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The physiological role and toxicological significance of the non-metal-selective cadmium/copper-metallothionein isoform differ between embryonic and adult helicid snails

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ABSTRACT

Metal regulation is essential for terrestrial gastropods to survive. In helicid snails, two metal-selective metallothionein (MT) isoforms with different functions are expressed. A cadmium-selective isoform (CdMT) plays a major role in Cd^{2+} detoxification and stress response, whereas a copper-selective MT (CuMT) is involved in Cu homeostasis and hemocyanin synthesis. A third, non-metal-selective isoform, called Cd/CuMT, was first characterized in *Cantareus aspersus*. The aim of this study was to quantify the transcriptional activity of all three *MT* genes in unexposed and metal-exposed (Cd, Cu) embryonic Roman snails. In addition, the complete Cd/CuMT mRNA of the Roman snail (*Helix pomatia*) was characterized, and its expression quantified in unexposed and Cd-treated adult individuals. In embryos of *Helix pomatia*, the *Cd/CuMT* gene was induced upon Cu exposure. Its transcription levels were many times higher than that of the other two *MT* genes, and also exceeded by far the *Cd/CuMT* mRNA concentrations of adult snails. In the hepatopancreas of adult Roman snails, no Cd/CuMT could be detected at the protein level, irrespective of whether the snails had been exposed to Cd or not. This contrasts with the situation in the near relative, *Cantareus aspersus*. It appeared that the 3′-UTR of the Cd/CuMT mRNA differed largely between *Cantareus aspersus* and *Helix pomatia*, being larger in the latter species, with a number of putative binding sites for proteins and miRNAs known to inhibit mRNA translation. We suggest this as a possible mechanism responsible for the lack of Cd/CuMT protein expression in adult Roman snails.

1. Introduction

The Roman snail (*Helix pomatia*) is a key species of terrestrial habitats in central Europe. Roman snails are in close contact with the soil substrate throughout their whole lifespan from eggs, buried a few centimeters in the substrate, to mature snails thriving on the soil surface. Due to fluctuating environmental meteorological conditions, the water supply and availability of metallic trace elements in the upper soil horizon may vary according to the intensity of precipitations. Moreover, the water balance of terrestrial snails is subjected to seasonal alterations due to intermittent periods of activity and aestivation (Pedrini-Martha et al., 2016). Hence, the handling and regulation of metallic trace elements within snail tissues becomes an important task for the species viability. The intracellular availability of essential trace elements needs to be regulated by homeostatic mechanisms. Copper (Cu) homeostasis, for example, is linked to the synthesis of hemocyanin, the respiratory pigment of terrestrial gastropods (Van Holde et al., 2001). Non-essential harmful metals, on the other hand, must be inactivated. This is the case for cadmium (Cd) which can negatively affect adult and embryonic snails, being able to cross the eggshell (Druart et al., 2010), as well as digestive epithelia of adult individuals. For example, Cd can reduce the fertility of terrestrial snails by decreasing the number of laid eggs and by delaying the egg laying cycle (Gimbert et al., 2008), by inhibiting the hatching of embryos (Druart et al., 2010; Baurand et al., 2014) and reducing their growth rate (Baurand et al., 2014).

Metallothioneins (MTs) are ubiquitous metal binding proteins, involved in metal homeostasis and detoxification (Dallinger et al., 1997; Egli et al., 2006), as well as in stress response (Chabicovsky et al., 2004; Fu and Miao, 2006; Egg et al., 2009; Pedrini-Martha et al., 2016). They are characterized by a low molecular weight, a low abundance or lack of aromatic amino acids and a high content of cysteines, arranged within the peptide chain in typical Cys-Xaa-Cys motifs. The sulfhydryl groups of the cysteine residues are essential for the formation of metal-thiolate clusters in which mono- and divalent metal ions are bound (Kojima et al., 1999). In helicid snails the development of

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metal-selective MT isoforms from a basal non-specific MT ancestor represents a unique evolutionary advance compared to MTs from all other animal phyla (Palacios et al., 2011; Pérez-Rafael et al., 2014). Accordingly, the Roman snail *Helix pomatia* possesses two metal-selective MT isoforms which are differentially expressed in diverse tissues and perform unequal, metal-specific functions in the snail organism (Dallinger et al., 1997; Chabicovsky et al., 2003). The helicid Cd-specific MT (CdMT) binds divalent metal ions with high selectivity, is mostly expressed in the hepatopancreatic and digestive tissues and is mainly involved in Cd detoxification and stress response (Dallinger and Berger, 1993; Chabicovsky et al., 2004; Egg et al., 2009). The Cu-selective MT isoform (CuMT), on the other hand, is exclusively expressed in one single cell type, the so-called rhogocytes, playing an important role in Cu homeostasis and hemocyanin synthesis (Berger et al., 1997; Dallinger et al., 1997; Dallinger et al., 2005). In *Cantareus aspersus*, an additional non-metal specific MT isoform (Cd/CuMT) was discovered, being able to bind both, Cu^{+} and Cd^{2+} ions simultaneously (Hispard et al., 2008; Höckner et al., 2011). Due to the rather low expression of the Cd/CuMT protein in the hepatopancreas of Cd-exposed adult snails in combination with low mRNA concentrations and the non-inducibility of the *Cd/CuMT* gene upon metal exposure, it was supposed that this intermediate MT isoform may only play a marginal role in the metal homeostasis and detoxification of adult *Cantareus aspersus* (Hispard et al., 2008; Höckner et al., 2011). However, high Cd/CuMT mRNA expression levels in embryonic snails of this species indicate a possible function of the Cd/CuMT in embryonic snail development (Baurand et al., 2015, 2016a). In contrast to *Cantareus aspersus*, the full characterization of this homolog isoform and its potential expression were so far unknown in the relative species *Helix pomatia*.

Hence, the complete Cd/CuMT mRNA sequence of *Helix pomatia* was identified and characterized, and their gene transcription and protein expression were measured in the hepatopancreas, the main site of Cd detoxification, in controls and Cd-exposed adult Roman snails. In addition, the transcriptional activity of this isoform was analysed for the first time in comparison with the two metal-specific *CdMT* and *CuMT* genes in unexposed and metal-treated (Cd, Cu) embryonic stages of *Helix pomatia*.

2. Materials and methods

2.1. Cadmium exposure and sampling of adult Helix pomatia

Adult Roman snails were obtained from a commercial dealer (Thüringer Weinbergschnecke, Germany) and acclimatized in transparent octagonal plastic boxes (diameter: 12 cm; height: 6 cm) on garden earth substrate (Bauhaus-Gartenerde, Austria, with a reported Cd concentration of <0.3 μg/g dry weight) (information provided by the supplier) containing lime powder ($CaCO_3$) under constant conditions (18 °C; 12:12 h light:dark). Prior to exposure snails were fed regularly with uncontaminated lettuce (*Lactuca sativa*) and sprayed with water for one week. Subsequently, control snails were fed with uncontaminated lettuce and Cd-exposed snails were fed with Cd-enriched lettuce every day as described in Pedrini-Martha et al. (2016). For MT expression studies by means of quantitative real-time PCR, five controls and five Cd-fed snails were dissected on a cooled aluminium-plate, which was cleaned with RNase AWAY (Sigma-Aldrich). Tissue aliquots from the hepatopancreas (approx. 1 mg fresh wt.) were transferred to RNA*later*™ Solution (Invitrogen by ThermoFisher Scientific, USA) and stored at −80 °C.

For MT protein purification, adult Roman snails were kept under laboratory conditions (18 °C; 12:12 h light:dark) in plastic boxes on moistened garden soil substrate. 15 animals were exposed to Cd over a period of 14 days by feeding on Cd-enriched lettuce every second day prepared as described in Dallinger et al. (2004). 15 control animals were reared under the same conditions as above by feeding on uncontaminated lettuce. Average Cd concentration in the metal-enriched feed was about 268.8 μg/g (2.4 μmol/g) dry weight, while the Cd concentration in the control salad was 4.48 μg/g (0.04 μmol/g) dry weight (Dallinger et al., 2004). At the end of the feeding experiment, all animals were sacrificed and dissected. Small hepatopancreas aliquots were used for metal analysis, the remaining samples of three individuals were pooled separately, yielding approximately 3–3.5 g fresh tissue mass for each pooled sample. Cd concentrations in the hepatopancreas of snails were about 29 μg/g dry weight for control snails and 324 μg/g dry weight for metal-exposed individuals.

2.2. Exposure design, metal concentrations and sampling of Helix pomatia eggs and embryos

Eggs of *Helix pomatia* were obtained from standardized laboratory rearing and were exposed using a liquid phase bioassay as previously described (Druart et al., 2010, 2012). Five clutches were used for the experiment. Each clutch was separated into groups of 6 to 9 eggs, which were placed in Petri dishes on four layers of paper (Quantitative filter paper grade 1 ashless, Whatman) dampened with 0.8 ml of control (demineralized water; pH = 6.2), Cd or Bordeaux mixture (BM) solution rapidly after egg laying within a maximum of 24 h after fertilization. For Cd exposure, eggs were incubated with a $CdCl_2$ solution (99.99%, Sigma Chemical Co., St. Louis, MO; C-2544) with a nominal Cd concentration of 10 mg/l for 24 h. For Cu exposure, eggs were incubated for 20 days with a solution of Bordeaux mixture made from powder of BM RSR Disperss (20% of Cu, Cerexagri, Cergy, France) with a nominal concentration tested of 0.5 g/l Cu (=2.5 g/l of Bordeaux mixture). The concentrations of Cd and Cu were measured using ICP-AES (ICAP 6000 series model radial, Thermo Scientific, France). The quality of the results was verified using a certified reference water (Hard Drink Water UK, ERM-CAO11a, LGC Promochem, Molsheim, France), Cd-certified at 4.94 μg/l (average recovery of 93%) and Cu-certified at 1970 μg/l (average recovery 97%). The actual verified concentrations of exposure solutions were 8.7 mg/l for Cd and 0.37 mg/l for Cu, respectively. Sampling of control and metal-exposed eggs was carried out at 7 and 20 days post fertilization (dpf). Three eggs were pooled to one sample on day 7 due to low RNA concentrations. Eggs were separated from albumen and stored in RNA*later*™ Solution (Invitrogen by ThermoFisher Scientific, USA) at −80 °C.

2.3. Characterization and quantification of Cd/CuMT mRNA from adult and embryonic Helix pomatia

2.3.1. RNA isolation

For RNA isolation of adult Roman snails, hepatopancreas tissue aliquots were homogenized in TRIzol® Reagent (Ambion™ by Life Technologies) using the Ultra-Turrax T25 (Janke & Kunkel IKA® Labortechnik, Germany). After DNase I digestion (Invitrogen) RNA was cleaned up with the RNeasy MiniElute Kit (Qiagen, Hilden, Germany). For RNA isolation of 7 day-old embryos, three eggs were pooled, whereas for 20 day-old embryos one individual was used. RNA was isolated using the RNeasy® Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

2.3.2. RACE PCR of the 5′ and 3′ UTR of the Cd/Cu-MT

The coding region of the *Cd/CuMT* gene was sequenced by us previously and is already available in GenBank as two allelic variants (V1 and V2) under the accession numbers GU111728 (V1) and GU111729 (V2). To obtain the full length cDNA of the *Cd/CuMT* isoform gene, the SMARTer™ Race cDNA Amplification Kit (Clontech, Canada) was applied. Total RNA (300–700 ng) from three adult Roman snails was used for generation of RACE-Ready cDNA according to the manufacturer's instructions. For the rapid amplification of the 5′ and the 3'UTRs, gene specific primers were employed as follows: GSP1, 5′-ATG TGG CAA GAT TCC TGT GCG GCT GTG G-3′; and GSP2, 5′-AAC AGC AA C CCC TGC AGC TGC GGC GAC G-3′. PCR was carried out