Ecotoxicology and Environmental Safety 103 (2014) 24–28

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

journal homepage: www.elsevier.com/locate/ecoenv

Behaviour of the oxidant scavenger metallothionein in hypoxia-induced neotropical fish

Rachel Ann Hauser-Davis [a,b,*], Frederico Freire Bastos [c], Rafael Ferreira Dantas [c,e], Santiago Alonso Leitão Tobar [c], Jayme da Cunha Bastos Neto [c], Vera Lucia Freire da Cunha Bastos [b], Roberta Lourenço Ziolli [d], Marco Aurélio Zezzi Arruda [a,b]

[a] Instituto de Química/UNICAMP, Grupo de Espectrometria, Preparo de amostras e Mecanização-GEPAM, C. Postal 6154, 13084-971 Campinas, SP, Brazil
[b] National Institute of Science and Technology for Bioanalitics, Instituto de Química/UNICAMP, Grupo de Espectrometria, Preparo de amostras e Mecanização-GEPAM, C. Postal 6154, 13084-971 Campinas, SP, Brazil
[c] UERJ, Instituto de Biologia Roberto Alcântara Gomes, Departamento de Bioquímica, Av. Manoel de Abreu 444, Maracanã, CEP 20550-900, Rio de Janeiro, RJ, Brazil
[d] Programa de Pós-Graduação em Biodiversidade Neotropical, Universidade Federal do Estado do Rio de Janeiro—UNIRIO, Av. Pasteur, 458 Urca, CEP 22290-240, Rio de Janeiro RJ, Brazil
[e] Fundação Oswaldo Cruz, Instituto Oswaldo Cruz, Laboratório de Bioquímica de Proteínas e Peptídeos, 21040-360 Rio de Janeiro, RJ, Brazil

ARTICLE INFO

Article history:
Received 22 June 2013
Received in revised form
9 January 2014
Accepted 10 January 2014
Available online 19 February 2014

Keywords:
Metallothionein
GSH
TBARS
Fish bile and liver
Hypoxia

ABSTRACT

The pacu (*Piaractus mesopotamicus*) is a hypoxia-tolerant neotropical fish species. There is little or no information in this species regarding biochemical adaptations to waters with different oxygen concentrations, such as the production of reactive oxygen species and antioxidant scavengers, which might be of interest in the study of antioxidant defense mechanisms. Metallothioneins (MT) have been widely applied as biomarkers for metal exposure in fish liver, and, recently, in bile. These metalloproteins, however, have also been reported as free radical scavengers, although studies in this regard are scarce in fish. In this context, normoxic and hypoxic controlled experiments were conducted with pacu specimens and MT levels were quantified in both liver and bile. Reduced glutathione (GSH) indicative of oxidative stress, and thiobarbituric acid reactive substances (TBARS), indicative of lipid peroxidation, were also determined in liver. The results demonstrate that hypoxic fish present significantly lower metallothionein levels in liver and bile and lower reduced glutathione levels in liver, whereas lipid peroxidation was not significantly different between hypoxic and normoxic fish. The results of the present study seem to suggest that metallothioneins may actively participate in redox regulation in hypoxic fish in both bile and liver. MT levels in these organs may be temporarily suppressed, supporting the notion that down-regulation of oxidant scavengers during the oxidative burst is important in defense signaling in these adapted organisms.

1. Introduction

The amount of dissolved oxygen in water is very important for aquatic organisms. This parameter affects the growth, survival, distribution, behavior and physiology of several aquatic organisms (Solis, 1988). For instance, only fish that tolerate low oxygen can survive in swamps and forest streams (Deekae et al., 2010).

Hypoxia is known to increase oxidative radical production when compared to normoxic conditions (Chandel and Budinger, 2007). This has been reported in some fish species, such as hypoxia-exposed Chinese sleeper (*Percottus glenii*) (Lushchak and Bagnyukova, 2007) and the hypoxia-tolerant medaka fish (*Oryzias latipes*) (Oehlers et al., 2007). The fact that hypoxia-induced oxidative stress occurs even in hypoxia-tolerant fish species is of interest, and the topic remains one of intensive discourse (Leveelahti, 2014).

The South American Pantanal area suffers cyclic inundation pulses, with annual floods and dry seasons. The seasonal variance in oxygen availability among the area's water bodies can result in periods of profound hypoxia (<2 mg L^{-1} O_2) lasting for up to several months at a time (Almeida-Val et al., 2000). The varying states of water levels throughout this area have led to several behavioral, physiological, biochemical and molecular adaptations in many Pantanal fish species that enable them to cope with hypoxia (Hamilton et al., 1995). The molecular mechanisms of hypoxia tolerance in fish in general, however, remain largely

* Corresponding author.
E-mail address: rachel.hauser.davis@gmail.com (R.A. Hauser-Davis).

0147-6513/$ - see front matter © 2014 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.ecoenv.2014.01.015

unknown, and there is little or no information regarding biochemical adaptations, such as the production of reactive oxygen species and antioxidant scavengers, to waters with different oxygen concentrations (Bastos et al., 2007). The pacu (*Piaractus mesopotamicus*) is a neotropical fish species of the South American Pantanal area which can survive under oxygen concentrations of less than 1 mg L^{-1} (Bastos et al., 2007), making it an ideal model species for studies on hypoxia effects in these organisms.

Several metalloproteins have been observed in fish (Santos et al., 2011), and some of them have recently been applied as biomarkers of exposure for environmental contamination, such as metallothioneins (MT) (Hauser-Davis et al., 2012). Metallothioneins have been widely applied in fish to indicate environmental metal exposure in several organs, such as muscle and liver, and, more recently, in bile (Hauser-Davis et al., 2012). In addition, these metalloproteins are also involved in the regulatory control of zinc metabolism, protecting neurons against toxic metals and protection and regeneration following neurological injury (Dittmann et al., 2005; Masters et al., 1994; Miyazaki et al., 2002). They have also been known to exhibit free radical scavenging activity, where the abundant cysteine residues present in these metalloproteins are actively involved in the capture of harmful oxidant radicals, such as superoxide and hydroxyl radicals (Kumari et al., 1998). In this reaction, the cysteine residues are oxidized and become cystine, and the metal ions eventually bound to the cysteine are released and radical uptake takes place (Kumari et al., 1998). Thus, it is now accepted that MT are also involved in intracellular protection against reactive oxygen free radical species which can cause lipid peroxidation, protein oxidation and DNA damage (Dittmann et al., 2005). This fact, however, has been mostly reported for mammals with regard to MT in the central nervous system. However, studies in fish in this regard are still scarce. Some reports indicate that MT is important as an overall antioxidant and is induced in response to factors promoting oxidative stress in these organisms (Kling and Olsson, 2005). For example, MT have been reported as reducing peroxidative damage to liver (Ahmad et al., 2000), and it has been suggested that both elevated GSH and MT afford H_2O_2 protection (Wright et al., 2000). Thus, this study aimed to (i) conduct a first-time investigation of whether hypoxic conditions would modify metallothionein levels in pacu in liver, (ii) verify if this protein is also present in pacu bile and (iii) investigate if changes in MT levels followed the same trend in liver and bile. Thiobarbituric acid reactive substances (TBARS) formed as the result of lipid peroxidation and total reduced glutathione (GSH), an antioxidant tripeptide, were also determined in liver, to verify oxidative stress and lipid peroxidation in a validated organ regarding these measurements, for comparisons with bile.

2. Material and methods

2.1. Fish specimens and laboratory setup

Pacu specimens (*P. mesopotamicus* Holmberg, 1887; Characiformes, Characidae) weighing between 400 and 500 g (approximately 30 cm total length) were purchased from the Sol Nascente fish farm located in the municipality of Laje de Muriaé, northern Rio de Janeiro, Brazil (lat: 21°12′24″ South, long: 42°7′57″ West). The animals were acclimated for 8 h at 25 °C (23 ± 2° C) before the trials. After acclimation, the fish were then maintained in 1000 L tanks at 6.5 mg O_2 L^{-1} (normoxic conditions, PO_2 = 28.6 ± 5.0 mg O_2 L^{-1} (means ± standard deviation), n = 10) and 0.5 mg O_2 L^{-1} (hypoxic conditions, PO_2 = 2.0 ± 0.9 mg O_2 L^{-1} (means ± standard deviation), n = 10) for 24 h with no food. These normoxic and hypoxic values were chosen because 6.5 mg O_2 L^{-1} are the mean values observed in Pantanal lagoons and 0.5 mg O_2 L^{-1} is the known concentration in which fish go into hypoxia, verified by the development of lower lip swellings. Also, prior experiments in our lab show that this species can support up to 42 h in extremely low oxygen levels (0.1 mg O_2 L^{-1}), while the Pcrit at 0.5 mg O_2 L^{-1} (hypoxia) for this species is of 2.0 ± 0.9 mg O_2 L^{-1} (Bastos et al., 2013, 2007; Bleich et al., 2009; Hamilton et al., 1995). Hypoxia was maintained constant by bubbling nitrogen gas in the water. Oxygen levels were measured with an oximeter (model HI 91410, Hanna instruments). Aerial surface respiration was avoided by covering all tanks flush at the water level with tight mesh nylon. Temperature was maintained constant at 25 °C (23 ± 2° C). At the end of the experiment, the fish were sacrificed in accordance with procedures recommended by COBEA 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 analyses.

2.2. Sample processing for MT extraction

MT extraction was carried out by thermal extraction in both bile and liver, according to the protocol proposed by Erk et al. (2002). Briefly, bile (n = 10 for the normoxic group, and n = 8 for the hypoxic group) and liver (n = 8 for the normoxic group, and n = 7 for the hypoxic group) samples were thawed and homogenized at a 3:1 ratio in sterile polypropylene tubes in a solution composed of Tris–HCl 20 mM pH 8.6, PMSF (phenylmethylsulphonylfluoride) 0.5 mM as an antiproteolytic agent and β-mercaptoethanol 0.01 percent (w/v) as a reducing agent. Samples were then centrifuged at 20,000 × g for 1 h at 4 °C in a Mikro 220R Hettich centrifuge (Germany). The supernatants were carefully separated from the pellet, transferred to new sterile polypropylene tubes and heated at 70 °C for 10 min. A second centrifugation was performed at 20,000 × g for 30 min at 4 °C and the final supernatants were then separated from the pellet and frozen at −80 °C until use. These purified samples were used in all further MT analyses.

2.3. MT quantification by spectrophotometry (Ellman's reaction)

The supernatant containing the MT was incubated for 30 min in the dark with 1 M HCl in 4 mM EDTA and 2 M NaCl containing 0.43 mM DTNB (5,5-dithiobis-2-nitrobenzoic acid) buffered with 0.2 M Na-phosphate, pH 8.0 (Duquesne and Richard, 1995; Ellman, 1959). The samples were then centrifuged at 3000 × g for 5 min and the supernatant absorbance was evaluated at 412 nm. Metallothionein concentrations were estimated using reduced glutathione (GSH) as standard. MT content was calculated by assuming the relationship of 1 mol MT = 20 mol GSH, as described by Kagi (1991) for fish.

2.4. TBARS assay

TBARS content in liver was determined according to Draper and Hadley (1990) (n = 10 for the normoxic group, and n = 6 for the hypoxic group). Approximately 300 mg of liver were homogenized in 5 mL of 5 percent (w/v) TCA and 0.5 mL of 0.5 g L^{-1} BHT using a Potter–Elvehjem glass/Teflon homogenizer (Potter, 1955). The homogenates were kept in boiling water for 30 min, cooled at room temperature and centrifuged at 1000 × g for 10 min. Supernatants were collected and mixed with 0.35 percent (w/v) TBA (1:1 v/v) and then kept in boiling water for 30 min. The mixture was cooled at room temperature and read in a Shimadzu UV-160 spectrophotometer at 532 nm. TBARS content in the sample was calculated using a standard curve of tetramethoxypropene and expressed as nmol g^{-1} wet tissue.

2.5. Total GSH assay

Total glutathione was determined according to Anderson (1985) in liver only (n = 10 for the normoxic group, and n = 6 for the hypoxic group). Livers were homogenized at 5 percent (w/v) 5-sulfosalicilic acid (1:5 w/v) using a Potter–Elvehjem glass/Teflon homogenizer (Potter, 1955). Homogenates were centrifuged at 12,000 × g for 5 min. The resulting supernatant containing glutathione was stored at −20 °C until use. An aliquot of supernatant was added to a reaction medium containing 0.8 mM EDTA, 0.6 mM DTNB, 0.175 mg mL^{-1} NADPH, 1.25 U mL^{-1} glutathione reductase in sodium phosphate buffer 0.1 M pH 7.5. The rate of 2-nitro-5-thiobenzoic acid (TNB) formation was continuously monitored in a Shimadzu UV-160 spectrophotometer at 412 nm. Total glutathione concentration was calculated using a standard curve of reduced glutathione and expressed as μmol g^{-1} wet tissue.

2.6. Statistical analyses

Data normality was tested using the Shapiro–Wilkes W test prior to additional statistical analyses. As the analytical data showed a normal distribution, parametric tests were used. The Student's t-test was used to verify statistical differences between MT expression of the controlled and exposed group, for both bile and liver and for GSH and TBARS in both groups for liver. A p value of less than 0.05 was considered as indication of statistical significance.