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Bile and liver metallothionein behavior in copper-exposed fish

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ABSTRACT

The present study analyzed metallothionein (MT) excretion from liver to bile in Nile Tilapia (*Oreochromis niloticus*) exposed to sub-lethal copper concentrations (2 mg L^{-1}) in a laboratory setting. MTs in liver and bile were quantified by spectrophotometry after thermal incubation and MT metal-binding profiles were characterized by size exclusion high performance liquid chromatography coupled to ICP-MS (SEC-HPLC–ICP-MS). Results show that liver MT is present in approximately 250-fold higher concentrations than bile MT in non-exposed fish. Differences between the MT profiles from the control and exposed group were observed for both matrices, indicating differential metal-binding behavior when comparing liver and bile MT. This is novel data regarding intra-organ MT comparisons, since differences between organs are usually present only with regard to quantification, not metal-binding behavior. Bile MT showed statistically significant differences between the control and exposed group, while the same did not occur with liver MT. This indicates that MTs synthesized in the liver accumulate more slowly than MTs excreted from liver to bile, since the same fish presented significantly higher MT levels in liver when compared to bile. We postulate that bile, although excreted in the intestine and partially reabsorbed by the same returning to the liver, may also release MT-bound metals more rapidly and efficiently, which may indicate an efficient detoxification route. Thus, we propose that the analysis of bile MTs to observe recent metal exposure may be more adequate than the analysis of liver MTs, since organism responses to metals are more quickly observed in bile, although further studies are necessary.

Introduction

Metallothioneins (MTs) are considered good biomarkers for metal exposure. Liver is expected to accumulate the highest metallothionein levels in the body, since it is the major detoxifying organ [1]. Previous research has indicated that metallothioneins are excreted in varying degrees in mammals. For example, Bremner et al. [2] verified that the metallothionein isoform MT-I is excreted in varying amounts into blood, urine and bile in rats, while Jaw and Jeffery [3] observed that, in rats administered zinc, mercury or cadmium, cadmium was excreted into the bile in inverse proportion to the hepatic metallothionein content, while metallothionein content did not appear to bear any relationship to biliary excretion of mercury or zinc. Mohan et al. [4], on the other hand, analyzing biliary copper excretion in the neonatal rat verified that biliary copper excretion per day in young rats is relatively low in the first week of life and is independent of MT secretion.

No studies regarding biliary MT in fish, however, were available until recently, when Hauser-Davis et al. [5] reported that MTs are excreted from the liver into bile in fish as an alternative detoxification route, and that they follow the same trend as hepatic MT in situations of environmental metal exposure. Previous studies show that sub-lethal copper concentrations directly affect hepatic MT [6], however no studies regarding bile MT in these situations are available.

Nile tilapia (*Oreochromis niloticus*) are good sentinel organisms for metal exposure, as they are particularly exposed to sediment-associated contamination such as metals [7], since metals in general adsorb to sediments and particulate matter and they are limnobenthofagous, feeding on suspended particles.

The biliary excretion of several compounds of environmental concern has been validated as an alternative indicator of environmental exposure in fish, since this matrix also excretes substances from blood and liver that have not been excreted by the kidneys, such as metals and organic compounds [8,9]. In between feeding bile accumulates in the gallbladder and, to accommodate the continual influx of fresh bile, water is continuously extracted from the gallbladder, concentrating bile and its constituents (i.e., biliary

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proteins, xenobiotics). This, allied to the fact that the rapid metabolism and elimination of several contaminants by vertebrates usually results in low concentrations in muscle and liver, indicating limited usefulness of the chemical analysis of fish tissues in environmental contamination situations [10]. Bile analysis in these cases, therefore, is more advantageous, since it is a less complex matrix than liver, concentrates and rapidly excretes xenobiotics and other compounds and, there may be no need to sacrifice animals, since bile can be obtained by chronic cannulation of the hepatic bile duct [6].

The aim of the present study, therefore, was to analyze hepatic and bile metallothionein in tilapia exposed to sub-lethal copper levels compared to a control group and investigate biliary and hepatic MT accumulation and metal binding behavior. Chronic copper contamination is of great concern in freshwater bodies in general, and more so in Brazil, where high levels of this environmental contaminant are present in several regions, justifying the choice of this metal in the present study [11].

Materials and methods

Sample collection and laboratory exposure experiment

Nile Tilapia specimens (*O. niloticus*) ($n = 20$) were obtained from a commercial aquaculture facility in southeastern Brazil, in the municipality of Silva Jardim, Rio de Janeiro, in which fish are raised specifically for human consumption in tanks under controlled conditions. The fish were taken to the laboratory and acclimated in two 500 L tanks containing dechlorinated tap water at pH 7.0. Copper, in the form of copper sulfate ($CuSO_4 \cdot 5H_2O$) was dissolved to obtain 2 mg L^{-1} of Cu in each tank. This concentration was chosen since it is an environmentally realistic value observed in certain contaminated urban areas of Rio de Janeiro [12] were this study takes place, and where this species is routinely consumed by the human population and also because this is the maximum allowed copper concentration in water by the Brazilian governmental CONAMA resolution [13].

The fish were maintained in these tanks in a static experiment for 96 h, indicated when the test substance is proven as stable in the environment, as is the case of copper and other metals [14]. The fish were then sacrificed by spinal cord severing and bile and liver were immediately removed, the former by direct puncture of the gallbladder, and stored at −80 °C in sterile polypropylene tubes until analysis.

Sample processing

Bile and liver metallothioneins were purified by thermal extraction in both bile and liver, according to a previously proposed protocol first used for mussels and recently applied for fish bile analyses [5,15]. Briefly, bile and liver samples were homogeneized at a 3:1 ratio in a buffer of Tris–HCl 20 mmol L^{-1}, pH 8.6, PMSF (phenylmethylsulphonylfluoride) 0.5 mmol L^{-1} as an antiproteolytic agent and β-mercaptoetanol 0.01% as a reducing agent and centrifuged at 20.000 × *g* for 1 h at 4° C. The supernatants were transferred to new sterile polypropylene tubes and purified by heating at 70 °C for 10 min, since MT are heat stable and will not denature at this temperature while other proteins will. The samples were centrifuged again for 1 h in the same conditions. The final supernatants were then separated from the pellet and used in all further analyses.

SDS-PAGE analyses for verification of the adequacy of the MT purification process

SDS-PAGE analyses were carried out in order to verify the adequacy of the purification process. Total protein content was quantified by the Lowry method modified by Peterson using Bovine serum Albumin (BSA) as standard [16] in order to apply the same amount of protein to each gel lane. Protein separations were carried out on 15% sodium dodecyl sulfate polyacrylamide gels (SDS-PAGE) following the Laemmli protocol [17]. This was done because previous studies indicate that metallothionein has a molecular weight of approximately 14 kDa, which is large enough to be separated in these conditions, making a tris-trycine separation (better suited for the separation of <10 kDa proteins) unnecessary [5]. Each sample (12 μg of total protein in each lane) was run in triplicate, to assure method reproducibility. Gels were stained by silver staining as described previously [18]. The molecular weights of the protein bands were determined using Biorad's Precision Plus Protein™ Dual Color Standards. Gels were scanned using an ImageScanner II (GE Healthcare, Uppsala, Sweden) with the densitometer operating at 300 dpi resolution. The Image-Master 2D Platinum 6.0 software (GeneBio, Geneva, Switzerland) was employed for gel imaging analyses.

Table 1
Instrumental operating SEC-HPLC–ICP-MS conditions.

SEC conditions	
Column	Superdex TM-75 (10 × 300 × 13 mm) (GE Healthcare, Uppsala, Sweden)
Effective resolution range	3–70 kDa
Exclusion limit	100 kDa
Mobile phase	Tris–HCl 0.02 mol L^{-1} (pH 7.4)
Flow rate	0.7 mL min^{-1}
Injection volume	20 μL
ICP-MS conditions	
Forward power	1100 W
Plasma gas flow rate	17.0 L min^{-1}
Auxiliary gas flow rate	1.2 L min^{-1}
Carrier gas flow rate	0.98 L min^{-1}
Sampling and skimmer cones	Pt
Dwell time	30 ms per isotope
Monitored isotopes	^{208}Pb, ^{202}Hg, ^{113}Cd, ^{64}Zn, ^{63}Cu

MT quantification by spectrophotometry (Ellman's reaction)

The purified supernatants were incubated with HCl 1 mol L^{-1} in EDTA 4 mmol L^{-1} and NaCl 2 mol L^{-1} containing 0.43 mmol L^{-1} DTNB (5,5-dithiobis-2-nitrobenzoic acid) buffered with 0.2 mol L^{-1} Na-phosphate, pH 8.0 [19]. The samples were centrifuged at 3000 × *g* for 5 min and the supernatant absorbance was then evaluated at 412 nm. Metallothionein concentrations were estimated utilizing reduced glutathione (GSH) as standard and MT content was calculated by assuming the relationship of 1 mol MT = 20 mols GSH, as described by Kagi for fish [20].

SEC-HPLC–ICP-MS analyses

Elemental detection was performed using a NexION 300X Perkin Elmer quadrupole inductively coupled plasma mass spectrometer (Perkin Elmer) equipped with a collision and reaction cell. Chromatographic separations were performed using a Model 1100 HPLC pump with UV detector (Agilent, Wilmington, DE, USA) as the delivery system. ICP-MS measurement conditions (Table 1) were optimized by using a HNO_3 2% (v/v) aqueous solution using a multielemental standard (Perkin Elmer).

The SEC-ICP-MS on-line coupling was performed by connecting the outlet of the chromatographic column to the nebulizer inlet of the ICP-MS by means of a 50-cm PEEK tubing. The instrumental operating conditions are given in Table 1. Five elements (^{208}Pb, ^{202}Hg, ^{113}Cd, ^{64}Zn, ^{63}Cu) known to bind with metallothioneins were analyzed: ^{208}Pb, ^{202}Hg, ^{113}Cd were monitored to verify if any contamination by these metals was present in the samples, and ^{64}Zn,