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β-NAPHTHOFLAVONE-INDUCIBLE CYTOCHROME P4501A1 ACTIVITY IN LIVER MICROSOMES OF THE MARINE SAFI FISH (*SIGANUS CANALICULATUS*)*

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Abstract—The cytochrome P450-dependent metabolism of benzo(*a*)pyrene and other xenobiotics has been investigated in liver microsomes prepared from a local marine safi fish, *Siganus canaliculatus*. The safi fish was found to have a well-developed microsomal monooxygenase system consisting of cytochrome P450, cytochrome b_5 and NADPH-cytochrome *c* reductase. The fish microsomal enzyme system was able to metabolize benzo(*a*)pyrene, 7-ethoxycoumarin and 7-ethoxyresorufin. Male fish were found to exhibit a higher monooxygenase activity than female fish. Treatment of fish with β-naphthoflavone was found to induce (2- to 4-fold) the activities of aryl hydrocarbon hydroxylase, ethoxycoumarin *O*-deethylase and ethoxyresorufin *O*-deethylase. HPLC analysis of the metabolites produced by incubation of benzo(*a*)pyrene with the liver microsomal preparation showed a predominant formation of 3-OH and 9-OH benzo(*a*)pyrene. There was an increased formation of benzo(*a*)pyrene 7,8-diol and benzo(*a*)pyrene 7,8,9,10-tetrol in liver microsomes prepared from β-naphthoflavone-treated fish. Western immunoblot analysis of liver microsomes from β-naphthoflavone-treated fish using an antibody to rat liver cytochrome P4501A1 (CYP1A1) suggested the presence of an inducible cytochrome P450 enzyme that was comparable with that of rat liver enzyme. Our results suggest that liver microsomes from the safi fish have multiple forms of cytochrome P450 with a specific β-naphthoflavone-inducible CYP1A1 homologous protein that can metabolize a variety of substrates.

Key words: marine fish; cytochrome P450; benzo(*a*)pyrene metabolism; HPLC; western blotting

In mammals, the CYP‡-dependent monooxygenase system has been shown to be responsible for the majority of cellular xenobiotic biotransformations [1–3]. Among the many compounds metabolized by the CYPs are endogenous substances, such as fatty acids, steroids, and prostaglandins, and exogenous compounds, such as alcohol, drugs, pesticides, and carcinogens [4, 5]. Today well over 200 different CYPs capable of catalyzing a variety of potential substrates [6] have been identified and cloned. The majority of these CYPs are selectively induced by a specific group of compounds that differ from species to species [2].

The multiplicity of CYPs and their selective induction have also been shown in marine and fresh water fish [7, 8]. Some of the earlier studies on CYP induction in fish were motivated by the search for biological indicators for environmental pollution. Exposure to petroleum products, polychlorinated biphenyls, dioxins and PAH has been reported to induce CYP in fish [9, 10]. It has also been shown that biotransformation of xenobiotics in fish occurs by many of the same enzyme systems and reactions that are found in mammalian species. Measurement of these activities has been suggested as a possible means of monitoring environmental pollution [11, 12].

The United Arab Emirates (U.A.E.) is a major oil producing country located in the Arabian Gulf. The possibility of marine pollution by petroleum products and other agents is a major concern to the region. To assess the possible toxic effects and carcinogenic risk associated with exposure of marine life to PAH, we have investigated the CYP-dependent monooxygenase system in the liver of a coastal fish, *Siganus canaliculatus* (safi fish). BNF induction of CYP activities in fish liver was studied and compared with the activity found in rat liver. Immunochemical characterization of the CYP enzyme of fish liver microsomes using polyclonal antibody raised against rat liver CYP1A1 was also undertaken.

MATERIALS AND METHODS

B(a)P, NADPH, 7-ethoxycoumarin, 7-ethoxyresorufin, and cytochrome *c* were purchased from the Sigma Chemical Co. (St. Louis, MO, U.S.A.). Solvents for HPLC analysis were from Fluka (Buchs, Switzerland). Chemicals and reagents for electrophoresis and western immunoblotting were purchased from Bio-Rad Laboratories (Richmond, CA, U.S.A.). All other chemicals used were in the purest forms commercially available.

Isolation of liver microsomal fractions

Safi fish (*S. canaliculatus*) raised in running sea water in the Marine Resources Research Center at Umm Al

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‡ Abbreviations: AHH, aryl hydrocarbon hydroxylase; B(a)P, benzo(*a*)pyrene; BNF, β-naphthoflavone; CYP, cytochrome P450; ECOD, 7-ethoxycoumarin-*O*-deethylase; EROD, 7-ethoxyresorufin-*O*-deethylase; and PAH, polycyclic aromatic hydrocarbons.

Quwain, U.A.E., were transported to Al Ain and maintained in a mini-aquarium at the Desert and Marine Environmental Research Center laboratory of the United Arab Emirates University. Both male and female fish (150–230 g body wt) were used in the present study. BNF induction was performed with a daily i.p. treatment of 40 mg BNF/kg body wt for 2 days. The BNF given (20 mg/mL) was dissolved in corn oil. Control fish received corn oil alone.

In experiments where comparisons between fish liver and rat liver activities were to be undertaken, male Wistar rats (200–250 g) were also treated i.p. with BNF (40 mg/kg for 2 days) with the control rats receiving corn oil alone.

Animals were decapitated, and the liver was removed rapidly. The tissue was washed immediately in ice-cold saline (0.9% sodium chloride) and homogenized (20%, w/v) in 100 mM potassium phosphate buffer, pH 7.4, containing 0.15 M potassium chloride and 0.1 mM EDTA. Microsomes were prepared from the homogenate as described earlier [13]. Protein concentrations were measured by the method of Bradford [14], using bovine serum albumin as a standard.

Microsomal monooxygenase activities

Fish liver enzyme activities were measured at 30°, while rat liver enzymes were assayed at 37° (the optimum temperature for fish and mammalian monooxygenase activities, respectively). CYP-dependent B(a)P AHH activity was determined fluorometrically according to the method of Nebert and Gelboin [15]. ECOD and EROD activities were determined as described by Greenlee and Poland [16] and Pohl and Fouts [17], respectively. Specific activity of the enzymes was expressed in terms of the fluorescence produced by metabolites, i.e. fluorescent units (FU) per minute per milligram of protein. CYP and cytochrome b_5 contents were measured according to the method of Omura and Sato [18]. NADPH-cytochrome *c* reductase activity was determined as described by Dignam and Strobel [19].

HPLC analysis of B(a)P metabolites

B(a)P metabolism in fish or rat liver microsomes isolated from BNF-treated and control animals was studied as described previously [20]. The assay system consisted of 50 nmol B(a)P, 1 mM NADPH, 1.0 mg bovine serum albumin, and 1–2 mg microsomal protein in 1.0 mL of 20 mM potassium phosphate buffer, pH 7.4. After a 15-min incubation at 30 or 37°, the reaction was stopped by the addition of 1.0 mL acetone, and the metabolites were extracted into 1.5 mL ethyl acetate. The extracted B(a)P metabolites were analyzed by reversed-phase HPLC (Waters Associates, Milford, MA, U.S.A.) using a Zorbox-ODS column (9.4 mm × 250 mm). A gradient of water and methanol (from 60 to 100% methanol) was used as the mobile phase at a flow rate of 1.5 mL/min. The details of the HPLC conditions, the identification of metabolites by UV monitoring at 254 nm, and the fluorescence analysis at an excitation wavelength of 364 and detection at 432 nm were as described previously [20]. The results are given as the area under the individual peaks and expressed as a percentage of the total peak area including the unchanged parent compound, as measured by UV absorption at 254 nm.

SDS–PAGE and western blot analysis

Purified CYP1A1 from rat liver microsomes [21] was used as a reference in this study. Polyclonal antibody raised against the rat liver CYP1A1 in rabbits [22] was used to characterize fish liver CYP. SDS–PAGE separation of purified CYP1A1 and microsomal proteins from the liver of fish and rats treated with BNF was performed according to the method of Laemmli [23], using a 12% polyacrylamide slab gel. Protein resolved by electrophoresis were transferred electrophoretically onto nitrocellulose membranes for subsequent analysis by western blotting as described by Towbin *et al.* [24]. The membrane was incubated with CYP1A1 polyclonal antibody to determine any specific antibody interaction with fish liver microsomal proteins as described before [22, 25].

Statistical significance of the data was assessed using Student's *t*-test. A value of $P \leq 0.05$ was considered to be significant.

RESULTS

Fish microsomal monooxygenase enzymes

The CYP content of fish liver microsomes was found to be in the range of 0.4 to 0.6 nmol/mg protein, which is comparable with that of rat liver microsomes (0.85 ± 0.15 nmol CYP/mg protein, rat data are not shown here and have been published elsewhere [20]). Male fish liver microsomes were found to have a moderately higher (30–40%) CYP content than female fish liver microsomes (Table 1). The sodium dithionite reduced-CO spectrum of fish liver CYP was found to exhibit an absorption maximum at 449.5 nm.

Table 1 shows the activities of the fish liver microsomal AHH, EROD and ECOD enzymes. The CYP-related activities in male fish were found to be moderately higher (25–50%) than in female fish. The NADPH–cytochrome *c* reductase activity and cytochrome b_5 contents were not appreciably different in male and female fish (118.4 vs 138.7 nmol/min/mg protein and 0.54 vs 0.52 nmol/mg protein, respectively).

BNF treatment of fish resulted in a significant ($P < 0.05$) increase in the CYP content of liver microsomes (Table 1). A 2- to 4-fold increase in microsomal AHH, ECOD and EROD activities was also seen after BNF treatment (Table 1). In contrast, NADPH–cytochrome *c* reductase activity was not altered, and a slight decrease in cytochrome b_5 content was observed. *In vitro* addition of α-naphthoflavone (0.1 to 2 mM) to the enzyme assay system caused a marked inhibition (40–80%) of CYP-dependent EROD activity in fish liver microsomes (Table 1).

HPLC analysis and identification of B(a)P metabolites

Figures 1 and 2 show the resolution by HPLC of the metabolites produced when BNF-induced fish and rat liver microsomes were incubated with B(a)P. The HPLC profiles of the metabolites were similar in BNF-treated rat and fish liver microsomes. The major metabolites [~15% of the total B(a)P metabolism] were monohydroxy (3-OH and 9-OH-) B(a)P with a smaller proportion [5–6% of the total B(a)P metabolites] of di- and tetrahydroxy (7,8-di- and 7,8,9,10-tetrahydroxy) B(a)P derivatives (Table 2). The untreated control liver mi-

Table 1. Cytochrome P450-dependent monooxygenase system in male and female fish liver microsomes and the effect of BNF administration

Monooxygenase system	Male fish	Female fish	BNF-treated fish
Cytochrome P450 content (nmol/mg protein)	0.64 ± 0.05* (36.2%)	0.47 ± 0.06	1.2 ± 0.09*
Cytochrome b_5 content (nmol/mg protein)	0.54 ± 0.06 (3.8%)	0.52 ± 0.01	0.45 ± 0.06
NADPH-cytochrome *c* reductase activity (nmol/min/mg protein)	118.40 ± 12.00	138.7 ± 5.00	118.31 ± 9.02
B(a)P AHH activity (FU/min/mg protein)	6.15 ± 1.30* (32.2%)	4.65 ± 0.26	13.25 ± 1.25*
ECOD activity (FU/min/mg protein)	160.40 ± 3.57* (24.8%)	128.52 ± 2.5	257.85 ± 34.0*
EROD activity (FU/min/mg protein)	102.50 ± 2.5* (49.6%)	68.5 ± 0.50	223.9 ± 30.5*
EROD + α-NF (1 mM) (FU/min/mg protein)		40.30 ± 2.9*	63.52 ± 4.9*

NADPH-cytochrome *c* reductase activity and cytochrome P450 and b_5 contents were determined using 0.1 to 0.2 mg microsomal protein. Monooxygenase activities were determined using 1 to 2 mg microsomal protein in the presence of 1 mM NADPH. α-Naphthoflavone (α-NF) inhibition of enzyme activity was determined after *in vitro* addition of 0.1 to 2 mM α-NF to the assay system. Only the results of a 1 mM concentration of α-NF are shown in the table. Values are the means ± SEM of three determinations. Values in parentheses are the percent higher than female fish.

* Significantly different ($P < 0.05$) from control female fish liver microsomes.

crosomes from fish or rat were also able to metabolize B(a)P to monohydroxy derivatives. However, in this case there was a very little [0.5 to 0.8% of the total B(a)P metabolism] di and tetrol formation (data not shown).

Immunocrossreactivity of fish liver cytochrome P450

Figure 3 shows the result of the crossreactivity of fish liver microsomal CYP with antibody developed against purified rat liver microsomal CYP1A1. Liver microsomes from fish treated with BNF had a major immunoreactive protein band in the region corresponding to a molecular mass of 53.5 kDa. This is comparable to the 54 kDa of rat liver CYP1A1. In liver microsomes from BNF-treated rats, an additional protein band of an apparent molecular mass of 53 kDa was also seen, which was assumed to reflect the induction of CYP1A2. This band was not detected in the liver microsomes from fish treated with BNF.

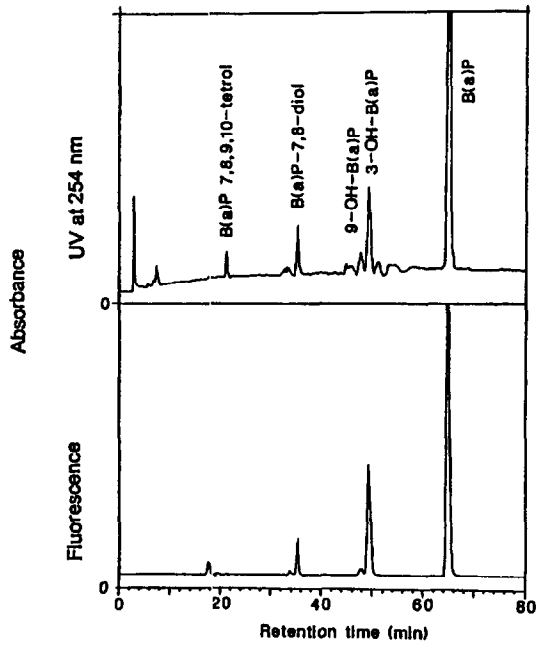

Fig. 1. HPLC profile of B(a)P metabolites extracted from BNF-treated fish liver microsomes. Microsomal enzyme assay conditions and HPLC analysis of B(a)P metabolites using fluorescent and UV detectors were performed as described in Materials and Methods.

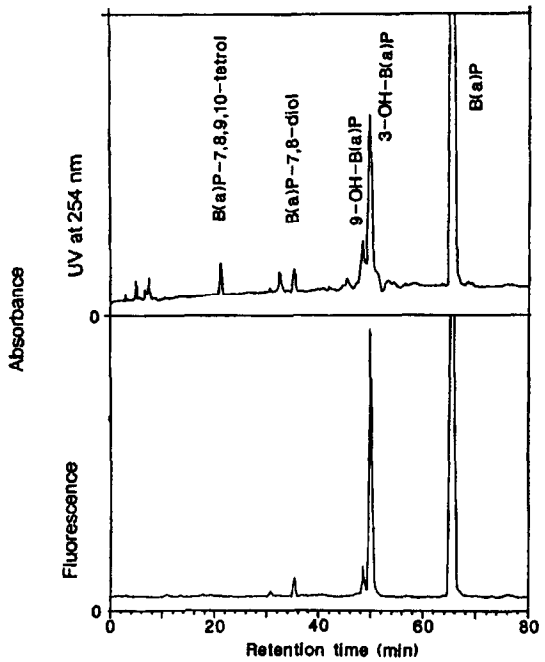

Fig. 2. HPLC profile of B(a)P metabolites extracted from BNF-treated rat liver microsomes.

Table 2. HPLC analysis of the products of benzo(a)pyrene metabolism and percent relative metabolite concentration in BNF-treated fish and rat liver microsomes

	B(a)P	3-OH-B(a)P	9-OH-B(a)P	B(a)P-7,8-diol	B(a)P-7,8,9,10-tetrol	Remainder
Fish	65.0	11.0 (5.2–6.0)	2.7 (1.3–1.6)	4.0 (2.0–2.4)	1.8 (0.85–1.0)	15.5 (7.5–8.5)
Rat	60.5	13.2 (6.5–7.0)	2.2 (1.0–1.5)	3.2 (1.5–1.8)	1.6 (0.75–0.94)	19.3 (9.0–10.5)

Values are expressed as a percentage of the total peak areas at 254 nm. Values in parentheses are the range of absolute values from duplicate assays of B(a)P metabolites as nanomoles of metabolite formed in 15 min at 30 or 37°.

DISCUSSION

Measurement of CYP-related activities is being used increasingly to monitor environmental pollution. The induction of CYP activities in marine and fresh water fish following the exposure to various pollutants has been reported [7, 9, 26, 27]. In the present study we have investigated the ability of a coastal marine safi fish, *S. canaliculatus*, to metabolize B(a)P, a marker for the exposure to PAH. In addition, we have also studied the CYP-dependent monooxygenase activities in fish liver microsomes, by measuring AHH, EROD and ECOD enzyme activities.

Safi fish liver microsomes gave a characteristic CO-spectrum for CYP with an absorption maximum at 449.5 nm (data not shown). The CYP-dependent activities towards a variety of carcinogenic and noncarcinogenic substrates were found to be NADPH dependent (Table 1). The NADPH omission from the assay system resulted in a substantial loss (>90%) of enzyme activities (data not shown). Male fish were found to have a higher CYP activity than female fish. These findings are in agreement with earlier reports that the majority of xenobiotics are preferentially metabolized by male fish liver microsomes [28]. Unlike mammalian CYP which have an optimum temperature of 37°, CYP-catalyzed activities in this marine fish were optimum at 30°. There are many reports which suggest that the environmental temperature has a great influence on xenobiotic metabolism in fish, with the majority of fish liver CYP studies exhibiting an optimum temperature for catalysis below 37° [29].

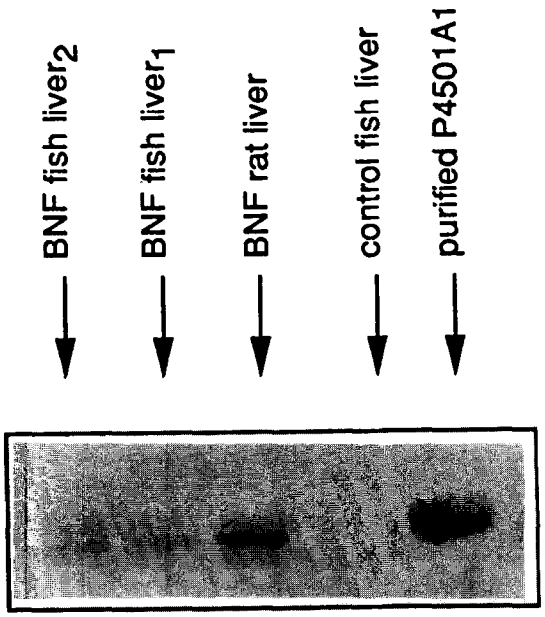

Fig. 3. Western blot analysis of fish liver microsomes using rat CYP1A1 antibody. Purified rat liver CYP1A1 (2 μg) and microsomal preparations (25 μg protein) from control fish and BNF-treated rat and fish (two fish) livers were subjected to 12% SDS–PAGE. Western blotting and immunoreactivity of proteins with CYP1A1 antibody were determined as described in Materials and Methods.

In the present study, BNF, a known inducer of CYP1A1 activity in mammals [22, 25, 30] and fish [26, 31, 32], was found to induce CYP-dependent activities (2- to 4-fold) in the *S. canaliculatus* microsomes (Table 1). An increase in the activities of AHH, EROD and ECOD enzymes suggests the presence of more than one form of CYP enzymes with a specific induction of the CYP1A1 isoenzyme. This was further confirmed by the observation of an inhibitory effect of α-naphthoflavone (a specific inhibitor for CYP1A1 [20] on EROD activity catalyzed by fish liver microsomes. However, neither induction of NADPH-cytochrome *c* reductase nor an increase in the concentration of cytochrome b_5 was seen. On the other hand, the total CYP content itself was increased significantly by a factor of 2.

HPLC analysis of B(a)P metabolites extracted from fish and rat liver microsomes treated with BNF indicated the formation of 7,8-B(a)P diol and 7,8,9,10-B(a)P tetrol. The 7,8-B(a)P diol is considered to be the precursor of a DNA-reacting carcinogenic metabolite, while 7,8,9,10-B(a)P tetrol is a non-reactive B(a)P metabolite formed by CYP action [20, 33]. However, the monohydroxy (3-OH and 9-OH) derivatives of B(a)P were seen as the predominant metabolites. Although not shown, the major metabolites analyzed in control untreated fish liver microsomes were 3-OH- and 9-OH-B(a)P, with little diol or tetrol formation. These observations further support the involvement of CYPs in xenobiotic metabolism in control and BNF-treated fish.

We have further characterized the BNF-inducible CYP in liver microsomes from safi fish by SDS–PAGE analysis and investigated the immunocrossreactivity with polyclonal antibody raised against rat liver CYP1A1 by western blotting. Liver from control fish did not have any detectable CYP1A1. However, the results in Fig. 3 clearly demonstrate the presence of a 53.5 kDa protein in fish liver microsomes crossreacting with the antibody raised against rat CYP1A1. This band corresponds to the purified rat liver BNF-inducible CYP1A1, which migrates in the region of protein with an apparent

molecular mass of 54 kDa. Our present observation suggests that the inducible fish CYP may have a structural homology with that of rat CYP1A1. BNF-induced rat liver microsomes exhibited another major protein band migrating at about 53 kDa, which may reflect the induction of CYP1A2. However, this was not detected in the fish liver.

In the present study, we have used a number of techniques to investigate the CYP-associated xenobiotic-metabolizing enzyme system in the marine fish *S. canaliculatus*. Our results suggest the existence of multiple forms of CYP in *S. canaliculatus* including a CYP1A1 homologous protein that can be induced by BNF. These results suggest that the safi marine fish could be used as a sensitive model for the monitoring of the Arabian Gulf region pollution of the coastal water.

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