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Identification and Induction by β-naphthoflavone of CYP1A1 in Liver of the Neotropical Fish Pacu, *Piaractus mesopotamicus* (Characiformes: Characidae)

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Cytochromes P450 (CYP) metabolizes many substances present in toxic waste, such as polychlorinated biphenyl hydrocarbons (PCB), dioxins and polycyclic aromatic hydrocarbons (PAH) (Ioannides and Parke 1990; Whitlock 1999). Some of these pollutants are not substrates only to P450, but are also capable of interacting with the Ah receptor, leading to increased transcription of the CYP genes (Whitlock 1999). Subfamily CYP1A is the most extensively found in fish (Stegeman 1989; Morrison et al. 1995). CYP1A1 hepatic activities are higher in fishes collected from contaminated water than in fishes collected from uncontaminated water (Gadagbui and Goksøyr 1996), which shows that high levels of CYP1A1 could indicate that fish has absorbed pollutants from water. It has been shown that administration of β-naphthoflavone (BNF) to some fishes produced a several-fold increase in their liver CYP1A1 levels, reflecting their hepatic ability to respond to PAH-like xenobiotics (Al-Arabi and Goksøyr 2002; Pretti et al. 2001).

Pacu, *Piaractus mesopotamicus*, is a characin endemic to the Paraguay-Parana basin in South America, including the rivers of the Brazilian Pantanal. This region is under steadily increasing risk of contamination by pesticides used to protect crop. Pacu is a fish intensively cultured, but there is no information on their CYP1A1 enzyme activities. Establishing if CYP1A1 is inducible in pacu exposed to xenobiotics is important because CYP1A1 activity found in specimens collected from rivers, lakes and fishery tanks would allow to determine if they are undergoing sublethal toxic effects.

We describe here the presence of CYP1A1 in liver microsomes from pacu and its major specific deethylase activity, which was inducible by β-naphthoflavone.

MATERIALS AND METHODS

7-Ethoxycoumarin, 7-ethoxyresorufin, resorufin, umbelliferone, α-naphthoflavone (α-NF), β-naphthoflavone (BNF), β-NADP, glucose-6-phosphate (monosodium salt) and glucose-6-phosphate-dehydrogenase (type XV) were purchased from Sigma Chem. Co. (USA). Electrophoresis reagents and nitrocellulose membranes were from BIO-RAD Co. (USA). Polyclonal BN-1 antibody against CYP1A1 was purchased from Biosense Laboratories in Bergen, Norway.

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Pacus of both sexes weighing around 400 g were donated by the National Centre for Research on Tropical Fish (CEPTA) of the Brazilian Environment and Renewable Natural Resources Institute (IBAMA). They were acclimated for three weeks in 500 L tanks equipped with biologic filter and filled with aerated dechlorinated freshwater at 24 °C. Fifteen fish were injected intraperitoneally with corn oil (control) and other fifteen with 50 mg of β-naphthoflavone in corn oil / kg of fish. Five control-fish and five β-naphthoflavone-injected fish were killed at 24, 48 and 96h after the injection and their livers were immediately removed, washed in ice-cold Tris 50 mM with 1.15% KCl, pH 7.4, and frozen in liquid nitrogen.

Microsomes were obtained as described (Lemaire et al. 1996). Protein concentration of the microsomal samples was determined according to Peterson (1977), using bovine serum albumin as standard.

Microsomal 7-ethoxyresorufin O-deethylase activity (EROD) was determined in a total volume of 2 mL as described before (Burke et al. 1985), except that the NADPH regenerating system consisted of 0.25 mM β-NADP, 2.5 mM $MgCl_2$, 5 mM glucose-6-phosphate, and one unit of glucose-6-phosphate dehydrogenase. The assay was carried out at 30 °C, for 5 minutes, and contained 0.2 mg microsomal protein and 0.5% DMSO used to dissolve 7-ethoxyresorufin. The rate of formation of resorufin was linear for up to 15 minutes with 0.5 mg microsomal protein. Resorufin was detected using a Hitachi F-3010 spectrofluorimeter at 550 nm excitation and at 582 nm emission.

Microsomal 7-ethoxycoumarin O-deethylase activity (ECOD) was determined as described by Aitio et al. (1978), with minor modifications. Assays were carried out for 5 min in a volume of 0.5 ml, at 37 °C, with 0.25 mg microsomal protein, the NADPH regenerating system described above and 0.5% DMSO used to dissolve 7-ethoxycoumarin. The reaction was stopped by adding 0.5 mL of 0.3 M trichloroacetic acid. Tubes were centrifuged at 1.000 x g for 15 minutes. Then, 0.5 mL of the supernatants were mixed with 2 mL of 1.6 M NaOH-glycine buffer, pH 10.3, and the fluorescence of the mixture was measured. Excitation wavelength was set at 390 nm and emission wavelength at 440 nm. Under these conditions, ECOD reaction was linear for up to 1.0 mg microsomal protein, for 10 minutes.

EROD V_{max} and K_M were determined varying ethoxyresorufin from 0.04 to 2.5 μM in the assays. ECOD V_{max} and K_M were determined varying ethoxycoumarin from 0.04 to 2.5 mM.

SDS-PAGE was carried out according to Laemmli (1970), using 8% polyacrylamide resolving gel. After transferring proteins to a nitrocellulose membrane (transblot tank, BIO-RAD), the immunostaining was performed using a first antibody against conserved regions of liver CYP1A1 from *Salmo salar, Oncorhynchus mykiss, Gadus morhua, Cyprinus carpio, Clarias anguillaris, Anguilla anguilla* and *Scophthalmus maximus*. The blots were developed using a commercially available alkaline phosphatase kit (BIO-RAD). Densitometry of the immunoreactive bands was performed using a Hitachi F-3010 spectrofluorimeter.

Data were analyzed for statistical significance by Student's test. Data analysis from control and BNF treated fish was accomplished using one-way ANOVA and the posttest of Dunnett.

RESULTS AND DISCUSSION

Figure 1-A shows the variation on the rates of EROD activity according to different concentrations of ethoxyresorufin, and the kinetic constants K_M and V_{max}. The K_M value (0.317 μM) was comparable to 0.194 μM described for carp liver microsomes (Marionnet et al. 1997). V_{max} value of 59.9 pmol x min^{-1} x mg^{-1} was higher than the values 19.8 and 18.15 pmol x min^{-1} x mg^{-1} obtained with microsomes prepared from liver of carp (*Cyprinus carpio*) (Marionnet et al. 1997) and trout (*Oncorhynchus mykiss*) (Haasch et al. 1994), respectively. Hepatic microsomes prepared from untreated pacus were also able to catalyze the deethylation of 7-ethoxycoumarin. Figure 1-B shows the variation on the rate of ECOD with different concentrations of 7-ethoxycoumarin, and the kinetic constants K_M and V_{max}. The K_M value of 0.312 mM for ECOD drew attention to the fact that liver microsomes of pacu needed a thousand more ethoxycoumarin to reach the same magnitude of EROD V_{max} values. Therefore, pacu liver might be less active to metabolize alkoxycoumarins than alkoxyresorufins. Nevertheless, ECOD V_{max} value of 31.7 pmol x min^{-1} x mg^{-1} we found is twice that of vendace (*Coregonus albula*) liver and comparable to the lowest levels of 37.6 pmol x min^{-1} x mg^{-1} found in liver from *O. mykiss* (Lindström-Seppä et al. 1981) and 28.8 pmol x min^{-1} x mg^{-1} found in liver of gilthead seabream (*Sparus aurata*) (Pretti et al. 2001).

To check if CYP1A1 was responsible for both ethoxyresorufin and ethoxycoumarin metabolism in liver of pacu, we measured EROD and ECOD activities in the presence of 5 and 10 mM α-naphthoflavone, which is a selective inhibitor of CYP1A1 at these concentrations (Wiebel et al. 1971). In fact, EROD was completely inhibited by α-naphthoflavone (Figure 2A), but ECOD was not significantly inhibited by either 5 or 10 mM α-naphthoflavone (Figure 2B). Furthermore, treatment of pacus with BNF, which is a typical inducer of CYP1A1, produced an increase of EROD activity (Figure 3A), with the maximum occurring within 24 hours (around 1,300 fold) remaining elevated 96 h after the treatment, thus showing that CYP1A1 is present in the liver of pacu and it is responsible for the deethylation of 7-ethoxyresorufin. Microsomal ECOD activity rates of BNF treated pacus (figure 3B) increased much less than the rate of EROD suggesting that CYP1A1 was not primarily involved in ethoxycoumarin metabolism in liver of pacu.

Figure 4 shows that the antibody against conserved sequences of CYP1A1 present in liver of other fishes reacted with a single polypeptide band, which was clearly induced in liver microsomes prepared from BNF treated pacus (lanes 3,4 and 5), when compared to controls (lanes 1 and 2). The molecular weight of the polypeptide band is around 50 kDa, which is similar to the 54 kDa of CYP1A1 present in Bermuda fishes (Stegeman et al. 1997) and to the 56 kDa CYP1A1 from *Sparus auratus* (Pretti et al. 2001).

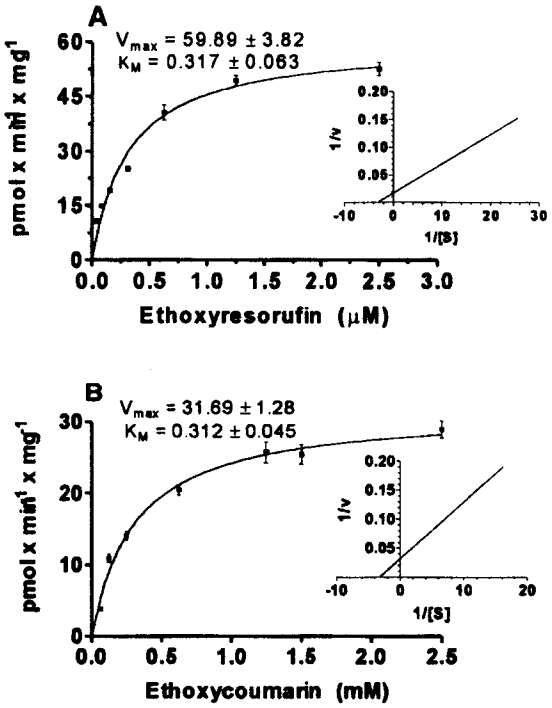

Figure 1. Velocity vs substrate concentration curves of microsomal EROD (A) and ECOD (B) liver activities of pacu. The inserted graphics show the corresponding $1/v$ vs $1/[S]$ curves. Each point is the mean ± SEM of five assays carried out in each microsomal preparation from five fish.

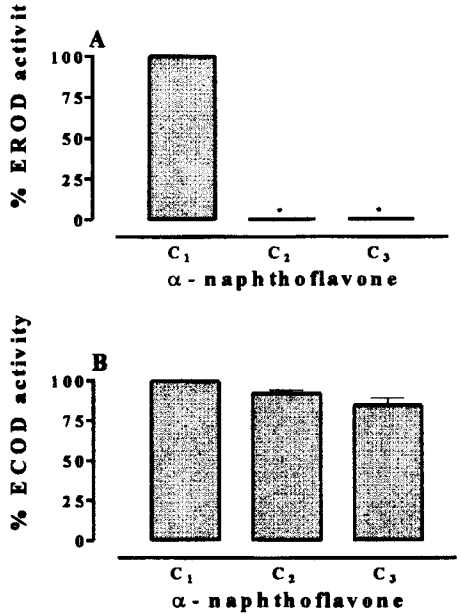

Figure 2. Effect of α-naphthoflavone different concentrations (C_1 = 0 μM; C_2 = 5 μM; C_3 = 10 μM) *in vitro* on activities of (A) EROD and (B) ECOD. 7-Ethoxycoumarin concentration was 3 mM and 7-ethoxyresorufin was 2.5 μM. Activities of 100% respective to V_{max} values shown in Figure 1. Bars represent the means ± SEM of five determinations. Significant different from C_1 is indicated *($P < 0{,}0001$).

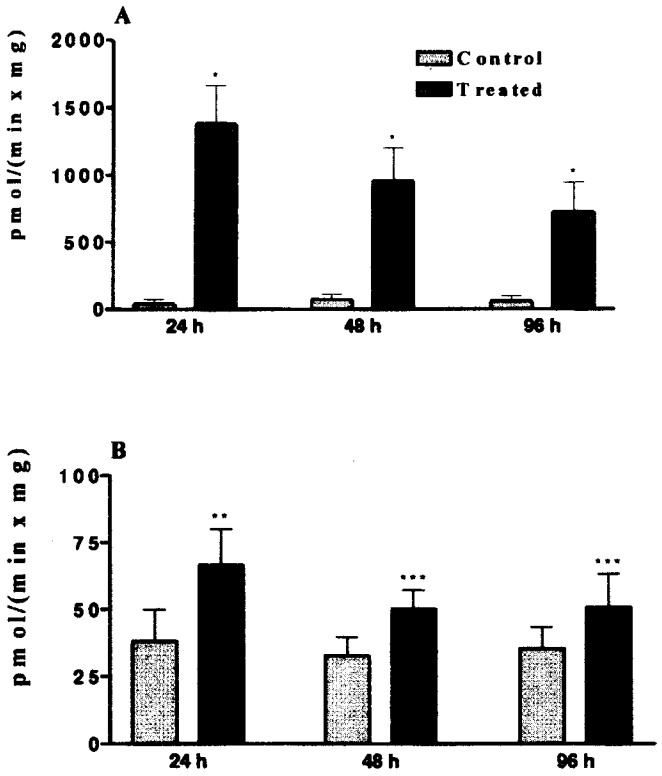

Figure 3. Effect of β-naphthoflavone on activities of (A) EROD and (B) ECOD after 24, 48 and 96 hr of injection. Bars represent the means ± SEM of determinations from 5 livers. Significant differences from control fish (Student test) are indicated as *($P < 0{,}0001$), ** ($P < 0{,}002$) and *** ($P < 0{,}05$).

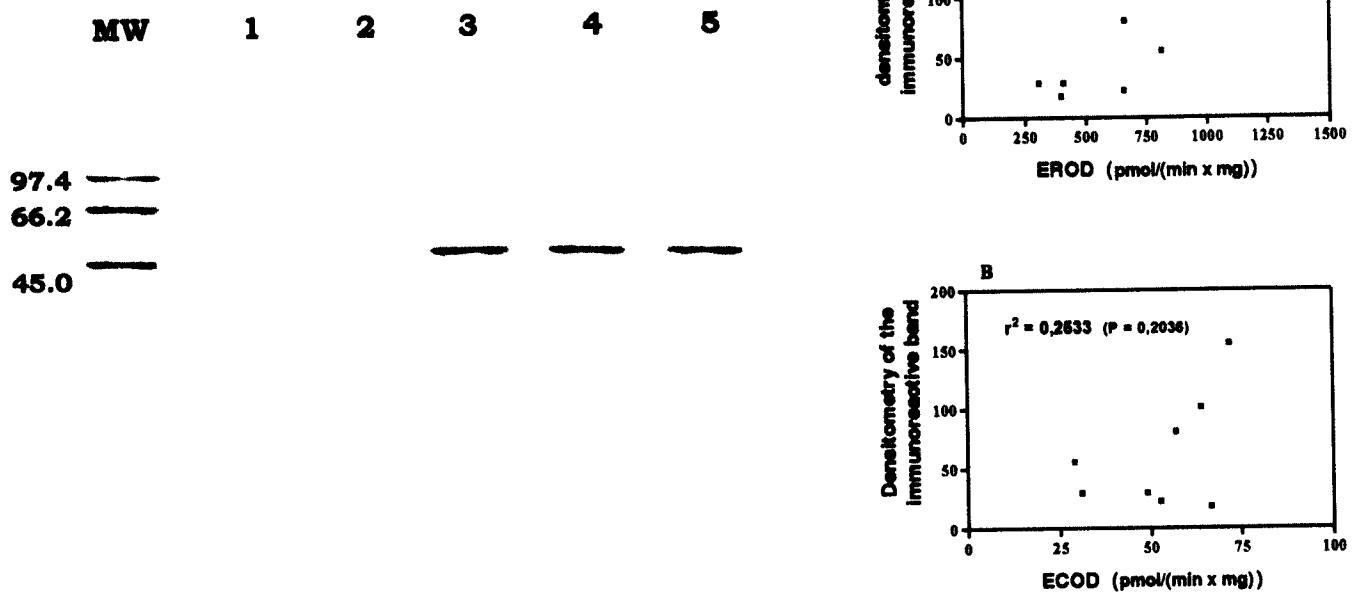

Figure 4: SDS-PAGE and immunoblot of CYP1A1 from liver microsomes of β-naphthoflavone-treated pacu. Lane MW indicates standards molecular weights. Lanes 1 and 2 contained 25 μg of microsomal proteins from controls. Lanes 3, 4 and 5 contained 12,5 μg of microsomal proteins from β-naphthoflavone-treated fish 24, 48 and 96 hr after injection, respectively. It is shown the correlation between densitometry of the immunoreactive band and activities of EROD (A) and ECOD (B).

Densitometric analysis of the immunoreactive band present in liver microsomes of BNF-injected pacus correlated well with levels of EROD, but not with levels of ECOD (figures 5A and 5B), which substantiates that 7-ethoxyresorufin is metabolized primarily by CYP1A1 and that this isoenzyme is not the main enzyme responsible for ethoxycoumarin metabolism in liver of pacu.

Our findings point out that EROD microsomal activity is present in liver of the Neotropical fish pacu and it is a better indicator of pacu exposure to PAH-like compounds than ECOD. In the other hand, hepatic ECOD activity is not CYP1A1-dependent in pacu.

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