Bull Environ Contam Toxicol (2012) 89:225–228
DOI 10.1007/s00128-012-0708-6

Hepatic Metallothionein and Glutathione-S-Transferase Responses in Two Populations of Rice Frogs, *Fejervarya limnocharis*, Naturally Exposed to Different Environmental Cadmium Levels

Mohd Sham Othman · Wichase Khonsue · Jirarach Kitana · Kumthorn Thirakhupt · Mark Robson · Marija Borjan · Noppadon Kitana

Received: 13 February 2011 / Accepted: 8 June 2012 / Published online: 22 June 2012

Abstract Glutathione-S-Transferase (GST) and metallothionein are important biomarker endpoints in studying the effect of Cd exposure. The purpose of this research was to study the correlation between hepatic GST and metallothionein with hepatic Cd in wild *Fejervarya limnocharis* exposed to environmental Cd. Results showed that frogs from contaminated sites had significantly higher hepatic metallothionein (3.58 mg/kg wet weight) and GST activity (0.259 µmol/min/mg total protein) than those from the reference site (2.36 mg/kg wet weight and 0.157 µmol/min/mg total protein respectively). There was a significantly positive correlation between hepatic Cd and GST activity ($r = 0.802$, $p = 0.009$) but not between hepatic Cd and metallothionein ($r = 0.548$, $p = 0.139$). The results concluded that while frogs from the contaminated site had higher GST and metallothionein, only GST showed significant positive correlation with hepatic Cd levels, indicating that hepatic GST activity may be used as a biomarker endpoint.

Keywords Cd · *Fejervarya limnocharis* · Metallothionein · GST · Biomarker

Biomarkers are sensitive tools for the assessment of biological effects of pollutants (Linde-Arias et al. 2008). Biomarkers such as stress proteins, enzymes and other biomolecules have been shown to be affected by metal pollutants and they can be regarded as either specific or non-specific biomarkers (Monserrat et al. 2007). One of the direct responses of metal exposure is the production of metallothionein. Free cystolic metal ions, especially Cd induce the production of metallothionein (Baykan et al. 2007), and the resulting metallothionein will bind to metal ions (Hansen et al. 2006). Loumbourdis et al. (2007) suggested that metallothionein is a good biomarker of exposure for heavy metal pollution and its use as a toxicant-specific biomarker has been widely employed (Monserrat et al. 2007). In this research, the traditional method of determining metallothionein concentration is chosen over the more contemporary approach of assessing metallothionein induction by measuring mRNA expression. Schmitt et al. (2007) stated that the traditional method documents cumulative and long term exposure history while

M. S. Othman
Environmental Health & Industrial Safety Program, Faculty of Health Sciences, Universiti Kebangsaan Malaysia, Kuala Lumpur, Malaysia

M. S. Othman
International Postgraduate Programs in Environmental Management, Graduate School, Chulalongkorn University, Bangkok, Thailand

M. S. Othman · W. Khonsue · J. Kitana · K. Thirakhupt · M. Robson · N. Kitana
National Center of Excellence for Environmental and Hazardous Waste Management (NCE-EHWM), Chulalongkorn University, Bangkok, Thailand

M. S. Othman · W. Khonsue · J. Kitana · K. Thirakhupt · N. Kitana (✉)
Department of Biology, Faculty of Science, Chulalongkorn University, Bangkok, Thailand
e-mail: noppadon.k@chula.ac.th

M. Robson · M. Borjan
School of Environmental and Biological Sciences, Rutgers, The State University of New Jersey, New Brunswick, NJ, USA

M. Robson · M. Borjan
Thai Fogarty International Training and Research in Environmental and Occupational Health Center, Chulalongkorn University, Bangkok, Thailand

metallothionein induction would give an account of the active or recent metallothionein synthesis. Metallothionein mRNA might not be detected in resident organisms that have acclimated to contemporary exposure conditions. Exposure to Cd may also lead to various physiological responses such as the increased production of reactive oxygen species responsible for oxidative stress in the body (Valdivia et al. 2007). As a result, antioxidants such as superoxide dismutase, catalase and glutathione-S-transferase (GST) will be produced.

In the agricultural areas of Tak Province, Thailand, Simmons et al. (2005) reported elevated Cd levels in the paddy soils and rice grain of the Mae Tao Creek vicinity, which is located downstream of a zinc mining area. Our previous studies have also found that Cd concentrations from the contaminated site (Mae Tao, 16°45′13″N; 98°35′25″E) ranged from 0.0019 to 0.0021 mg/L in water samples, and 2.92 to 3.29 mg/kg in sediment samples (Othman et al. 2009). The concentration ranges at the reference site (Mae Pa, 16°40′43″N; 98°35′36″E) were 0.0018–0.0020 mg/L (water) and 0.101–0.221 g/kg (sediment). The purposes of this study were to correlate hepatic metallothionein and GST in *Fejervarya limnocharis* caught from reference and contaminated sites and to correlate hepatic GST and metallothionein with hepatic Cd in wild *Fejervarya limnocharis* exposed to environmental Cd.

Materials and Methods

A visual encounter survey (Crump and Scott 1994) was conducted and 97 frogs were collected on a monthly basis during November 2007 and October 2008 from several rice fields in Mae Tao and Mae Pa in Mae Sot District, Tak Province. The livers of the frogs were removed for analysis. For hepatic Cd concentration, the liver samples were subjected to a microwave digestion procedure with concentrated nitric acid followed by cadmium determination using graphite furnace atomic absorption spectrometer (AAS ZEEnit 700 by Analytik Jena, Thuringia, Germany). The standard curve range used was 0–10 µg/L and the detection limit of this instrument is 0.020 µg/L. Recovery of spiked liver ranged from 72 % to 93 %. Cd-hemoglobin affinity assay was used to determine metallothionein. The tissues were subjected to metallothionein preparation according to Eaton and Cherian (1991) with modification by Kuroshima (1995). The Cd in the supernatant was determined with graphite furnace atomic absorption spectrometer. Metallothionein concentrations were calculated by the assumption that 7 g-atoms of Cd are bound to one mole of metallothionein, and the molecular weight of Cd-metallothionein complex is 7,000. Total protein concentrations in liver tissue were determined by total protein assay (Bradford 1976). This was then used as a tissue dilution guideline for the determination of the activities of hepatic GST. Glutathione-S-transferase activities were determined by GST assay (Habig et al. 1974).

All data were statistically analyzed with Two-way ANOVA and Student-Newman Keuls tests by using the SigmaStat 2.0 program (Systat Software, California, USA). The Spearman-Rank Order correlation was also used to determine correlation between hepatic metallothionein concentration and hepatic GST activities with hepatic Cd concentration.

Results and Discussion

Hepatic metallothionein levels of *F. limnocharis* caught from Mae Pa range from 1.50 to 2.76 mg/kg wet weight (Fig. 1a). For those caught from Mae Tao, the range is 2.99–3.91 mg/kg wet weight. The differences in hepatic metallothionein between the stations are statistically significant ($p = 0.048$). The overall average hepatic metallothionein for

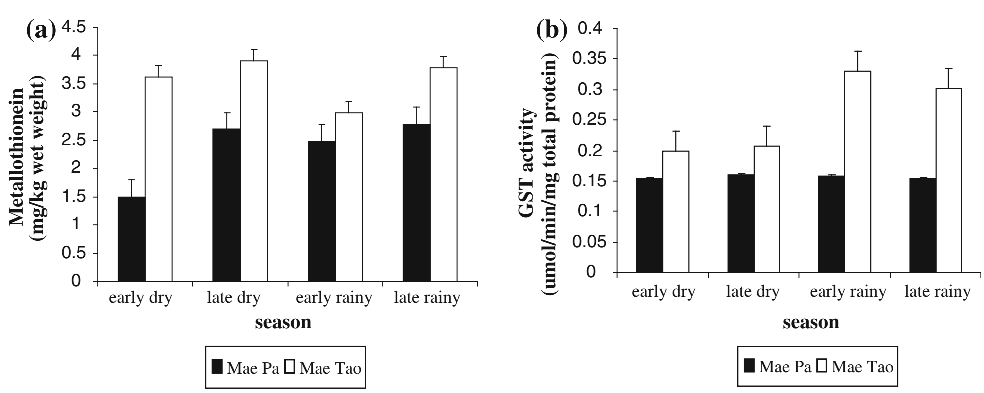

Fig. 1 Quarterly average **(a)** hepatic metallothionein and **(b)** glutathione-S-transferase activity in *Fejervarya limnocharis* caught from Mae Sot, Tak. All mean differences between stations are statistically significant ($p < 0.05$)

frogs caught from Mae Pa is 2.36 mg/kg wet weight, while the value is 3.58 mg/kg wet weight for frogs caught from Mae Tao.

Figure 1b shows the activities of GST in the livers of F. *limnocharis* caught from Mae Pa and Mae Sot. GST activities of the frogs caught from Mae Pa range from 0.154 to 0.159 μmol/min/mg total protein. For Mae Tao, the range extends from 0.199 to 0.331 μmol/min/mg total protein. The difference of GST activity between the sites is statistically significant ($p < 0.001$). The overall average GST activity for frogs caught from Mae Pa is 0.157 μmol/min/mg total protein, while for Mae Tao, the overall average value is 0.259 μmol/min/mg total protein.

A strong positive correlation was found between hepatic Cd and hepatic GST activity ($r = 0.802$, $p = 0.0096$). For the hepatic Cd-hepatic metallothionein pairing, there is a modest positive correlation ($r = 0.548$). However, this correlation is not statistically significant ($p = 0.139$). This may indicate that while increase in hepatic Cd is likely to result in an increase in hepatic GST activity, the same assumption cannot be made for the hepatic Cd-hepatic metallothionein interaction.

Hansen et al. (2006) reported that in *Salmo trutta*, metallothionein is an important mechanism in Cd acclimatization because Cd-acclimatized *Salmo trutta* had increased intercellular concentrations of metallothionein-like proteins. A number of other studies also have found that organisms exposed to Cd were induced to produce metallothionein. Other amphibians that have shown this response include include *Xenopus laevis* (Mouchet et al. 2006) and *Rana ridibunda* (Loumbourdis et al. 2007). Figure 1a of this study confirms the findings of the research mentioned above because *F. limnocharis* living in the Cd contaminated site (Mae Tao) had higher hepatic metallothionein concentration as opposed to those living in the reference site (Mae Pa). This indicated that frogs exposed to high Cd concentrations in the environment would react by producing metallothionein as a line of defense from the toxic effect of Cd. This is because induction of metallothionein would provide more binding sites for Cd, hence would limit latent damage (Linde-Arias et al. 2008). This would limit the possibility for cationic Cd to impact its toxicity against the organ in which it had accumulated.

Isani et al. (2008) reported that Cd that was absorbed from the environment would be transported to the liver and would induce the production of hepatic metallothionein. Therefore, it may be inferred that there is a positive correlation between the concentration of Cd in the liver and the amount of metallothionein produced by the liver. There was a strong and significant positive relationship between hepatic metallothionein and hepatic Cd concentration in *Anguilla anguilla* (Bird et al. 2008). However in this research, despite having a positive correlation between hepatic Cd and hepatic metallothionein, the correlation was not significant. This could be attributed to the insufficiency of metallothionein induction due to a spillover effect (Mouchet et. al 2006). Spillover occurs when the body's capacity to sequester metal is overwhelmed. As a result, the spilled-over metal would remain as free metals rather than sequestered metals. In this case, there would be an initial positive correlation between both parameters. However, after a certain threshold level, the liver would no longer be able to produce enough metallothionein to bind with the increasing amount of Cd. Therefore, in *F. limnocharis* caught from Mae Sot, the insignificant, but positive correlation between hepatic Cd and hepatic metallothionein may be explained by an overwhelmed defense capacity that leads to spillover effect. Due to the overwhelmed accumulation of Cd, further increase in Cd uptake may not result in the increase of Cd being sequestered to metallothionein to form the Cd-thiolate clusters in the metallothionein-Cd complex. Instead, this would increase the concentration of free Cd in the liver.

Figure 1b shows that *F. limnocharis* caught from the contaminated site (Mae Tao) had higher GST activity than those caught from the reference site (Mae Pa). Also, there was a strong correlation between hepatic Cd and the hepatic GST activity. This is in line with results from studies on *Rana ridibunda* (Loumbourdis et al. 2007), *Salmo trutta* (Hansen et al. 2006), *Xenopus laevis* (Mouchet et al. 2006), *Sparus aurata* (Isani et al. 2008), *Sander vitreus* and *Perca flavescens* (Larose et al. 2008). The higher activity of GST in *F. limnocharis* caught from Mae Tao and the strong correlation between hepatic Cd and hepatic GST was anticipated because Cd is known to induce oxidative stress by the formation of reactive oxygen species (Isani et al. 2008). This leads to an increase in glutathione concentration (Kuroshima 1995) and the production biotransformation enzyme GST. According to Larose et al. (2008), research has shown that high activity of GST can be induced by exposure to Cd. Glutathione-S-transferase is one of the key enzymatic players in the defense mechanism against reactive oxygen species and oxidative stress (Hermes-Lima and Zenteno-Savin, 2002).

This research found that rice frogs from a site contaminated with Cd had elevated levels of Cd in the liver as well as significantly higher concentrations of hepatic metallothionein and GST, when compared to rice frogs from a reference site. However, only GST showed a significant strong positive correlation with hepatic Cd concentration. Therefore it would appear that the biomarker of hepatic GST activity would be more useful than the biomarker of hepatic metallothionein concentration in cases where sentinel species are chronically exposed to Cd.

Acknowledgments This study was supported by the National Center of Excellence for Environmental and Hazardous Waste Management

(NCE-EHWM), the 90th Anniversary of Chulalongkorn University Fund, a new staff development grant (Ratchadaphiseksomphot Endowment Fund), and the MUA-TRF research grant (MRG4980120) to NK. An educational grant from the Malaysian Ministry of Higher Education to MSO is fully acknowledged. Additional support was also obtained from NIH Fogarty ITREOH NIH FIC D43TW007849, NIEHS P30ES005022 and NIEHS P32ES007148.

References

Baykan U, Atli G, Canli M (2007) The effects of temperature and metal exposures on the profiles of metallothionein-like proteins in *Oreochromis niloticus*. Environ Toxicol Pharmacol 23:33–38

Bird DJ, Rotchell JM, Hesp SA, Newton LC, Hall NG, Potter IC (2008) To what extent are hepatic concentrations of heavy metals in *Anguilla anguilla* at a site in a contaminated estuary related to body size and age and reflected in the metallothionein concentrations? Environ Pollut 151:641–651

Bradford MM (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254

Crump ML, Scott NJ (1994) Visual encounter surveys. In: Heyer WR, Donnely MA, McDiarmid RW, Hayek LC, Foster M (eds) Measuring and monitoring biological diversity: standard methods for amphibians. Smithsonian Institution Press, Washington DC, pp 84–92

Eaton DL, Cherian MG (1991) Determination of metallothionein in tissues by Cd-hemoglobin affinity assay. Methods Enzymol 205: 83–88

Habig WH, Pabst MJ, Jacoby WB (1974) Glutathione-S-Transferase, the first enzymatic step in mercapturic acid formation. J Biol Chem 249:7130–7139

Hansen BH, Rømma S, Garmo ØA, Olsvik PA, Andersen RA (2006) Antioxidative stress proteins and their gene expression in brown trout (*Salmo trutta)* from three rivers with different heavy metal levels. Comp Biochem Physiol Part C 143:263–274

Hermes-Lima M, Zenteno-Savin T (2002) Animal response to drastic changes in oxygen availability and physiological oxidative stress. Comp Biochem Physiol Part C 133:537–556

Isani G, Andreani G, Cocchioni F, Carpené E, Falcioni G (2008) Cd accumulation and biochemical responses in *Sparus aurata* following sub-lethal Cd exposure. Ecotoxicol Environ Saf 72:224–230

Kuroshima R (1995) Hepatic metallothionein and glutathione levels in Red Sea bream. Comp Biochem Physiol Part C 110:95–100

Larose C, Canuel R, Lucotte M, Di Giulio RT (2008) Toxicological effects of methylmercury on walleye (*Sander vitreus)* and perch (*Perca flavescens*) from lakes of the boreal forest. Comp Biochem Physiol Part C 147:139–149

Linde-Arias AR, Inácio AF, de Alburguergue C, Freire MM, Moreira JC (2008) Biomarkers in an invasive fish species *Oreochromis niloticus* to assess the effects of pollution in a highly degraded Brazillian river. Sci Total Environ 339:186–192

Loumbourdis NS, Kostaropoulos I, Theodoropoulou B, Kalmanti D (2007) Heavy metal accumulation and metallothionein concentration in the frog *Rana ridibunda* after exposure to chromium or a mixture of chromium and Cd. Environ Pollut 145:787–792

Monserrat JM, Martínez PE, Geracitano LA, Amado LL, Martins CMG, Pinho GLL, Chaves IS, Ferreira-Cravo M, Ventura-Lima J, Bianchini A (2007) Pollution biomarkers in estuarine animals: critical review and new perspectives. Comp Biochem Physiol Part C 146:221–234

Mouchet F, Baudrimont M, Gonzalez P, Cuenot Y, Bourdineaud JP, Boudou A, Gauthier L (2006) Genotoxic and stress inductive potential of Cd in *Xenopus laevis* larvae. Aquat Toxicol 78:157–166

Othman MS, Khonsue W, Kitana J, Thirakhupt K, Robson MG, Kitana N (2009) Cd accumulation in two populations of rice frogs (*Fejervarya limnocharis)* naturally exposed to different environmental Cd levels. Bull Environ Contam Toxicol 83: 703–707

Schmitt CJ, Whyte JJ, Roberts AP, Annis LM, May TM, Tillitt DE (2007) Biomarkers of metal exposure in fish from lead-zinc mining areas of Southeastern Missouri, USA. Ecotoxicol Environ Saf 67:31–47

Simmons RW, Pongsakul P, Saiyasitpanich D, Klinphoklap S (2005) Elevated levels of Cd and zinc in paddy soils and elevated levels of Cd in rice grain downstream of a zinc mineralized area in Thailand: implications for public health. Environ Geochem Health 27:501–511

Valdivia PA, Zenteno-Savin T, Gardner SE, Aguirre AA (2007) Basic oxidative stress metabolites in eastern Pacific green turtles (*Chelonia mydas agassizii*). Comp Biochem Physiol Part C 146:111–117