Pesticide Biochemistry and Physiology 108 (2014) 8–15

Contents lists available at ScienceDirect

Pesticide Biochemistry and Physiology

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

Antioxidant, phase II and III responses induced by lipoic acid in the fish *Jenynsia multidentata* (Anablapidae) and its influence on endolsulfan accumulation and toxicity

J.M. Monserrat [a,b,*], M.L. Garcia [a,b], J. Ventura-Lima [a,b], M. González [c], M.L. Ballesteros [c], K.S.B. Miglioranza [c], M.V. Amé [d], D.A. Wunderlin [d]

[a] Curso de Pós-graduação em Ciências Fisiológicas – Fisiologia Animal Comparada, Universidade Federal do Rio Grande – FURG, Cx. P. 474, CEP 96.201-900, Rio Grande, RS, Brazil
[b] Instituto de Ciências Biológicas (ICB), FURG, Brazil
[c] Laboratório de Ecotoxicología, Universidad Nacional de Mar del Plata, Instituto de Investigaciones Marinas y Costeras (IIMyC)-CONICET, Buenos Aires, Argentina
[d] Universidad Nacional de Córdoba – CONICET, Facultad Ciencias Químicas, Córdoba, Argentina

ARTICLE INFO

Article history:
Received 30 April 2013
Accepted 22 October 2013
Available online 1 November 2013

Keywords:
Lipoic acid
Endosulfan
Pgp expression
Antioxidant defenses
Oxidative damage
Fish

ABSTRACT

Antioxidants like lipoic acid (LA) are known to trigger augmented antioxidant and phase II and III responses. This study aimed to evaluate the effect of LA in P-glycoprotein (Pgp) expression, glutathione-S-transferase (GST) activity, total antioxidant competence, levels of lipid peroxides (TBARS) and accumulation of the organochlorine insecticide endosulfan (Endo: α-, β-isomers and sulfate metabolite) in different organs of the fish *Jenynsia multidentata*. One hundred and twenty females (1.55 ± 0.07 g) were fed during 8 days with (n = 60) or without (n = 60) a LA enriched ration (6000 mg/kg). Four experimental groups were defined: −LA/−Endo; +LA/−Endo; −LA/+Endo; and +LA/+Endo. Endo groups were exposed during 24 h to 1.4 μg of insecticide/L. Results showed that only LA induced a significant increment in liver Pgp expression. GST activity was augmented in liver after exposure to LA or Endo. TBARS levels were lowered in liver and gills after LA pre-treatment. Total antioxidant capacity was lowered in liver of Endo exposed fish, a result that was reversed by LA pre-treatment. It is concluded that LA induced the expected effects in terms of Pgp expression, GST activity and reduced TBARS levels although favored α-Endo accumulation in brain. However, the Endo metabolism to the more persistent endosulfan sulfate was not facilitated by LA pre-treatment.

1. Introduction

Lipoic acid (LA) is a well-known cofactor of mitochondrial dehydrogenases with excellent antioxidant properties [1–3]. In fish, many authors showed biochemical effects of LA in terms of circulating aminoacids levels, lipid metabolism and ascorbic acid content [4–6]. *Corydoras paleatus* fed with a LA enriched diet, showed lower levels of carbonylated proteins in liver and muscle, demonstrating the antioxidant effect of this molecule [7]. Other aquatic organisms like the gastropod *Haliotis discus hannai* showed augmented levels of glutathione (GSH) in the hepatopancreas when fed with LA added to diet (up to 3200 mg/kg) [8].

Suh et al. [3] showed that LA induces the antioxidant system through the control of the expression of key genes as, for example, those that code for both subunits of glutamate–cysteine ligase (GCL), the rate-limiting enzyme for the synthesis of glutathione (GSH). Antioxidant genes and other involved in phase II and III reactions present motifs known as antioxidant response elements (ARE) at the promoters or upstream of them. Transcription factors like Nrf2 together with partners like Jun or Fos can bind to ARE regions, regulating the constitutive and inducible expression of genes like GCL subunits, glutathione-S-transferase (GST) and multidrug resistance proteins [3,9]. The influence of LA on the antioxidant and detoxification systems has prompted its use as a chemoprotectant. In fish, Amado et al. [10] reported that carp *Cyprinus carpio* injected i.p. with LA (40 mg/kg) augmented the expression of several GST forms. Moreover, LA treatment showed to be effective to counteract liver GST inhibition elicited by a cyanotoxin, microcystin. As several molecules induce toxicity by direct or indirect generation of oxidative stress [11], the pre-treatment with LA should protect or ameliorate these toxic effects.

As mentioned above, Nrf2-reponsive genes include multidrug resistance-associated proteins (Mrp) and multidrug resistance proteins (Mdr) [9]. Amé et al. [12] have characterized the P-glycoprotein multidrug transporter protein (Pgp) in the fish

* Corresponding author at: Instituto de Ciências Biológicas (ICB), Universidade Federal do Rio Grande – FURG, Cx. P. 474, CEP 96200-970, Rio Grande, RS, Brazil.
E-mail address: josemmonserrat@pesquisador.cnpq.br (J.M. Monserrat).

0048-3575/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.pestbp.2013.10.009

Jenynsia multidentata (Anablapidae), a protein known to be involved in the protection against toxins like microcystin. Sreeramulu et el. [13] verified that Pgp activity in reconstituted proteoliposomes was inhibited by several pesticides, including the organochlorine endosulfan, meaning that pesticides can affect Pgp functionality, in turn affecting the clearing of xenobiotics from the cells, an effect that should augment the toxicity of these molecules.

Endosulfan is a widespread insecticide that has been used in many countries in different crops such as cotton, fruits, tomato and cereals. Due to its massive use and volatility it occurs on several environmental compartments [14]. Moreover, endosulfan represents one of the main pesticide used in Argentina, being their residues (i.e., α- and β-isomers and endosulfan sulfate) found in ground and stream water at concentration that in some cases are above international limits for the protection of aquatic organisms (3 ng/L) [14]. Toxic effects induced by this chemical on fish at biochemical (enzyme induction) and hematological levels [15] as well as on reproduction and behavior, are well known. Particularly, previous results, using the model fish, *J. multidentata*, showed that sub-lethal concentrations of technical endosulfan (1.4 μg/L) augmented lipid peroxidation in liver and brain [16].

Thus, considering the modulation of LA in the antioxidant and detoxifying systems, the present study aimed to evaluate its potential effect as chemoprotectant agent against endosulfan toxicity in *J. multidentata* by measuring several parameters associated with antioxidant defense system and oxidative damage in conjunction with changes in the expression of Pgp protein induced by LA, as well as the effect on endosulfan accumulation pattern.

2. Materials and methods

2.1. Fish sampling

Female adult fish (1.55 ± 0.07 g; n = 120) were sampled using a backpack electrofisher equipment from a non-polluted area at San Antonio River (64°13′20″W, 31°12′80″S-Córdoba, Argentina), as in previous studies [12,16,17]. Fish were transported to the laboratory in water tanks (20 L) within 4 h after capture. Once in laboratory, fish were maintained during 8 days at 21 ± 1 °C with photoperiod fixed in 12 L:12 D in 40 L tanks with 100% oxygen saturated water and pH of 7.85 ± 0.02.

2.2. Preparation of lipoic acid-enriched ration

The one hundred and twenty fish were randomly divided in two groups: fed with (+LA) or without (−LA) lipoic acid enriched diet during eight days. Racemic lipoic acid (ACROS Organics, purity >98%) was added to a commercial fish diet (TetraColor, 47.5% of protein), mixed with ultrapure water (Millipore, Milli Q system) and dried at 50 °C to obtain pellets. Fish were fed twice a day (morning: 8:30 h; afternoon: 15:30 h), in order to get a daily ingestion of 2% of body weight.

In order to measure the effective dose of LA in the ration, 0.25 g were dissolved in 2 ml of methanol (HPLC grade) and extracted overnight at room temperature. LA was quantified by HPLC–ESI-MS using a Varian 1200 triple quadrupole, equipped with an ESI ion source operated in negative mode with nitrogen as main gas (74 psi), drying gas (21 psi, 250 °C) and synthetic air (5.0 grade, 99.9999%) as nebulizing gas (58 psi); needle 5000 V, shield at 600 V, capillary −50 V. The detector was set at 1900 V, with a requested scan of 0.5 s. LA was separated using a column Varian Polaris 5 μm C18-A (50 × 2.0 mm). Solvent delivery was performed at 0.25 ml/min by two pumps Varian Prostar 210 Dynamax using ammonium acetate (4 mM) (A) and methanol (B), starting with 50% B, changing to 90% B within 8 min, held by 2 min, returning to 50% B in one minute and keeping this condition for four additional minutes to achieve column stabilization before next run (total run time 15 min). Quantification was performed using external standard method, using pure LA dissolved in the starting mobile phase (50%A: 50%B). Both samples and standard solutions were analyzed by triplicate.

The −LA group was fed only with the commercial ration, which was prepared with the same procedure cited above but omitting the adding of LA. Visual examination confirmed that fishes accepted very well both kinds of rations, feeding even when the pellets sank to the bottom of the aquariums.

2.3. Exposure to technical endosulfan (Endo)

After eight days of feeding fish with (+LA) or without (−LA) enriched ration, fish were split into four groups: −LA/−Endo; +LA/−Endo; −LA/+Endo; and +LA/+Endo. +Endo groups were exposed to a commercial formulation of endosulfan (6,7,8,9,10,10-hexachloro-1,5,5a,6,9,9a-hexahydro-6,9-methano-2,4,3-:benzodioxathiepine-3-oxide), Galgofan 35 EC®, with a α-/β-ratio equal to 70:30. A concentration of 1.4 μg/L during 24 h exposure was selected on the basis of Ballesteros et al. [16], that demonstrated antioxidant and oxidative damage responses. In a posterior study, Ballesteros et al. [18] determined that the measured total endosulfan concentration in water represented almost 23–45% of the nominal assayed concentration (1.4 μg/L) after 24 h exposure. Fish exposure of +Endo group was performed by immersion and the organisms were starved during exposure.

2.4. Biochemical analysis

After Endo exposure, fish were sacrificed and organs (brain, gills and liver) dissected and homogenized (1:5 for brain, 1:10 for the other organs) in Tris–HCl (100 mM, pH 7.75) buffer plus EDTA (2 mM) and Mg^{2+} (5 mM) [7]. Samples were centrifuged at 10,000×g during 20 min at 4 °C and the supernatant used for antioxidant and oxidative damage measurements. Previously, total protein content was determined by the Biuret method (550 nm), in triplicate, using a microplate reader (BioTek LX 800).

The activity of the enzyme glutathione *S*-transferase (GST) was determined following the conjugation of 1 mM glutathione and 1 mM 1-chloro-2,4-dinitrobenzene (CDNB) at 340 nm [19,20]. Total antioxidant competence against peroxyl radicals was determined through reactive oxygen species (ROS) determination in sample tissues treated or not with a peroxyl radical generator. Peroxyl radicals were produced by thermal (35 °C) decomposition of 2,2′-azobis(2-methylpropionamidine)dihydrochloride (ABAP; 4 mM; Aldrich) [21]. ROS concentration was measured along 30 min with the fluorogenic compound 2′,7′-dichlorofluorescin diacetate (H_2DCF-DA) at a final concentration of 40 μM, in according to the methodology reported by Amado et al. [22]. These authors integrated the fluorescence units (FU) along the time of the measurement, after adjusting FU data to a second order polynomial function. The results were expressed as area difference of FU min^{-1} in the same sample, with and without ABAP addition, and standardized to the ROS area without ABAP as follows:

$$(\text{ROS area}_{\text{with ABAP}} - \text{ROS area}_{\text{without ABAP}})/\text{ROS area}_{\text{without ABAP}}$$

However, data analysis using this methodology is cumbersome and time-consuming. A new approach was defined to calculate the relative area, using only FU data for 30 min measurement and the following expression:

$$(\text{FU 30min}_{\text{with ABAP}} - \text{FU 30min}_{\text{without ABAP}})/\text{FU 30min}_{\text{without ABAP}}$$