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EFFECTS OF SOME COMMON INDUCERS ON THE HEPATIC MICROSOMAL METABOLISM OF ANDROSTENEDIONE IN RAINBOW TROUT WITH SPECIAL REFERENCE TO CYTOCHROME P-450-DEPENDENT ENZYMES

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Abstract—The effects of 16α-cyanopregnenolone, 3-methylcholanthrene (3-MC), Clophen A 50 (Cl 50) and phenobarbital on the total amount of cytochrome P-450 and the metabolism of 4-androstene-3,17-dione in liver microsomes from rainbow trout were studied. Only 3-MC and Cl 50 caused marked changes in the cytochrome P-450 levels and the cytochrome P-450-dependent steroid hydroxylase activities. A varying response to 3-MC and Cl 50 was seen in fish of different ages and sex. Different responses of 6β-hydroxylase towards α-naphtoflavone, SKF 525-A and metyrapone *in vitro* were seen in 3-MC- and Cl 50-treated fish when compared to control fish. It is suggested that endocrine factors may be involved in the regulation of cytochrome P-450-mediated metabolism in fish and the presence of multiple forms of cytochrome P-450 in trout liver is indicated.

Recently, cytochrome P-450-dependent reactions in fish liver have received a great deal of attention [1]. In contrast to earlier reports [2], it is now established that fish liver possesses drug metabolizing enzyme systems similar to those of the mammalian liver. Induction of hepatic cytochrome P-450 in fish by environmental pollutants, such as PCB's and polycyclic polyaromatic hydrocarbons (PAH), has been reported [3–6]. However, attempts to induce cytochrome P-450 by barbiturates and related compounds have been unsuccessful in several species of fish [6–9].

In mammals, the liver microsomal hydroxylase system participates in detoxification reactions of a variety of xenobiotics as well as in metabolism of endogenous compounds, such as steroid hormones [10]. It has been shown that cytochrome P-450, the terminal oxidase of the microsomal hydroxylase system, exists in multiple forms differing with respect to substrate specificity, sensitivity to specific inhibitors and degree of inducibility by a variety of compounds, such as drugs and environmental pollutants [11–13]. In a previous report we demonstrated the involvement of cytochrome P-450 in steroid hormone metabolism in fish liver [14]. Whether liver microsomal hydroxylation of steroid hormones in fish is catalysed by a species of cytochrome P-450 similar to that involved in detoxification reactions is not known. Since it has been suggested that many environmental pollutants interfere with animal reproduction by disturbing the metabolism of steroid hormones [15–17], it was considered of interest to study the effects of some common inducers on the hepatic metabolism of androstenedione in rainbow trout of different age and sex.

The present study describes the effects of 16α-cyanopregnenolone, phenobarbital, Clophen A 50 and 3-methylcholanthrene on the hepatic microsomal metabolism of androstenedione in rainbow trout with special reference to cytochrome P-450-mediated metabolism.

MATERIALS AND METHODS

Chemicals. 4-[4-^{14}C]Androstene-3, 17-dione (specific radioactivity, 57.5 mCi/mmol) was purchased from New England Nuclear, Boston, MA, and was purified by thin-layer chromatography prior to use. Unlabelled androstenedione and 16α-cyanopregnenolone (PCN) were supplied by Dr. John Babcock (The Upjohn Co., Kalamazoo, MI). Clophen A 50 (Cl 50) was donated by Bayer Chemicals, Germany. SKF 525-A (SKF) (2-diethylaminoethyl-2,2-diphenylvalerate HCl) was a gift from Smith, Kline & French Laboratories, Welwyn Garden City, England. Metyrapone (MP) was supplied by Ciba Geigy Chemical Co (Hässle, Gothenburg, Sweden). NADPH, DL-isocitric acid (trisodium salt) and isocitric dehydrogenase (Type IV, 36 units/ml) were purchased from Sigma Chemical Co, St. Louis, MO. 3-Methylcholanthrene (3-MC) and α-naphtoflavone (ANF) were obtained from Fluka AG, Buchs SG, Switzerland. Phenobarbital (PB) was purchased from Apoteksbolaget, Gothenburg, Sweden.

Fish. Cultured rainbow trout, *Salmo gairdnerii*, were obtained from a local hatchery close to Gothenburg (in September–March). The fish used in the experiment weighed from 70 to 700 g and were taken at successive times in their sexual cycle, spanning the period from immaturity to spawning (yearling, 0–1 yr old; juvenile, 1–1.5 yr old; maturing, 1.5 yr old; prespawning and spawning, 1.5–2 yr old). Groups A and B, taken in September, consisted of yearling trout (70–100 g) and 1-yr-old juvenile trout (200–300 g) of both sexes, respectively. Group E, taken in November, consisted of spawning males

(500 g). Group C, taken in December, consisted of maturing females (600–700 g) and group D, taken in March, consisted of prespawning and spawning female trout (500–600 g). The trout were kept in basins with filtered, recirculating and aerated fresh water at a temperature of 10°. Under these conditions the fish were acclimatized and starved for at least 7 days prior to the experiment.

Treatment of fish. Trout were injected (i.p.) on day 1 and day 3 with Cl 50 (500 mg/kg in corn oil), 3-MC (20 mg/kg in corn oil) and PCN (40 mg/kg in saline), respectively. Control trout received i.p. injections of corn oil. Preliminary data showed saline and corn oil controls to yield similar results. One group of 1.5-yr-old juvenile trout, taken in March, was injected (i.p.) daily for 7 days with sodium phenobarbital (PB) (80 mg/kg in saline). Control trout received vehicle only. After treatment the fish were kept in separate acquaria, filled with 50 l non-circulating but aerated water at a temperature fluctuating between 10 and 15°. The water was changed daily. Sampling was performed on day 14 for the Cl 50, PCN and 3-MC experiments and on day 7 for the PB experiment.

Preparation of liver microsomes. The fish were killed by a blow on the head and the livers were isolated and washed with ice cold 0.154 M KCl. Individual livers were weighed. Microsomes were prepared as described previously [14] and resuspended in 0.1 M sodium phosphate buffer, pH 7.4, to give a final concentration of about 15 mg microsomal protein per ml. The whole procedure was performed at 4°.

Assays. The total amount of cytochrome P-450 was measured by the method of Omura and Sato [18] using a Beckman DB-GT spectrophotometer.

Microsomal protein content was determined by the method of Lowry *et al.* [19], using bovine serum albumin as standard. The incubations of androstenedione were carried out in a total volume of 3.0 ml, containing 2.5 ml Bucher medium [20] and liver microsomes corresponding to 2–6 mg of microsomal protein in the presence of an NADPH-regenerating system consisting of 0.03 μmole $MnCl_2$, 3.0 μmole NADP, 12.5 μmole isocitrate and 0.4 i.u. isocitric dehydrogenase.

After preincubation for 3 min at 27° the reaction was started by the addition of androstenedione (300 μg, 4×10^5 cpm) in 50 μl acetone. The incubation was terminated after 15 min by adding 10 ml chloroform:methanol (2:1, v/v). The incubation conditions were designed to give linear total conversion of substrate with time and enzyme concentration. In addition to the standard incubations, additional tubes containing ANF (10^{-4} M), SKF (10^{-3} M) and MP (10^{-3} M), respectively, were incubated to test for enzyme activation or inhibition. The inhibitors were added to the incubations in 25 μl acetone. The same volume of acetone was also added to the respective control incubations. A series of incubations containing different amounts of steroid (from 25 to 200 μg) was performed in the absence or presence of inhibitors with microsomes from untreated and treated male trout. These incubations were carried out with liver microsomes pooled from 2 to 4 fish from each group.

Analysis of incubation extracts. The incubation mixtures were extracted and analysed by thin-layer chromatography and autoradiography as described previously [14]. The radioactive zones were localized, scraped off separately and measured for radioactivity in a Packard Tri-Carb scintillation spectrometer. The steroid metabolites in the various zones were identified by gas chromatography–mass spectrometry (LKB 2091 instrument) as described before [21]. Statistical analysis was performed by means of Student's *t*-test and the significance level was set at 0.05.

RESULTS

The metabolites formed on incubation of [4-^{14}C]-androstenedione with trout liver microsomes have been described and identified in a previous study [14]. The compounds identified and measured in the present investigation were 6β- and 16-hydroxyandrostenedione (no attempt was made to separate the 16β- and 16α-hydroxylated metabolites), testosterone and 5α-androstane-3,17-dione. Quantitation of these metabolites made it possible to assay the 6β- and 16-hydroxylase, 17-hydroxysteroid oxidoreductase and 5α-reductase activities.

Effects of PCN, Cl 50 and 3-MC on total cytochrome P-450 content. The effects of PCN, Cl 50 and 3-MC on the total amount of cytochrome P-450 in liver microsomes from trout of different age and sex are presented in Fig. 1. Whereas PCN treatment had no effect on the cytochrome P-450 content in any of

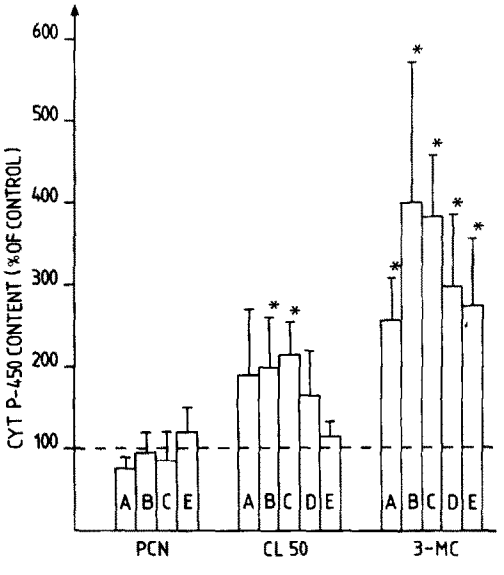

Fig. 1. Effects of PCN, Cl 50 and 3-MC on total amount of cytochrome P-450 in liver microsomes from trout of different age and sex. Cytochrome P-450 content was measured as nmoles per mg protein (mean ± S.D.) and the values for the untreated fish were: 0.16 ± 0.06 for yearling trout (A); 0.19 ± 0.09 for 1-yr-old juvenile trout (B); 0.15 ± 0.08 for maturing female trout (C); 0.17 ± 0.04 for prespawning and spawning female trout (D); 0.29 ± 0.05 for spawning male trout (E). Cytochrome P-450 content is expressed as a percentage of the content in the control group which is set at 100. Bars indicate S.D. for 4–9 individuals. *$P < 0.05$ when compared to controls.

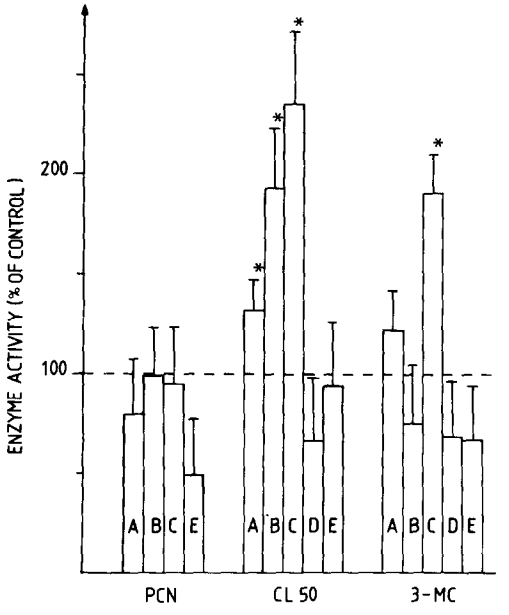

Fig. 2. Effects of PCN, Cl 50 and 3-MC on the 6β-hydroxylase activity in liver microsomes from trout of different age and sex. 6β-Hydroxylase activity was measured as nmoles per min per mg protein (mean ± S.D.) and the values for the untreated fish were: 0.23 ± 0.06 for yearling trout (A); 0.21 ± 0.05 for 1-yr-old juvenile trout (B); 0.11 ± 0.02 for maturing female trout (C); 0.29 ± 0.07 for pre-spawning and spawning female trout (D); 0.29 ± 0.10 for spawning male trout (E). Enzyme activity is expressed as a percentage of the activity in the control group which is set at 100. For further explanations see Fig. 1.

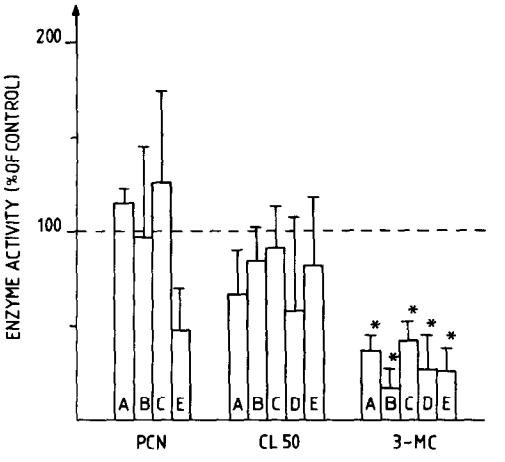

Fig. 3. Effects of PCN, Cl 50 and 3-MC on the 6β-hydroxylase activity in liver microsomes from trout of different age and sex. 6β-Hydroxylase activity was measured as nmoles per min per nmol cytochrome P-450 (mean ± S.D.) and the values for the untreated fish were: 1.62 ± 0.76 for yearling trout (A); 1.29 ± 0.58 for 1-yr-old juvenile trout (B); 0.92 ± 0.33 for maturing female trout (C); 1.83 ± 0.60 for prespawning and spawning female trout (D); 1.02 ± 0.39 for spawning male trout (E). Enzyme activity is expressed as a percentage of the activity in the control group which is set at 100. For further explanations see Fig. 1.

the investigated groups, 3-MC significantly increased the total amount of cytochrome P-450 in all groups of fish. In yearling trout the increase was twofold, in female trout at different stages of development and mature male trout, threefold, and 1-yr-old juvenile fish, fourfold. However, the CO difference spectra of 3-MC-induced cytochrome P-450 did not show the spectral shift to 448 nm characteristic of the mammalian system [22]. This observation is consistent with other studies on cytochrome P-450 in fish [6, 23, 24]. Cl 50, a mixed type of inducer in the rat, significantly increased the total amount of cytochrome P-450 twofold in 1-yr-old juvenile and maturing female trout, but had no effects on the cytochrome P-450 content in liver microsomes from yearling or mature trout.

Effects of PCN, Cl 50 and 3-MC on the hydroxylation of androstenedione. 16-Hydroxylation, which is a minor pathway in the trout, was unaffected by Cl 50 or 3-MC (data not shown). However, these inducers significantly stimulated 6β-hydroxylation of androstenedione twofold in maturing female trout, while no effects were seen in female fish at an advanced state of maturity (Fig. 2). In addition, Cl 50 significantly increased the rate of 6β-hydroxylation in juvenile trout while male trout did not respond to 3-MC or Cl 50. PNC treatment did not affect 6β-hydroxylation in trout. Figure 3 shows the effects of PCN, Cl 50 and 3-MC on the 6β-hydroxylase activity expressed per min per nmol cytochrome P-450. The 6β-hydroxylase activity was significantly lower in all 3-MC treated groups when compared to the corresponding control groups. PCN or Cl 50 did not significantly change the specific 6β-hydroxylase activity.

Effects of ANF, SKF and MP in vitro *on the 6β-hydroxylase activity in liver microsomes from control and PCN-, Cl 50- and 3-MC-treated trout.* In order to further characterize the effects of PCN, Cl 50 and 3-MC on trout liver microsomal 6β-hydroxylation, the effects of known inhibitors of cytochrome P-450-dependent enzymes were tested. The compounds were chosen on the basis of their specific effects on different forms of cytochrome P-450. Table 1 shows

Table 1. *In vitro* inhibition of trout liver microsomal 6β-hydroxylase activity from yearling trout by SKF, MP and ANF. Results are expressed as per cent of the corresponding non-inhibited control. Values in brackets represent activity expressed as nmoles per min per mg protein in the non-inhibited control

Treatment		SKF (10^{-3} M)	MP (10^{-3} M)	ANF (10^{-4} M)
None	[0.23 ± 0.06]	58	63	65
PCN	[0.18 ± 0.06]	64	109	75
Cl 50	[0.30 ± 0.03]	37	39	43
3-MC	[0.28 ± 0.06]	36	48	37

the effects *in vitro* of ANF, SKF and MP on the 6β-hydroxylase activity in microsomes from yearling trout treated with different inducers. In general, the 6β-hydroxylase activity in control and PCN-treated fish was less sensitive to inhibition when compared to the enzyme activity in 3-MC and Cl 50-treated fish.

Effects of PCN, Cl 50 and 3-MC on the reductive metabolism of androstenedione. The effects of PCN, Cl 50 and 3-MC on the reductive metabolism of androstenedione in liver microsomes from trout were very small (data not shown). The only observed effect was a significant decrease (50%) in 17-hydroxysteroid oxidoreductase activity in liver microsomes from PCN treated mature trout (data not shown).

Effects of PB on the liver microsomal metabolism of androstenedione and total cytochrome P-450 content. PB treatment had no effect on the cytochrome P-450 content in 1.5-yr-old juvenile trout (data not shown). Furthermore, in contrast to Cl 50 and 3-MC, PB did not affect the hydroxylation or reduction of androstenedione (data not shown).

DISCUSSION

In mammals, and presumably in fish, microsomal hydroxylation is mediated by different species of cytochrome P-450 which exhibit individual responsiveness towards inducing agents belonging to the PB and PAH classes of inducers [25]. The presence in fish of microsomal hydroxylases, e.g. aryl hydrocarbon hydroxylase (AHH), responsive to the PAH type of inducers is well documented [5, 6, 23] but there is very little information on the heterogeneity of the cytochrome P-450 system in these animals. Since steroid hormones are hydroxylated at multiple positions around the steroid skeleton and different forms of cytochrome P-450 seem to be involved in these hydroxylations, steroid substrates may be used to characterize the specificity of different forms of cytochrome P-450 [12, 26].

In a recent publication we have described the presence of cytochrome P-450-dependent steroid hydroxylases in liver microsomes from fish [14]. While in the rat androstenedione is mainly hydroxylated in positions 6β, 16α and 7α [21], the predominating hydroxylation in trout liver is 6β-hydroxylation, 16-hydroxylation being a minor pathway [14].

In the present study, 3-MC and Cl 50 were shown to increase liver microsomal 6β-hydroxylase activity in trout, whereas no effects were observed with PCN or PB. 16-Hydroxylation, which is PB- and PCN-inducible in the rat [27, 28], was not affected by any of the inducers in trout. The response of 6β-hydroxylase to 3-MC and Cl 50 in fish was shown to vary with age and sex. While the enzyme was inducible in maturing female trout (by 3-MC and Cl 50) and in juvenile trout (by Cl 50) it was unaffected by either inducer in prespawning female trout and spawning fish of both sexes. The observed variability of induction in female fish of different maturity is most probably due to hormonal factors. It is known that changes in steroid balance can influence the metabolism of drugs and steroid hormones [29, 30] and the response of the rat liver to inducing agents [31, 32]. In trout, seasonal reproductive development requires the integrated activities of the pituitary, the liver and the gonads [33–35]. A vitellogenic protein is produced in the liver of the female fish under estrogen stimulation and dramatic increases in gonadotropin are usually found in spawning trout [33]. These hormonal changes and the active vitellogenin synthesis in the liver in the developing fish may have profound effects on the hepatic steroid metabolism, as well as the inducibility of the cytochrome P-450 system, and may explain the great variability in enzyme inducibility in maturing fish.

Although 3-MC was the most potent inducer of the total amount of cytochrome P-450 in all groups of fish (2 to 4-fold), indicating a high responsiveness of trout cytochrome P-450 to the PAH type of inducers, the 6β-hydroxylase activity was only increased in maturing female fish. Furthermore, whereas the effects of Cl 50 on the cytochrome P-450 content in trout liver were not as pronounced as those observed after 3-MC treatment, the effects of Cl 50 on 6β-hydroxylation were marked in several groups of fish. These differences in inducibility of total amount of cytochrome P-450 and 6β-hydroxylase may be explained by different responsiveness of various types of cytochrome P-450 to inducers.

The lower induction of the total amount of cytochrome P-450 by Cl 50 (a mixed type of inducer in the rat) when compared to 3-MC, may be explained by the presence of a higher proportion of PAH- than PB-inducible forms of cytochrome P-450 in trout. This is in line with earlier reports [6–8] and our own present results showing a lack of induction of the total amount of cytochrome P-450 by PB in fish, as well as with the suggestion by Ahokas *et al.* [36] that trout liver cytochrome P-450 resembles the PAH-inducible form in the rat.

The large increase in the total amount of cytochrome P-450 in the absence of a concomitant increase in 6β-hydroxylase activity in 3-MC-treated juvenile and mature fish of both sexes indicates that a novel type of cytochrome P-450 with a low specificity for steroid substrates is induced by 3-MC. In contrast to the 6β-hydroxylase activity, the AHH activity is increased 20 to 30-fold in trout pretreated with 3-MC [23]. Furthermore, the higher sensitivity of 6β-hydroxylase in Cl 50- and 3-MC-treated fish than in control and PCN-treated fish to inhibitors of cytochrome P-450 supports the contention that a novel type of cytochrome P-450 has been induced or that the ratio between different cytochrome P-450 species has been changed.

A differential effect of ANF on AHH activity in liver microsomes from control and PAH-treated rats has been well established [37]. Thus, ANF preferentially inhibits AHH activity in 3-MC-treated rat liver and enhances AHH activity in control liver microsomes *in vitro*, while MP and SKF preferentially inhibit control and PB-inducible monooxygenase activities [11, 37]. In trout, ANF inhibits both control and PAH-induced AHH activity while MP and SKF are less potent inhibitors [36, 38]. However, the 6β-hydroxylase activity in control and induced trout liver was not differentially inhibited by ANF, MP and SKF, suggesting the presence of multiple forms of cytochrome P-450 in liver microsomes from control as well as induced trout. The presence of

multiple forms of cytochrome P-450 in fish is an important concept for the understanding of variations in cytochrome P-450-mediated reactions in fish of different age and sex as well as in fish exposed to environmental pollutants. Therefore, efforts to isolate and characterize cytochrome P-450 from fish liver microsomes are essential for further advances in this field.

The variable response of cytochrome P-450 to 3-MC and Cl 50 with age and sex suggests that pollutants may affect the fish differently depending on their stage of development and thus the physiological consequences of the exposure may vary in different endocrinological situations. In view of this it seems essential to investigate the hormonal regulation of liver microsomal cytochrome P-450 in fish.

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