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Pituitary as a target organ for toxic effects of *P4501A1* inducing chemicals

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Summary

We report here that cytochrome *P4501A1* in the male rainbow trout pituitary is highly inducible by β -naphthoflavone. Pituitary cells containing inducible *P4501A1* were identified by double immunostaining as gonadotrophs containing gonadotropin II. Thus, the pituitary gonadotrophs may be target cells for polyaromatic hydrocarbons. Elevated plasma levels of gonadotropin II (GTH II) and testosterone in the induced fish indicated that the functioning of the pituitary was disturbed. Because GTH II regulate the final stage of sexual maturation the results implies that exposure to *P4501A1* inducing compounds may disturb this development stage.

Introduction

Chlorinated dioxins, biphenyls and related aromatic hydrocarbons interfere with several important physiological functions including reproduction (Poland and Knutson, 1982; Safe, 1984). Such interference is probably due to toxin-induced disturbances to the hormonal systems regulating reproduction. This contention is supported by reports of major hormone imbalances, pathological alterations in reproductive organs, and depression of steroid biosynthesis in rodents exposed to this group of chemicals (Silberberg and Mattison, 1987). In nature, at high trophic levels predators are exposed to high concentrations of persistent aromatic hydrocarbons bioaccumulated in the food chain. The low reproductive rates reported for Baltic seals and salmon as well as birds from the Great Lakes have been tentatively attributed to the contamination of the aquatic environment by dioxins, polychlorinated biphenyls (PCB) and structurally related chemicals (Bergman and Olsson, 1985; Mac et al., 1985; Fox et al., 1992). In some of these animal populations, fertility has been completely abolished.

It has been suggested that the various toxic re-

sponses induced by dioxins and related chemicals are mediated by a common initial event, i.e., the binding of hydrocarbons to the cytosolic Ah receptor (Landers and Bunce, 1991). The ligand-receptor complex is translocated to the nucleus where it stimulates gene transcription. The role of this receptor in inducing cytochrome *P4501A1* (*P4501A1*) is well understood. The large diversity of toxic effects produced by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and related compounds may be due to the fact that the ligand-Ah receptor complex regulates different sets of genes in each cell type.

To determine whether the pituitary would be a target organ for dioxins and dioxin-like compounds, we examined the inducibility of *P450*-dependent ethoxyresorufin-*O*-deethylase and the localization of *P4501A1* in the tissue. The results show that *P4501A1* is induced specifically in gonadotropin II (GTH II) containing cells. Furthermore, increased plasma levels of GTH II and testosterone were measured in trout treated with *P4501A1* inducing compounds.

Materials and methods

Male rainbow trout in their second reproductive cycle and ranging in weight between 1–2 kg, were obtained from a local fish farm. The fish were acclimated to laboratory conditions for 2 weeks prior weekly i.p. injections of β -naphthoflavone (BNF) (5 mg/kg)

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for 10 weeks. Samples were collected when the trout were spermiating. We chose to use BNF as our model inducing compound since this chemical is metabolized faster and is less toxic than TCDD, but both compounds induce the *P4501A1* system through the same receptor mechanism (Boobis et al., 1977).

At sampling the whole pituitary was homogenized in 0.1 M phosphate buffer containing 0.2 mM butylated hydroxytoluene, 1 mM dithiothreitol, 0.1 mM EDTA, and 0.1 mM phenylmethylsulfonyl fluoride. Activities were measured immediately in the homogenate as described previously (Andersson et al., 1985).

Immunoblotting was performed after electrophoresis of samples of 7.5% acrylamide separating slab gels. Transfer of the protein to nitrocellulose sheets was performed in a Min-protean II blotting cell (Bio-Rad) according to manufacturers procedure. Antiserum containing anti-perch *P4501A1* IgG (Celerander and Förlin, 1991) was used to study crossreacting proteins. Horseradish-peroxidase conjugated goat anti-rabbit IgG (GAR-HRP) was used as secondary antibody. Color development was performed as described by the manufacturers.

To fix tissue sections for immunohistochemical analysis, they were kept immersed in 0.1 Na-cacodylate buffer (pH 7.3) containing 0.4% (w/w) *p*-benzoquinone for 4 h followed by immersion in 0.1 M Na-cacodylate buffer containing 20% sucrose for 12 h. Cryostat slices (10 μ m) were incubated with antiserum containing perch anti-*P4501A1* (Celerander and Förlin, 1991) (diluted 500 times) in phosphate-buffered saline containing albumin (4%) and Na-azide (2%) in a moist chamber overnight at room temperature. Swine anti-rabbit labeled with fluorescein isothiocyanate was used as a secondary antibody for fluorescence microscopy.

Sections were double stained using an indirect immunofluorescence method including an intermediate step in which rabbit anti-IgG activity was blocked by paraformaldehyde vapor treatment (Wang and Larsson, 1985). Sections were first incubated with anti-*P4501A1* serum (diluted 500 times) and the second antibody was fluorescein isothiocyanate (FITC)-labeled sheep anti-rabbit IgG. After treatment with formaldehyde vapor the sections were incubated with anti-GTH II serum, anti-GTH I serum or anti-GH serum diluted 1000 times. The second antibody was tetramethyl rhodamine isothiocyanate (TRITC)-labeled anti-rabbit IgG. Sections were also stained with the primary antibodies in reverse order which gave the same results. Antibody specificities has been characterized by Suzuki et al. (1988) and Kawauchi et al. (1986). Conditions were optimal for the formaldehyde treatment and primary antibody concentrations used. Immersion of sections from induced fish with preimmune serum or sections from uninduced fish with anti-*P4501A1* gave no staining. The sections were examined in a fluores-

Fig. 1. EROD activity in pituitary homogenate from control and BNF-treated rainbow trout. Values are the mean $\pm$ SD of seven individuals. *, $P < 0.05$ compared with control (Mann-Whitney U test).

cence microscope with selective filters at 490 nm (FITC) and 546 nm (TRITC).

Plasma levels of GTH II were determined by radioimmunoassay (RIA) as described by Breton et al. (1978), using an antibody developed against the rainbow trout GTH₂ and a standard purified from chinook salmon (*Oncorhynchus tshawytscha*). This gonadotropin was equipotent to Kawauchi's GTH II in RIA as demonstrated by Sumpter and Scott (1989). The testosterone levels were measured by RIA without extraction, as previously described (Fostier and Jalabert, 1986).

Results

The cytochrome *P450* enzyme system in the male rainbow trout (*Oncorhynchus mykiss*) pituitary was induced about 4-fold by BNF (Fig. 1). Activity was recorded as 7-ethoxyresorufin-*O*-deethylation (EROD) which is catalyzed by the *P4501A1* isoenzyme in teleosts

Fig. 2. Western blot analysis of pituitary homogenates from control (C) and BNF-induced male rainbow trout. The lanes contained 10 μ g protein of pituitary homogenate from control trout (lane 1), 5 and 10 μ g protein of pituitary homogenate from BNF treated trout (lanes 2 and 3, respectively), and molecular weight standards (lane 4).

Fig. 3. Immunohistochemical localization of *P4501A1* in rainbow trout pituitary slices incubated with a polyclonal antibody diluted 500 times. Distinct staining could be seen in the proximal pars distalis from BNF-treated fish (A and B, magnification $350\times$ and $2100\times$, respectively), whereas no staining was seen in untreated fish (C, magnification $2100\times$).

Fig. 4. Fluorescence microscopical double staining of the proximal pars distalis of rainbow trout pituitary slices. Double exposure of the sections using contrasting filters revealed that anti-P4501A1 recognizes the same cells as anti-GTH II (A1 and A2), whereas no co-localization of P4501A1 and GTH I (B1 and B2) or P4501A1 and GH (C1 and C2) could be seen. Magnification is 1250 $\times$.

Fig. 4 (continued).

(Stegeman, 1989). Untreated fish contained a low but detectable EROD activity.

Western blot analysis with anti-perch *P4501A1* gave a weak signal at 58,000 in pituitary from BNF treated trout (Fig. 2). No signal was obtained from control trout pituitary homogenates. A molecular mass of 58,000 has previously been reported for hepatic *P4501A1* proteins in several teleosts (Goksøyr et al., 1991).

Immunohistochemical examinations of control fish did not reveal any staining after incubation with *P4501A1* antibodies, indicating that the enzyme level

was low. In BNF-treated fish, however, anti-*P4501A1* clearly stained distinct cells in the proximal pars distalis section of the pituitary (Fig. 3). Double immunostaining revealed that these cells contained gonadotropin II, a peptide hormone in salmonids which is important for the final maturation of the germ cells. The same area also contains GTH I and growth hormone producing cells. However, double immunostaining did not reveal any co-localization of *P4501A1* with the two peptide hormones GTH I and growth hormone.

Plasma levels of GTH II were higher in BNF-ex-

Fig. 5. Plasma levels of GTH II (A) and testosterone (B) in control and BNF-treated rainbow trout. The values $\pm$ SD of 7 individuals. *, $P < 0.05$ compared with control (Mann-Whitney *U* test).

posed fish than in untreated fish (Fig. 5A). The plasma testosterone level was also increased by BNF treatment (Fig. 5B), possibly because GTH II stimulates gonadal steroid biosynthesis.

Discussion

The function and toxicological significance of cytochrome *P*450 isoenzymes in the pituitary are poorly understood. In mammals the only evidence for the presence of *P*4501A1 in the pituitary is that 7-ethoxycoumarin-*O*-deethylase activity is inhibited by polyclonal antibodies raised against the *P*4501A1 protein in BNF treated rat but not in control rat (Näslund et al., 1988). The present study clearly shows that BNF treatment of male rainbow trout induce the pituitary EROD activity, a *P*4501A1 catalyzed reaction in teleosts (Stegeman, 1979). Western blot analysis gave further evidence for induction of *P*4501A1 in the pituitary by BNF. Antibodies against *P*4501A1 recognized a protein in pituitary from BNF treated trout with the same electrophoretic mobility as hepatic *P*4501A1 whereas no signal could be detected in control trout. However the signal from pituitary homogenates from BNF treated trout was weak indicating an overall low concentration of the protein. The immunohistochemical analysis showed that inducible *P*4501A1 was confined to certain cells identified with double immunostaining as gonadotrophs containing GTH II. The concentration of *P*4501A1 in the cell population containing the enzyme may therefore be high.

The toxicological and pathological effects of polyaromatic hydrocarbons, including reproductive impairment, are well known. However, their mechanisms of action are not well understood. In male rats TCDD caused a pronounced androgenic deficiency (Moore et al., 1985). The reduction in levels of serum androgens could have been due to TCDD-induced inhibition of the steroidogenic enzymes 17 α -hydroxylase and 17,20-lyase in the testis (Mebis et al., 1987). Recently, Bookstaff et al. (1990) suggested that TCDD alters the negative feed back system by which steroids regulate pituitary hormone secretion by, in some unknown way, increasing the potency of the circulating steroids. In mature male rainbow trout and carp PCB treatment for 4 weeks (weekly injections with 25 mg/kg body weight) resulted in a 45% reduction in levels of circulating androgens (probably measuring both 11 ketotestosterone and testosterone) and morphological alterations of the head region of the spermatozoa (Sivarajah et al., 1978a, b). Good correlations between the decreased amount of steroidogenic tissues and plasma steroid levels were seen in Atlantic croaker treated with PCB (Thomas, 1990). In Atlantic croaker PCB also altered the in vitro secretion of gonadotropin from isolated pituitaries. Field investigations on fish

living in water polluted with a complex mixture of organic pollutants have also indicated a perturbation in plasma hormone levels which probably caused the observed abnormal reproductive development (Munkittrik et al., 1991; McMaster et al., 1991). In the present study plasma testosterone levels increased by 80% and GTH II levels by 30% in response to BNF. These hormonal changes are within the range previously reported to affect reproductive functions in fish (Sivarajah et al., 1978a, b; Thomas, 1990; McMaster et al., 1991; Munkittrik et al., 1991). The study started during the late stage of maturation and sampling was performed after 10 weeks when the fish were spermiating. In spermiating rainbow trout the levels of gonadotropins, measured by a radioimmunoassay preferentially measuring GTH II, in plasma and pituitary reach a plateau whereas the level of androgens rapidly decreases after peak values which are reached before or at the onset of spermiation (Scott and Sumpter, 1989; Sumpter and Scott, 1989). The mechanism for the elevated plasma GTH II and testosterone levels caused by BNF treatment, seen in the present study, is not clear but may be due to a direct effect on the gonadotrophs resulting in an increased secretion of the peptide hormone from the pituitary.

The double immunostaining technique used indicated that trout gonadotrophs containing GTH II were particularly susceptible to induction of *P*4501A1 by BNF. This reflect their capacity to take up aromatic hydrocarbons which can lead to formation of reactive and cytotoxic metabolites formed during *P*450 catalyzed oxidations of xenobiotics (Gelboin, 1980). Furthermore, other cellular functions involved in gonadotropin synthesis or release could be affected by *P*4501A1 induction. For example, TCDD exposure has been reported to alter the intracellular Ca²⁺ homeostasis in heart cells and thymocytes, and it has been suggested that *P*4501A1 participate in the regulation of plasma membrane permeability to Ca²⁺ (Canga et al., 1988; McConkey et al., 1988; Alvarez et al., 1991). Any effects on Ca²⁺ regulation in gonadotrophs could directly influence the secretion of the hormone since this ion serves as a second messenger required for hormone release (Conn et al., 1986).

An increase in *P*4501A1 level could also lead to an elevated metabolism of essential compounds or to the production of metabolites modifying the cascade of intracellular events leading to the release of gonadotropin. In this context, the metabolism of arachidonic acid is of special interest since the metabolic conversion of arachidonic acid seems to be involved in the regulation of hormone release by the pituitary (Fitzpatrick and Murphuy, 1989). Rifkind et al. (1991) showed that TCDD causes a major increase in the NADPH-dependent metabolism of arachidonic acid in chick embryo liver microsomes. Purified rat *P*4501A1

was recently shown to be especially active in monohydroxylating arachidonic acid in the 19 position (Falck et al., 1990). Thus the arachidonic acid metabolism in organisms exposed to polyaromatic hydrocarbons may be altered which could result in the impairment of important physiological functions.

In summary, the present study shows that gonadotrophs containing GTH II are target cells for P4501A1 inducing compounds. Elevated plasma levels of GTH II and testosterone suggests that the functioning of the gonadotrophs were disturbed leading to a hormonal imbalance. Because GTH II regulates the final stage of sexual maturation these results implies that exposure to P4501A1 inducers may disturb this development stage.

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