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Effects of ß-Naphthoflavone on the Levels of Glutathione S-Transferase from Liver of Pacu, *Piaractus mesopotamicus*

L. Camacho,[1] M. V. Alves,[1] J. Cunha Bastos,[1] I. Felzenszwalb,[2] V. L. F. Cunha Bastos[1]

[1] Department of Biochemistry, Biology Institute, Rio de Janeiro State University, Av. Prof. Manuel de Abreu, 48, Rio de Janeiro, RJ, 20550-170, Brazil
[2] Department of Biophysics and Biometry, Biology institute, Rio de Janeiro State University, Av. Prof. Manuel de Abreu, 48, Rio de Janeiro, RJ, 20550-170, Brazil

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Glutathione transferases, E.C. 2.5.1.18, (GST) are detoxication enzymes expressed in cells as a family of isoforms that conjugate electrophilic compounds with reduced glutathione, rendering more water-soluble elimination products. GST can also perform the important task of protecting cells from oxidative damage, and act as hormonal binding proteins (Hayes and Pulford 1995). The expression of multiple isoforms of GST differs from one tissue to another and evidence has been accumulated indicating that the expression of GST isoenzymes responds to different drugs such as organochlorine insecticides (Martínez-Lara et al. 1996; Syvanen et al. 1996) and polycyclic aromatic hydrocarbons, like β-naphthoflavone (Andersson et al. 1985). Four classes of GST can be characterized in biological material by assaying their activity using different molecules as specific substrates. These classes have been referred to as a (alpha), μ (mu), π (pi) and θ (theta) glutathione transferases (Kunze 1997). The normal substrate used for assaying GST in various biological species is 1 -chloro-2,4-dinitrobenzene (CDNB), which is recognized as a general substrate for all GST isoforms. Class alpha glutathione S-transferase shows higher activity to cumene hydroperoxide (CHP) (Carmagnol et al. 1983). Class mu has high activity upon epoxides, and 1,2-dichloro-4-nitrobenzene (DCNB) has been employed as the substrate to determine its activity (Mannervik and Danielson 1988). Class pi shows higher affinity to the herbicide atrazine (Egaas et al. 1995a, 1995b) and the substrate used to measure its activity is ethacrynic acid (ETHA), which is structurally related to atrazine. Class theta is thought to be an ancestral form of GST that gave origin to the others and no specific substrate has yet been described for it (Pemble and Taylor 1992).

There is no published biochemical information concerning GST isoenzymes in the fish "pacu" *(Piaractus mesopotamicus)* despite its economic importance for aquaculture in Brazil, its abundance in the Pantanal central-western rivers of the Paraguai river basin, and the steadily increasing risk of contamination of these rivers by pesticides used to protect crops. In this work we describe the presence of GST activities with four different substrates in liver cytosolic fraction from fish inoculated with β-naphthotlavone as an attempt to learn which hepatic pacu GST classes might be particularly sensitive to induction by substances of the polycyclic aromatic hydrocarbon (PAH) group.

Correspondence to: J. Cunha Bastos

MATERIALS AND METHODS

Chemicals used for substrate specificity were 1-chloro-2,4-dinitrobenzene (CDNB), ethacrynic acid (ETHA), cumene hydroperoxide (CHP), β–naphthoflavone (β-NF), purchased from Sigma Chemicals, and 1,2-dichloro-4-nitrobenzene (DCNB), purchased from Fluka.

Fish used in this study were pacu *(Piaractus mesopotamicus,* Holmberg 1887) weighing around 200-240 g and measuring approximately 20-25 cm. Pacus were kindly donated by the National Center for Research on Tropical Fish (CEPTA) of the Brazilian Environment and Renewable Natural Resources Institute (IBAMA). They were maintained under standard laboratory conditions in tanks equipped with biologic filter and containing 500 L of aerated dechlorinated water. Fish were acclimated for at least 10 days before being used.

The animals were divided into three groups of ten fish each, named group 1, group 2 and group 3. In each group, five pacus received 0.5 mL of the vehicle (corn oil) and were used as control. The other five fish of each group received a single dose of 0.5 mL of a β-NF solution, corresponding to 50 mg of B-NF per kg of body weight, injected intraperitoneally. Five fish were kept per tank. Fish of group 1 were sacrificed at 24h, fish of group 2 at 48h and fish of group 3 at 96 h after injection of β-NF or corn oil. The liver of each fish was removed immediately after the sacrifice.

Each liver was washed in ice-cold saline and homogenized in 0.05 M Tris-HCl buffer, pH 7.4, containing 1.15 % KCl. The homogenate was centrifuged at 9,000 x g for 30 minutes at 4 °C and the resulting supernatant was centrifuged further at 105,000 x g for 60 minutes at 4 °C. This 105,000 x g supernatant was recentrifuged at 131,000 x g for 60 min. Then, the new supernatant - the cytosolic subfraction - was collected for assaying GST activities upon specific substrates, as described below.

Protein concentration was determined according to Peterson (1977) using bovine serum albumin as standard.

GST activities were determined in the cytosolic subfraction of each liver using a Shimadzu UV-160A spectrophotometer in a kinetic reaction with four different substrates: CDNB, ETHA, DCNB (Habig et al. 1974) and CHP (Carmagnol et al. 1983). The general ordinary activity of GST was determined with 15 µg of protein, 2.0 mM CDNB in ethanol 4%, 1.8 mM GSH in 0.1 M of phosphate buffer, pH 6.0, and the conjugates were detected at 340 nm. The activity of pi (π) class was determined with 500 µg of protein, 0.25 mM ETHA in ethanol 4%, 0.2 mM GSH in 0.025 M of phosphate buffer, pH 7.0, and the conjugates were detected at 270 nm. The alpha (a) class was determined by measuring the decrease of NADPH at 340 nm, using 15 µg of protein, 2.5 mM CHP in ethanol 4%, 2.0 mM GSH, 1.0 mM NADPH, 1.5 mM of potassium cyanide and 1.4 U of glutathione reductase/mL in 0.1 M of phosphate buffer, pH 7.0. The activity of mu

(μ) class was determined with 500 μg of protein, 4.0 mM DCNB in ethanol 1%, 5.0 mM GSH in 0.1 M of phosphate buffer, pH 6.0, and the conjugates were detected at 345 nm. All the assays were performed at 25 °C.

Comparisons among GST activities of each group of five fish were made by one-way analysis of variance (ANOVA) with Dunnett's post test using GraphPad Prism version 3.00 for Windows, GraphPad Software, San Diego, California, USA.

RESULTS AND DISCUSSION

Brazilian fishes of the Pantanal have been under increasing risk of exposure to xenobiotics from different sources, including effluents from industry and runoff from agriculture. Understanding the biochemistry of these chemicals in species suitable for pisciculture is essential for defining strategies to avoid biodiversity and economic losses, mainly because such knowledge is particularly useful for substantiating more precise bioindicators.

Liver cytosolic GST activities increased in injected fish using CDNB, ETHA or CHP as substrates (Figure 1).

Figure I-A shows that the activity on CDNB increased 81% 24 h after β-NF injection. This activity increased even more, to 120% after 48 h, remaining elevated until 96 h. It might be that these CDNB levels of GST activity could remain elevated for more than a week in pacu, since it has been published (Andersson et al. 1985) that increased CDNB-GST activities provoked by 100 mg/kg body wt. β-NF in trout *(Salmo gairdneri)* reached 100% induction, and stayed at these maximum levels for 2-3 weeks.

Figure I-B shows that pacu liver GST activity measured with ETHA also increased, but only 48 h after the treatment, to levels (39% higher than controls) that are not as large as those assayed with CDNB. It is noteworthy that this increase seems to be reflected by the heightened CDNB-GST levels that occurred between 24 and 48 h after β-NF injection, since CHP-GST levels (Figure 1-D) changed 24 h after treatment and remained elevated.

When GST was assayed using DCNB (Figure I-C), a class mu GST-epoxide substrate, we did not find a significant decrease in activity, indicating that in pacu this GST isoenzyme might be insensitive to the dose of β-NF we used.

We found that GST activity with CHP as substrate increased to levels 42% higher than those of the controls. As this was the highest activity assayed in the cytosol of pacu liver cells and appeared as early as 24 h after β-NF injection we suggest that a-GST should be always assayed to evaluate if a pacu specimen might have been recently contaminated with PAH substances.

No information concerning the effects of β-NF on fish liver GST activities upon other substrates than CDNB is available to compare with our results using ETHA,

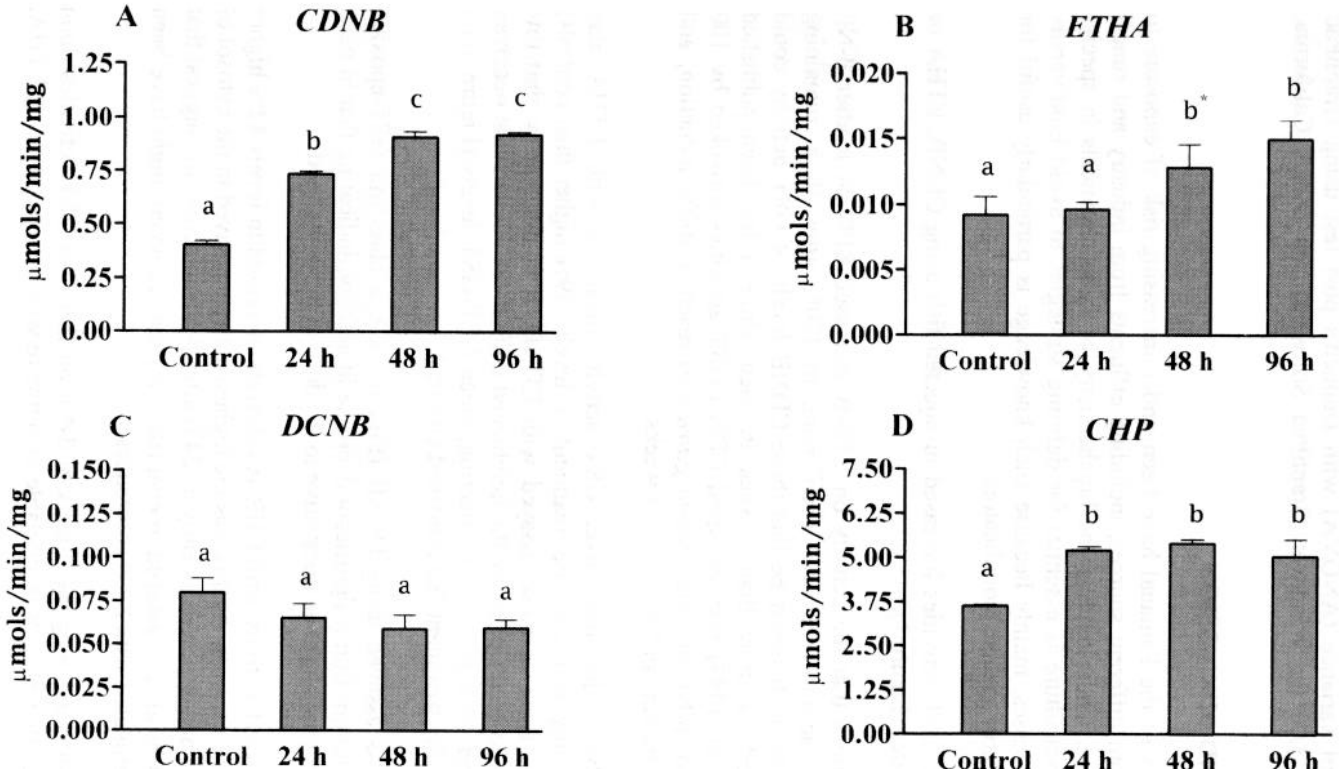

Figure 1. Cytosolic GST activities from livers of fish injected with 50 mg/kg body weight β-NF. Assays were carried out using different substrates (A) CDNB, (B) ETHA, (C) DCNB and (D) CHP. Each control bar presents the means ± SEM of GST determinations from 15 livers and the other bars show means ± SEM of five livers. Bars showing different superscripts are significantly different, ($P < 0.001$), * ($P < 0.010$).

DCNB or CHP. Authors who used only CDNB as GST substrate did not find alterations of activity in hybrids of male bluegill sunfish *(Lepomis macrochirus)* and female green sunfish *(Lepomis cuyanellus)* (Oikari and Jimenez 1992) treated with β-NF. Tilapias *(Oreochromis niloticus)* from the Billings Reservoir, located in São Paulo State, Brazil, had no observable alteration in their CDNB-GST levels in spite of dwelling in waters in which sediment PCB levels ranged from 7 to 101 μg/Kg (Leitão 1999). It is possible to speculate, though, that if other substrates had been used to assay GST activity, the authors could have found some specific response.

Altogether, these findings point out that discriminating which GST substrate activity is affected by a particular pollutant should be part of any precise protocol that is aimed at evaluating if a given species is being exposed to important sublethal concentrations.

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