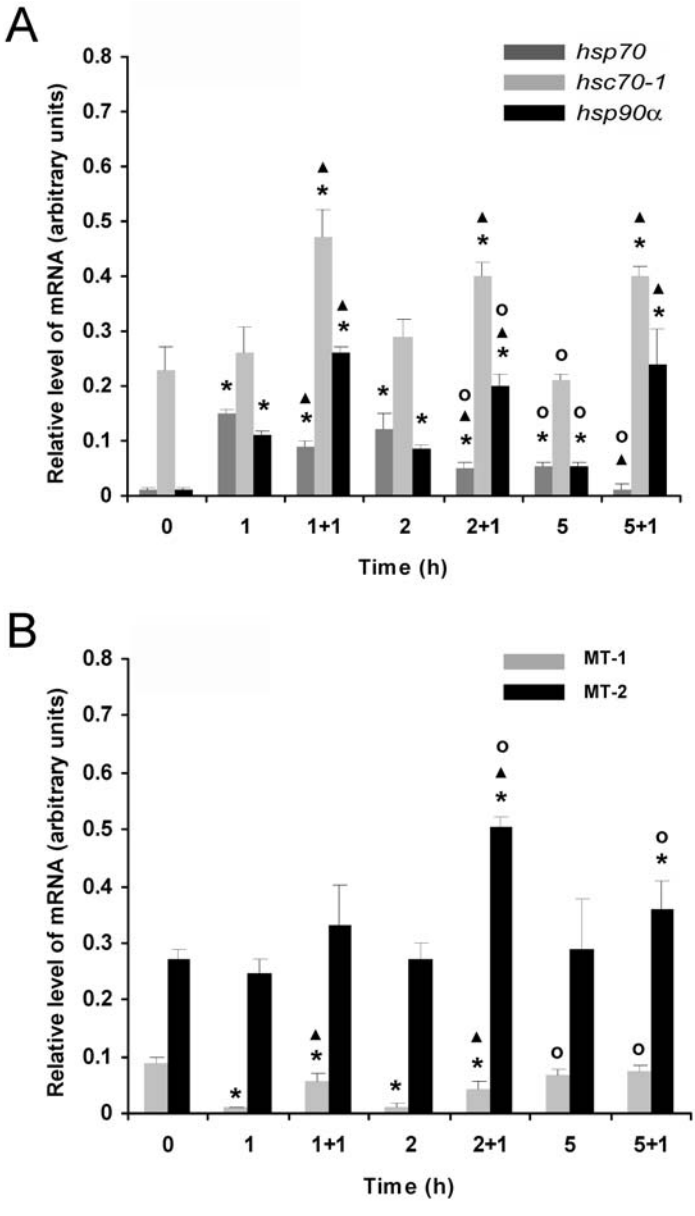

Fig. 4. Effects of hypothermia on the expressions A: of hsp: and B: MT genes in the heart. The 1, 2 and 5 indicate the duration in hours of cold treatment. The 1 + 1, 2 + 1 and 5 + 1 indicate the same treatments followed by a 1-h recovery period at the acclimatization temperature. Significant difference: *, between the control (0) and the value at a given time point; ▲, between the values directly after heat shock and after the corresponding recovery period (1, 1 + 1; 2, 2 + 1 and 5, 5 + 1); ○, between the values at consecutive time points directly after cold treatment (1, 2 and 5) or after the recovery period (1 + 1, 2 + 1 and 5 + 1), respectively

was 50% of that measured directly after the cold shock, and after 5 + 1 h it was barely detected. The *hsp90α* expression increased to 26-fold of the basal mRNA level after 1 + 1 h of incubation, and it was not reduced significantly after 2 + 1 and 5 + 1 h. The *hsc70-1* expression was not affected directly after the cold treatment. However, a further 1 h of recovery at the acclimatization temperature led to a modest, but measurable 1.5–2-fold induction (Fig. 4A).

In contrast with the heat shock, a 7 °C decrease in temperature induced the expressions of the two MT genes; their altered expressions differed markedly. The MT-1 mRNA level had decreased to below the threshold of detectability directly after 1 or 2 h of cold shock, and it was about 75% of the basal level after 5 h of incubation. The MT-2 expression was not altered at these time points. The 1-h recovery at the adaptation temperature resulted in a detectable MT-1 expression, but the measured level did not reach the basal expression level at any time point. The MT-2 mRNA levels were increased in a time-dependent manner during the recovery, with peak expression (1.8–2-fold) after 2 + 1 h (Fig. 4B).

DISCUSSION

The three heat shock and the two MT genes examined in this study exhibited a unique, tissue-specific basal expression pattern and a characteristic sensitivity to metal treatments and temperature shocks.

One of the major differences between the heart and other organs was found in the basal and induced expression patterns of MT-1 and MT-2. A 2:1 and a 3:1 MT-1/MT-2 ratio were found previously in the liver and kidney of the unstressed animals, while in the brain and the skeletal muscle the ratios were close to 1, with a slight excess of MT-1. In the heart, significantly less MT-1 than MT-2 mRNA was detected, resulting in a 1:3 ratio [12]. The total MT level measured in the carp heart is comparable to that in the liver or the kidney, while in other fish species for which detailed tissue-specific expressions are available, the heart MT level is about one-fourth of that in the kidney or liver [2].

Both MT genes were found to be almost insensitive to Cd^{2+} treatment in the heart. Since the amount of Cd^{2+} accumulated in the heart is not even comparable to that measured in the liver or kidney, this could be one of the reasons for the poor MT upregulation. Unlike in other fish tissues (i.e. the gill, liver and kidney), in the heart Cu^{2+} proved to be a more potent inducer of MT expression than was Cd^{2+} [2, 12].

Ten mg/l As^{3+} treatment had contrasting effects on the MT-1 expression; while this gene proved to be insensitive to this treatment in the liver and kidney, a 10-fold increase in its expression was found in the heart after a 24-h exposure. In contrast, the inducibility of MT-2 by As^{3+} was similar in all three organs; a 2–3-fold increase was detected in the mRNA at 24–48 h in the heart and the kidney, and a 4.5–5-fold induction in the liver [14].

Both MT genes were transiently induced by 10 mg/l Cu^{2+} treatment, with some quantitative differences only between the three organs: the inducibility of MT-1 was

highest in the heart (8.5–10-fold) and lowest in the kidney (3-fold), while the induced level of MT-2-specific mRNA was lowest in the heart (3-fold) and highest in the liver (6–7-fold) [14].

We have previously examined the expressions of MT-1 and MT-2 in the liver after heat and cold shocks. A 14 °C jump in temperature did not result in significant changes in the expression of either MT gene, and this is what we have now found in the heart. MT also proved to be unaffected in fish cell lines exposed to elevated temperature [22]. Cold shock, however, altered the expression in a tissue and gene-specific manner. In the heart, the expression of MT-1 was downregulated to less than 10% of the basal level after 1 h of cold exposure and remained at this level at 2 h but had recovered to 70% by 5 h. This sharply contrasts with the effect of cold shock in the liver, where a 4–6-fold induction of MT-1 was detected by 2-h. MT-2 was also induced 2-fold in the liver, directly after hypothermia, but we did not observe this effect in the heart. We suggest that the different extent of regulation of the MTs by cold shock in the heart and liver are rather indirect effects of the sudden temperature drop. Such an indirect signal could be provided by the Zn^{2+} concentration. After 1 h and 5 h of cold shock plus 1 h of recovery, the Zn^{2+} content in the liver was increased by 100%, while that in the heart was decreased to 80% (Hermesz, unpublished).

In the heart, both the *hsp70* and the *hsp90*α genes displayed a low basal expression level and a strong transient induction by As^{3+} and Cu^{2+}. Direct cold shock also resulted in a similar response: a strong, 10–14-fold induction. An increased *hsp70* expression associated with cold exposure has also been described in the brain and muscle tissues of catfish [15, 26].

In contrast, the expressions of *hsp70* and *hsp90*α differed markedly when the animals were kept for 1 h at the adaptation temperature after the cold shock: the level of *hsp70* mRNA declined fast to the uninduced level, while further increased levels of *hsp90*α mRNA were detected at all time points of recovery. This difference could not be explained by the possible heat shock caused by the 5 °C temperature shift, since both genes were sensitive to heat shock; in fact, *hsp70* was induced more than *hsp90*α by heat treatment. We speculate that factors regulating the heat and cold shock responses of *hsp90*α may function independently, while in the case of *hsp70* the two pathways interfere with each other at some point. It is well known that heat shock transcription factors (HSFs) mediate the induction of *hsp*s through binding to heat shock elements in the promoter region of the *hsp* genes. Though relatively little knowledge is available on cold-induced gene regulation, it has been reported that cold induction of the expression of *hsp*s is also mediated by HSFs. However, the cold activation of HSF lacks the hyperphosphorylation characteristic of the heat activation of HSF. The mechanism involved in the cold activation step is as yet unknown [3, 9].

In the heart, *hsc70-1* exhibited an expression pattern expected from a *bona fide* cognate heat shock gene; a substantial basal expression level and no (Cd^{2+}) or only moderate (As^{3+}, Cu^{2+} and heat or cold shock) inducibility by stressors. However, even this moderate inducibility resulted in the highest relative mRNA levels among the three heat shock genes tested, due to the high basal levels in the control animals. The Cd^{2+} insensitivity contrasts with the strong inducibility (11–13-fold by 10 mg/l

Cd^{2+}) of *hsc70-1* in the liver. A considerable difference in the basal expression levels was also noted between the heart and other organs, i.e. no *hsc70-1* mRNA was detected in the liver and kidney previously [1].

In conclusion: The five stress genes examined exhibited characteristic sensitivity to metal treatments and temperature shocks; *hsp90α* proved to be the most inducible by cold shock (26-fold), and heat treatment (15-fold); *hsp70* responded best to Cu^{2+} loading with a 24-fold increase in expression. The MT-1 and MT-2 genes were most affected by metal treatment, in an isoform-specific manner (11- and 3-fold, respectively).

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