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Induced alkoxyresorufin-*O*-dealkylases in tilapias (*Oreochromis niloticus*) from Guandu river, Rio de Janeiro, Brazil

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Abstract

The activity of fish monooxygenases has been extensively used as a monitoring tool to detect contamination of water bodies by cytochrome P450-inducing agents. In this study we evaluated the activities of ethoxy- (EROD), methoxy- (MROD) and pentoxy- (PROD) resorufin-*O*-dealkylases in the liver of Nile tilapias (*Oreochromis niloticus*) collected at the Guandu river, at a reference clean site (Lake 1) and at two other sampling sites (Lakes 2 and 3) in Rio de Janeiro state, Brazil. Alkoxyresorufin-*O*-dealkylases were measured fluorimetrically in the hepatic S9 fraction. EROD (17.7-fold), MROD (14.2-fold) as well as PROD activities were considerably higher in tilapias from Guandu river. A moderate increase of EROD (5.0-fold) and MROD (5.4-fold) was also found in tilapias from Lake 3. These findings suggest that Guandu river watershed, the main source of urban drinking water supply in Rio de Janeiro, is polluted with CYP1A-inducing xenobiotics. Furthermore, we also found a good linear relationship between EROD and MROD, a finding that agrees with the hypothesis that the two reactions are catalysed by the same CYP1A isoform in *O. niloticus*.

Keywords: Monooxygenases; EROD; Cytochrome P450; Biomarkers; Aquatic pollution; Freshwater fish

1. Introduction

A variety of highly toxic environmental pollutants such as polycyclic aromatic hydrocarbons (PAHs), polychlorinated dibenzo-*p*-dioxins and -furans (PCDD/Fs), and coplanar polychlorinated biphenyls (PCBs), are known to markedly induce cytochrome P4501A isoforms (CYP1A) in mammalian as well as in fish tissues (Haasch et al., 1993; Parkinson, 1996). Owing to this fact, activity of CYP1A subfamily in fish liver has been extensively used as a biomarker of aquatic contamination by industrial pollutants in freshwater as well as in marine environments (Narbonne et al., 1991; Haasch et al., 1993). Considerably less is known, however, on the induction of other fish cytochrome P450 isoenzymes by aquatic pollutants.

The Guandu river is the main source of drinking water supply in the Greater Metropolitan Rio de Janeiro, the second largest and most populated urban area in Brazil. During the last three decades, dump landfills, sand mining activities, hazardous chemicals waste sites and many industries and human settlements

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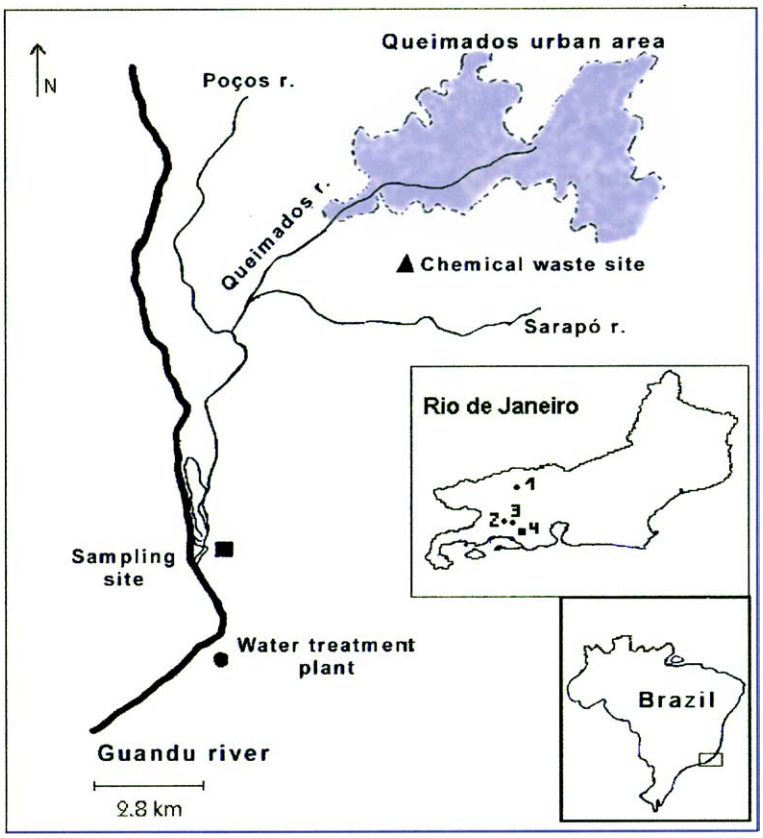

Fig. 1. Sampling site (■) and the water-treatment plant (●) on the Guandu river. A hazardous chemicals waste site (▲) and the urban area of Queimados county (Rio de Janeiro state, Brazil) are located at a short distance or on the banks of Queimados river and its tributaries. Reference sites (Lakes 1, 2 and 3) and Guandu river sampling site (4) are shown in the Rio de Janeiro box.

have been established nearby or on the banks of Guandu river and its tributaries, upstream the site from where water is pumped into the main water-treatment plant (Fig. 1). Since the existing sewage system does not cover most of this area, and there has been no strict control on industrial discharges, worsening of Guandu river pollution has caused several interruptions of water pumping into the water-treatment plant during the last years.

The present study was undertaken to investigate to what extent Guandu river fish are contaminated by CYP1A-inducing pollutants. Contamination by monooxygenase-inducing pollutants was evaluated by measuring the activities of alkoxyresorufin-*O*-dealkylases (MROD, EROD and PROD) in the liver of Nile tilapias (*Oreochromis niloticus*).

2. Materials and methods

2.1. Fish capture

In the summer of 2001/2002, Nile tilapias (*O. niloticus*) were caught by using cast nets at the Guandu river (March 20th and 27th), at a reference clean site (Lake 1, Experimental Aquaculture Center of EMATER-RJ, in February, 6th) and at two other sites (Lake 2, March 23rd, and Lake 3, January 10th) located in Rio de Janeiro state, Brazil. All fish were caught in the morning between 9:00 and 12:00 h. Male and female fish were collected in all sampling sites and the number of samples taken from each site depended on the abundance of tilapias.

The Guandu river sampling site (22°45′45″S, 43°41′46″W) was less than 500 m far from the place where water is pumped into the main water-treatment plant (Fig. 1). Lakes 2 and 3 are located within the campus of Federal Rural University of Rio de Janeiro (UFRuRJ). Lake 2 is used for aquaculture and fish farming and lake 3 (Lago Açu) is an ornamental water body. Lake 3 receives discharges of domestic organic pollutants and it has most of its surface covered by water lilies (*Eichhornia azurea*).

2.2. Chemicals

Substrates (ethoxyresorufin, methoxyresorufin and pentoxyresorufin), the reaction product (resorufin),

β-NADP, glycose-6-phosphate, glucose-6-phosphate-dehydrogenase, bovine serum albumin and the Bradford reagent were all purchased from Sigma Chemical Company, St Louis MO, USA. TRIS, $MgCl_2$ and other salts were of analytical grade and supplied by Merck SA Indústrias Químicas, Rio de Janeiro, Brazil.

2.3. Preparation of S9 fraction

All fish were measured, weighed and killed with a cephalic blow. Livers were then rapidly excised, weighed and frozen in liquid nitrogen until further use. Three frozen livers from fish collected at the same sampling site were thawed on ice and homogenized in a cold buffer solution (50 mM Tris, 1 mM EDTA, 250 mM sucrose, 20% glycerol, pH 7.4) by using a motor-driven glass Potter-Elvejhem homogenizer equipped with a Teflon™ pestle. Hepatic homogenates were subsequently centrifuged at 9000*g* for 30 min at 4 °C. Aliquots (1 ml) of the supernatant (liver S9 fraction) were transferred to cryotubes and stored in liquid nitrogen until they were assayed for monooxygenase activity. Protein concentrations in the S9 fractions were measured by a colorimetric method (absorbance at 595 nm) using Coomassie brilliant Blue G dye and bovine serum albumin as the standard. Details of the method used for protein quantification were reported by Bradford (1976).

2.4. Enzyme assays

Ethoxyresorufin-*O*-deethylase (EROD), methoxyresorufin-*O*-demethylase (MROD) and pentoxyresorufin-*O*-depenthylase (PROD) activities in the hepatic S9 fractions were assayed essentially as described by Burke et al. (1985) except for the use of a NADPH regenerating system. Reactions were carried out in quartz cuvettes at 37 °C and were started by addition of the regenerating system which consisted of 0.25 mM β-NADP, 2.5 mM $MgCl_2$, 5 mM glycose-6-phosphate, and 0.5 units of glucose-6-phosphate-dehydrogenase per ml of incubation mixture. The rate of resorufin formation was measured fluorimetrically. The spectrofluorimeter (Shimadzu RF-5000) settings were as follows: excitation at 550 nm, emission at 582 nm and a 5 nm band slit width.

2.5. Statistical analysis

Enzyme activities were compared by a non-parametric analysis of variance (Kruskal–Wallis test) followed by the Mann–Whitney *U* test. In any case a difference was considered statistically significant when $P < 0.05$. Statistical calculations were made by using either Minitab® or Excell® (linear regression analysis) software.

3. Results and discussion

As shown in Table 1, activities of EROD, MROD and PROD in tilapias from Guandu river were all markedly higher than those found in fish collected at the reference clean site (Lake 1). Activities of liver monooxygenases in Guandu river tilapias were also higher than those activities measured in fish caught at Lakes 2 and 3. The rates of EROD and MROD dealkylations in fish from Lake 2 were similar to those found in tilapias from the reference unpolluted site (Lake 1), but activities

Table 1
Activities of liver monooxygenases in Nile tilapias (*O. niloticus*) collected at the Guandu river, at a clean reference site (Lake 1), and at two other sampling sites (Lakes 2 and 3) located in Rio de Janeiro state, Brazil

Sampling sites	Fish parameters		Activity of alkoxyresorufin-*O*-dealkylases (pmoles resorufin/mg protein/min)			
	Length (cm)	Weight (g)	*N*	EROD	MROD	PROD[a]
Lake 1 (reference site)	19.62 ± 1.23	148.64 ± 31.53	7	16.70 ± 3.84 (1.0)	13.72 ± 4.47 (1.0)	nd
Lake 2 (induction factor)	18.14 ± 1.91	102.22 ± 28.47*	6	23.07 ± 8.64 (1.4)	25.45 ± 10.02 (1.8)	nd
Lake 3 (induction factor)	20.06 ± 2.42	120.08 ± 37.49	3	83.53 ± 18.14* (5.0)	73.49 ± 2.88* (5.4)	nd
Guandu river (induction factor)	17.22 ± 1.86*	112.24 ± 33.63*	5	295.23 ± 33.63* (17.7)	194.53 ± 99.17* (14.2)	23.31 ± 19.78*

Values are shown as means ± S.D. Enzyme activities were measured in fish liver S9 fractions. N = number of pools assayed. Each pool was made with livers from three fishes collected at the same site. Induction factor = (activity in fishes from the sampling site)/(activity in fishes from Lake 1). nd = not detected.

* Different ($P < 0.05$) from Lake 1 values.

[a] Since PROD activity was not detected in the hepatic S9 fraction obtained from fishes caught at the reference site (Lake 1), the induction factor was not calculated for this monooxygenase. Data were analysed by the Kruskal–Wallis test followed by the Mann–Whitney *U* test.

of hepatic monooxygenases in fish collected in Lake 3 were somewhat higher than those observed in Lake 1. The foregoing results thus indicate that hepatic EROD and MROD were markedly induced (induction factors 17.7 and 14.2, respectively) in fish collected at the Guandu river. Moreover, data from the present study also showed that M/EROD induction in Guandu river tilapias was accompanied by a marked enhancement of PROD activity.

These increases in M/EROD activities suggest that Guandu river tilapias were exposed to CYP1A-inducing contaminants. Besides industrial discharges, a possible source of contamination of Guandu river by CYP1A-inducing agents, such as polychlorinated biphenyl congeners (PCBs) and PAHs, is a hazardous chemicals waste site located nearby its tributary river 'Queimados', the mouth of which is only a few meters upstream the sampling site (Pinto, 2001). Askarel oil—a mixture of PCBs congeners—was formerly employed as an insulator fluid in most transformers used in Rio de Janeiro and elsewhere. Since askarel-containing transformers have been gradually replaced by others without PCBs, a large amount of these persistent chlorinated compounds has been left on this chemical waste site (Pinto, 2001). Owing to inadequate storage under open sky conditions, weathering led to corrosion of askarel containers thereby causing a leakage of PCBs into soil and water bodies. Additionally, this chemical waste site has burned at least twice in recent years (Pinto, 2001), and these fires may have produced a substantial amount of polychlorinated-dibenzo-dioxins and -furans. Levels of PCBs and PCDD/Fs have not yet been determined in soil, sediments and water in the Guandu river area. Nonetheless, since some PCDD/Fs and PCBs congeners are among the most potent CYP1A inducers, data presented here suggest that Guandu river may be markedly contaminated with these chlorinated compounds.

Hepatic EROD and MROD activities also seemed to be somewhat induced (approx. 5-fold) in fish from Lake 3 (Table 1). Lakes 2 and 3 are polluted by local domestic sewage discharges but not by industrial effluents and, contrasting with the former site (no induction), the latter lake (moderate induction) has most of its surface covered with water lilies (*E. azurea*). Some plant xenobiotics have been found to induce CYP1A (e.g. cruciferous vegetables) and CYP2B (e.g., a variety of terpenoid compounds) in mammals but it is not clear whether this would occur in fish as well. *O. niloticus* has been reported to be an omnivorous fish but some studies have suggested that it is primarily a herbivorous feeder (Dempster et al., 1993). Whether a greater intake of certain types of plants is responsible for the moderate induction of CYP1A noted in tilapias from Lake 3 remains to be investigated.

Only a few studies using fish monooxygenases as a tool for monitoring aquatic pollution in Brazil and South America have been published so far. Although being an alien species, the Nile tilapia (*O. niloticus*) was employed in most of the monitoring studies performed in Brazilian freshwater sites. *O. niloticus*, a cichlidae fish native to Eastern Africa, was introduced, primarily as a source of food protein, in reservoirs and dams in poor and semiarid areas of Northeastern Brazil in 1971 (Bizerril and Primo, 2001). Today, *O. niloticus* and other related species (popularly known as tilapias) are among the species most frequently found in fish farming activities and in reservoirs, dams, lakes and even in rivers in Southeastern and Southern Brazil as well. Besides its wide geographic distribution and abundance in the tropics, an additional advantage of using *O. niloticus* for monitoring purposes is its well known resistance to highly polluted environments.

Induced activities of hepatic alkoxy-resorufin-*O*-dealkylases were previously found in liver microsomes of *O. niloticus* caught at the Billings reservoir, a highly polluted—by domestic sewage as well as industrial effluents—water body located in the metropolitan area of São Paulo, Brazil (Bainy et al., 1999; Leitão et al., 2000). EROD induction factors as high as 22.8-fold and 26.5-fold in Billings *O. niloticus* were reported by Bainy et al. (1999) and Leitão et al. (2000), respectively. Bainy et al. (1999) also reported that MROD activity of Billings tilapias was higher (19.9-fold) than that of fish from a reference unpolluted site. The magnitude of EROD (17.7-fold) and MROD (14.2-fold) induction in Guandu river tilapias therefore was not very different from that noted in *O. niloticus* from the highly polluted Billings reservoir. It should be noted that, in contrast with Leitão et al. (2000) and Bainy et al. (1999), we measured enzyme activities in the S9 fraction. As demonstrated by O'Hare et al. (1995) EROD activity is somewhat lower in the S9 fraction, but the level of enzyme induction in the S9 fraction seems to be similar to that in microsomes. The advantage of using S9, instead of using microsomal fraction for monitoring purposes is that the former is less costly and time consuming than the latter.

EROD seems to be predominantly catalysed by CYP1A1 in mammals as well as in fish liver microsomes (Burke et al., 1994; Stegeman et al., 1997). MROD, on the other hand, has generally been considered as a marker for CYP1A2 in rodents. In mammals both CYP1A isoforms are inducible by Ah-receptor agonists such as polycyclic aromatic hydrocarbons and polyhalogenated aromatic hydrocarbons (Parkinson, 1996). Compared to mammals, however, much less is known about CYP1A isoenzymes expressed in fish tissues. It has been reported that some salmonids (e.g. *Oncorhynchus mykiss*, *Salvelinus alpinus*) possess two CYP1A isoforms (Berndtson and Chen, 1994; Wolkers et al., 1996) but there have also been indications that other fish species express only one CYP1A isoenzyme (Smeets et al., 2002). It is not known whether EROD and MROD are catalysed by the same

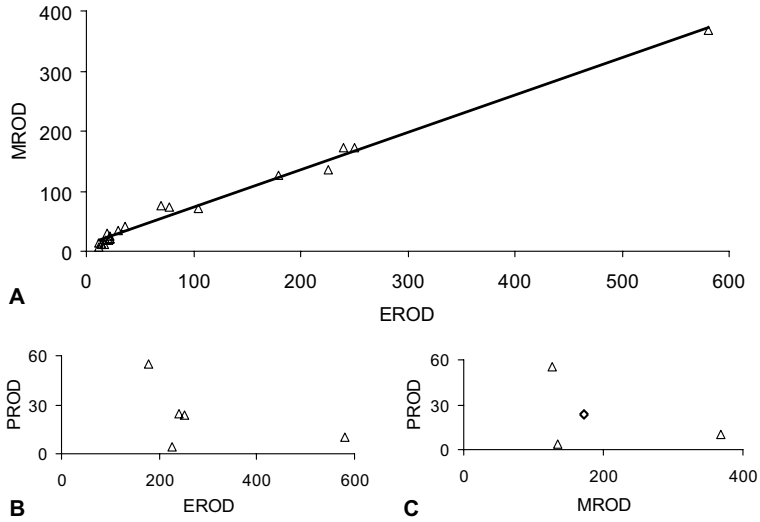

Fig. 2. Plotting of EROD versus MROD (A), EROD versus PROD (B) and MROD versus PROD (C) activities (pmoles resorufin/mg protein/min) in the Nile tilapia (*O. niloticus*). In panel A (EROD versus MROD), the line was fitted by linear regression of values obtained for all liver S9 preparations analysed. In panels B and C, a smaller number of values were plotted because PROD activity was detected only in Guandu river tilapias. Linear correlation coefficients (R^2) were as follows: $A = 0.99$; $B = 0.24$ and $C = 0.19$.

isoform (i.e. a CYP1A1-like isoenzyme) in *O. niloticus*. Recently, Smeets et al. (2002) found a good linear relationship between EROD and MROD in cultured hepatocytes of four fish species (*O. mykiss*, *P. flesus*, *L. limanda* and *M. kitt*), thereby suggesting that the same isoenzyme metabolises the two substrates (methoxy- and ethoxyresorufin) in these fish species. An excellent linear relationship between EROD and MROD ($R^2 = 0.99$) was noted in this study (Fig. 2), a finding that agrees with the hypothesis that both substrates are predominantly metabolised by the same CYP1A isoform in *O. niloticus* hepatic S9 fraction as well. Nonetheless, the alternative possibility that both substrates are metabolised by distinct isoforms that were similarly induced by substances found in the collection sites cannot be ruled out.

Even less clear is what cytochrome P450 isoenzyme is responsible for pentoxyresorufin-*O*-depentylation (PROD) in fish. In rats and mice, PROD seems to be predominantly catalysed by isoforms belonging to CYP2B subfamily (Burke et al., 1994) but in fish, as far as we are aware, such a substrate specificity has not been demonstrated yet. CYP2B-like proteins have been identified (by using antibodies to rat CYP2B1 or to scup P450B) in tropical marine fish (Stegeman et al., 1997) but it is not clear whether these proteins catalyse pentoxyresorufin-*O*-depentylation. It has been demonstrated, for instance, that pentobarbital, a classical CYP2B-inducer in mammals induces EROD and CYP1A gene expression, but not PROD activity, in primary cultures of rainbow trout hepatocytes (Sadar et al., 1996). On the other hand, a high correlation was found between EROD and PROD activities in the cod (*Gadus morhua*) liver, a finding suggestive that both reactions are catalysed by a single isoform (CYP1A) in this marine fish (Ruus et al., 2002).

PROD activity was not detected in tilapias from lakes 1, 2 and 3 (Table 1). In Guandu river tilapias, however, PROD was measured in all cases but it was not correlated with EROD and MROD activities (Fig. 2). The lack of correlation between EROD/PROD and MROD/PROD suggests that pentoxyresorufin is not predominantly metabolised by CYP1A in *O. niloticus*.

In conclusion, data presented here indicated that activities of EROD, MROD and PROD are induced in the liver of Guandu river tilapias. Moreover, our results are also in agreement with the hypothesis that EROD and MROD are catalysed by the same CYP1A isoform in *O. niloticus*. Since tilapias are consumed by local population and Guandu river is the main source of water supply in Rio de Janeiro, levels of CYP1A-inducing pollutants, including carcinogenic PAHs and PCBs, should be further investigated in its sediments, water and biota.

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