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SEX DIFFERENCES IN CYTOCHROME P-450 ISOZYME COMPOSITION AND ACTIVITY IN KIDNEY MICROSOMES OF MATURE RAINBOW TROUT

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Abstract—Kidney microsomes from sexually mature male, as opposed to female, rainbow trout displayed an approximately 20-fold higher cytochrome P-450 specific content, NADPH–cytochrome *c* reductase activity, and rates of hydroxylation of lauric acid, testosterone, progesterone and aflatoxin B_1. Little or no sex difference in metabolism was observed with benzo[*a*]pyrene or benzphetamine as substrates. A similar pattern was observed in hepatic microsomes from these fish, but the difference was much less striking (approximately 2-fold higher activity in males). Juvenile trout (both sexes) possessed activities intermediate between mature males and females. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of kidney and liver microsomes of juvenile and sexually mature male and female trout suggested that the striking sex difference in kidney could be due to the high amount of trout P-450 isozyme LM_2 in sexually mature males. Immunoquantitation of LM_2, performed by Western Blotting and immunostaining with rabbit anti-trout LM_2-IgG, confirmed that mature male kidney contained much higher levels of P-450 LM_2 than juvenile or female kidney, or even of liver microsomes of all three groups. The amount of P-450 LM_2 in mature female kidney microsomes was barely detectable. The high amount of LM_2 in male trout kidney is consistent with the high activity of these microsomes towards lauric acid and aflatoxin B_1, which have been shown previously to be preferentially metabolized by trout P-450 LM_2. It is suggested that rainbow trout may serve as an alternative to the rat as an animal model for the study of sex-dependent differences in cytochromes P-450.

The liver microsomal cytochrome P-450-dependent MFO‖ system in fish is active towards numerous xenobiotics [reviewed in Refs. 1–3]. The MFO system in fish is very responsive to induction by the PAH type of inducer, such as 3-MC, but is refractive to the phenobarbital class of inducers [reviewed in Refs. 3 and 4].

The importance of the further study of the properties and regulation of fish cytochromes P-450 can be appreciated as rainbow trout are being utilized as an animal model for AFB_1-induced hepatocarcinogenesis [5]. Formation of the ultimate carcinogenic metabolite, AFB_1-2,3-epoxide, is a cytochrome P-450-dependent reaction [6]. In addition to AFB_1, rainbow trout have been demonstrated recently to develop liver tumors following dietary exposure to BP [7]. These findings could have important environmental implications as recent studies have demonstrated high incidences of hepatic neoplasms in some populations of fish which appear to be correlated to environmental exposure to PAHs [8, 9].

Recent success in the purification of cytochromes P-450 from rainbow trout [10, 11] and the marine teleost, scup [12], has demonstrated the existence of multiple forms. The major P-450 isozyme purified from BNF-induced rainbow trout, LM_{4b}¶, and the major isozyme purified from scup, $P\text{-}450_E$, are both active towards BP and display immunochemical cross-reactivity. In addition to cross-reacting with scup $P\text{-}450_E$, antibody to trout LM_{4b} inhibits liver microsomal BP-hydroxylase activity catalyzed by winter flounder and brook trout.** Immunochemical analysis by Western Blotting has demonstrated that, in liver, trout LM_{4b} is induced many-fold following exposure to BNF or PCBs [13].

A major constitutive P-450 in rainbow trout, LM_2, is active towards lauric acid [14] and is very effective at activating AFB_1 to the carcinogenic and DNA-binding metabolite, AFB_1-2,3-epoxide [15]. Trout P-450 LM_2 is not induced following exposure to BNF

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‖ Abbreviations: MFO, mixed-function oxidase; 3-MC, 3-methylcholanthrene; AFB_1, aflatoxin B_1; BP, benzo[*a*]pyrene; PAH, polycyclic aromatic hydrocarbon; BNF, β-naphthoflavone; PCBs, polychlorinated biphenyls; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; PMSF, phenylmethylsulfonyl fluoride; DTT, dithiothreitol; and HPLC, high performance liquid chromatography.

¶ The authors have previously [11, 13] adopted a nomenclature system patterned after that developed for rabbit cytochromes P-450. Trout P-450s are designated LM_{1-4} with the subscript reflecting relative mobility on SDS–PAGE gels. Trout LM_2 has a molecular weight of 54,000 and is a major constitutive P-450. Trout LM_{4b}, molecular weight 58,000, is the major isozyme obtained following induction with BNF. This terminology does not imply any structural or functional similarity between trout P-450 LM_2 and LM_{4b} and rabbit LM_2 and LM_4.

** Personal communication, cited with permission of John Stegeman, Woods Hole Oceanographic Institution.

or PCBs when assayed immunochemically [13]. On the contrary, its activity appears to be repressed as evidenced by lower microsomal activity towards lauric acid [14] and AFB_1 [15].

As in the case with rat [reviewed in Ref. 16], rainbow trout, brook trout and winter flounder exhibit marked sex differences in cytochrome P-450 levels and activities towards certain substrates [17, 18]. In both rat and fish, activity is higher in males. Androgens appear to be stimulatory and estrogens inhibitory [19–21]. In the rat, this sex difference is due to the synthesis of male- and female-specific isozymes of cytochrome P-450, requires an intact hypothalamus-pituitary-liver axis and is neonatally imprinted [reviewed in Ref. 22]. The studies with trout suggest a somewhat different mechanism as an intact pituitary does not appear to be required for the sex difference to be expressed [23].

We now present evidence that sexually mature rainbow trout kidney exhibits a sex difference in cytochrome P-450 specific content and activity towards lauric acid, AFB_1 and sex steroids greater than observed previously in any other animal. Furthermore, we attribute this sex difference to higher levels of a particular form of trout P-450, LM_2, in males compared to females or juveniles.

MATERIALS AND METHODS

Chemicals and equipment. [G-^{3}H]AFB_1 was from Moravek Biochemicals. [7,10-^{14}C]BP, [1-^{14}C]lauric acid, [4-^{14}C]testosterone and [4-^{14}C]progesterone were all obtained from Amersham. The unlabeled compounds, which were used to dilute the above to known specific activities, were all obtained from Sigma. Benzphetamine-HCl was provided by the Upjohn Co. [^{125}I]Protein A was purchased from ICN.

Nitrocellulose, utilized in Western Blotting and the [G-^{3}H]AFB_1–DNA binding experiments, was from Schleicher & Schuell. The apparatus and chemicals used for SDS–PAGE were from Bio-Rad and the equipment for Western Blotting was from the E-C. Apparatus Corp. Salmon sperm DNA, PMSF, cytochrome *c*, isocitrate and human serum albumin were from Sigma. Isocitrate dehydrogenase was obtained from Boehringer Mannheim and NADPH was from P-L Biochemicals.

Trout. Rainbow trout *Salmo gairdneri* were obtained from Hide Away Springs Hatchery of Kewaskum, WI. Sexually immature juvenile trout were 8 months of age and weighed an average of 200 g. Sexually mature trout were over 2 years old, weighed an average of 1200 g, and possessed ripe eggs or milt. All trout were maintained at a water temperature of 10°.

Trout were killed by a blow to the head, and the livers and kidneys were removed immediately and placed in either 0.1 M Tris-acetate, pH 7.5, 0.1 M KCl, 1 mM EDTA, 0.1 mM PMSF for liver, or 10 mM potassium phosphate, pH 7.5, 0.25 M sucrose, 1 mM EDTA, 0.1 mM PMSF for kidney. The tissues were then minced, rinsed, and homogenized with 3 vol. of the respective buffers, and microsomes were prepared by ultracentrifugation.

The microsomal pellets were washed once and resuspended in 0.1 M potassium phosphate, pH 7.25, 30% glycerol, 1 mM EDTA, 1 mM DTT and 0.1 mM PMSF and frozen in aliquots at −80°. Cytochrome P-450 content and [^{14}C]laurate hydroxylase activity were determined with fresh microsomes. All other assays were performed with microsomes that had been stored for 1–2 weeks at −80°.

Enzyme assays. Cytochrome P-450 was determined from the CO-reduced versus CO difference spectrum [24] to eliminate hemoglobin interference. NADPH–cytochrome *c* reductase was assayed at 20° [25]. The hydroxylation of [1-^{14}C]lauric acid was measured by a previously described HPLC technique [14], modified from Orton and Parker [26]. [^{14}C]BP hydroxylase was assayed by the method of DePierre *et al.* [27]. The N-demethylation of benzphetamine was assayed by the formation of formaldehyde with Nash reagent [28]. The NADPH-dependent covalent binding of [G-^{3}H]AFB to DNA was determined as previously described [6, 15]). Metabolite profiles of [^{14}C]testosterone and [^{14}C]progesterone were examined by HPLC, utilizing conditions described previously [20], or by autoradiography following TLC [29]. All assays were performed at 29°.

Other assays. SDS–PAGE was performed by the method of Laemmli [30]. The procedure for Western Blotting was from Burnette [31]. In this procedure, proteins separated by SDS–PAGE were transferred electrophoretically (20 V for 3 hr) to nitrocellulose and immunostained with rabbit anti-trout P-450 LM_2-IgG followed by [^{125}I]Protein A. Visualization and quantitation were performed by autoradiography followed by densitometry.

Rainbow trout cytochromes P-450 LM_2 and LM_{4b} were purified from hepatic microsomes of BNF-induced fish [10, 11]. Rabbits were immunized and IgG fractions prepared as described previously [11]. Protein was assayed by the method of Lowry *et al.* [32] with human serum albumin as standard.

RESULTS

Sex differences in the specific content and activity of the MFO system of rainbow trout kidney microsomes. The specific content of cytochrome P-450 and specific activity of NADPH–cytochrome *c* reductase were approximately 20-fold higher in kidney microsomes of sexually mature male trout compared to female (Table 1). This level of cytochrome P-450 of approximately 1.7 nmoles/mg is, to our knowledge, the highest reported for kidney of any animal and is higher than that reported for hepatic cytochrome P-450 levels in fish.

The specific content of kidney microsomal cytochrome P-450 in sexually immature juvenile trout was intermediate between that of sexually mature males and females. When kidney microsomal activity towards a number of endogenous substrates and xenobiotics was examined, the males displayed higher activity towards lauric acid, AFB_1, progesterone and testosterone (Table 1). The higher activity towards [^{14}C]testosterone was reflected by an increase in the formation of one of the three major polar metabolite peaks observed upon HPLC or on autoradiograms of the TLC plates (data not shown). The identity of this peak was not confirmed but is tentatively designated as 6β-hydroxytesto-