Inhibition of ovarian microsomal aromatase and follicular oestradiol secretion by imidazole fungicides in rainbow trout

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Inhibition of Ovarian Microsomal Aromatase and Follicular Oestradiol Secretion by Imidazole Fungicides in Rainbow Trout

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ABSTRACT

The knowledge of critical sites of interaction between pollutants and fish is a major means of investigation in order to obtain early advance knowledge of adverse alterations and ecotoxicological risks. In fish, the ovarian secretion of oestradiol-17β has been demonstrated to govern hepatic synthesis of vitellogenin, a lipoprotein corresponding to the major part of embryonic trophic reserves. Oestradiol synthesis is catalysed by aromatase, a cytochrome P450-dependent enzymatic activity. Effects of different imidazole fungicides, well known as potent inhibitors of cytochrome P450s, were tested in vitro. *A concentration-dependent inhibition of the microsomal aromatase was observed with an inhibiting potency order as follows: clotrimazole (Inhibition 50%: $5{\cdot}10^{-7}$ M) > prochloraz and imazalil (I 50%: $5{\cdot}10^{-6}$ M) > ketoconazole (I 50%: $5{\cdot}10^{-4}$ M). Secretion of oestradiol from cultured follicles was reduced after exposure to 10^{-6} M prochloraz in the culture medium.*

Exposure of fish to pollutants may occur at different levels of the reproductive cycle. The biosynthesis of steroid hormones provides enzymatic targets for xenobiotics, particularly the steps catalyzed by cytochrome P450-dependent enzymes. Although xenobiotic-metabolizing cytochrome P450s and steroid-metabolizing cytochrome P450s correspond to quite different members in the P450 super-family, it may be hypothesized

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that xenobiotics metabolized by the former could interact with the second as well and thus interfere with endogenous metabolism. In this perspective, aromatase cytochrome P450, which catalyzes the terminal step in the biosynthesis of oestrogens (aromatization of androgens into oestrogens), is a potential target of great ecotoxicological significance. Indeed, in fish, the ovarian secretion of oestradiol-17β has been demonstrated to govern hepatic synthesis of vitellogenin, a lipoprotein corresponding to the major part of embryonic trophic reserves.[1] In mammals, extensive research has been conducted on the inhibition of aromatase for therapeutic agents against oestrogen-dependent breast tumors.[2] Aromatase-inhibiting activity of nitrogen heterocyclic compounds such as imidazole derivatives has recently been reported.[3] Such compounds have also been demonstrated to be inhibitors of hepatic xenobiotic-metabolizing cytochrome P450s in mammals[4,5] as well as in rainbow trout.[6] In fish, characterization of microsomal-aromatase activity was recently performed in rainbow trout.[7] The present study investigates the inhibition of rainbow trout aromatase by xenobiotics. We tested imidazole derivatives, two antimycotic drugs (ketoconazole and clotrimazole) previously studied in mammals, and two agricultural fungicides (prochloraz and imazalil), on ovarian microsomal aromatase *in vitro*. Additionally, the impact of xenobiotics on oestradiol-17β secretion by cultured ovarian follicles was assessed.

For the preparation of microsomes, ovaries of rainbow trout in final vitellogenesis were homogenized in phosphate buffer (10 mM), saccharose (0·25 M), NaCl (12‰), and PMSF (1 mM) at pH 7·5 by using Ultra-Turrax and Potter-Elvehjem homogenizers. Homogenate was centrifuged for 20 min at 10 000*g* and post-mitochondrial supernatant was ultracentrifuged for 90 min at 150 000*g*. Microsomes were resuspended in phosphate buffer (10 mM), NaCl (12‰), KCl (0·15 M), saccharose (0·25 M), dithiothreitol (5 mM), and EDTA (1 mM) at pH 7·5 and stored at −80°C. Aromatase activity was assayed for 25 min at 12°C in a final volume of 500 μl with 450 μl microsomes (6·75 mg protein), 35 μl NADPH (0·5 mM final), 5 μl fungicide in ethanol, and 10 μl [1β,2β-^{3}H]androstenedione in ethanol (45 nM final). Reaction was stopped with TCA (33%) and 500 μl distilled water were added. After centrifugation, 750 μl of supernatant were collected and mixed with activated charcoal for 2 h at 4°C for removal of [^{3}H]androstenedione. After centrifugation, the supernatant was collected and [^{3}H]H_2O generated by aromatase activity was measured by scintillation counting.[8] With these conditions, the appearance of [^{3}H]H_2O is linear with time and is totally inhibited by androstatrienedione (1 μM final), a well-known aromatase inhibitor in mammals.[9] The tritiated-water assay was validated with respect to the classical one by measuring [^{3}H]oestradiol and [^{3}H]oestrone, [1,2,6,7-^{3}H]androgenes being used as precursors.[10] Culture of follicles was carried

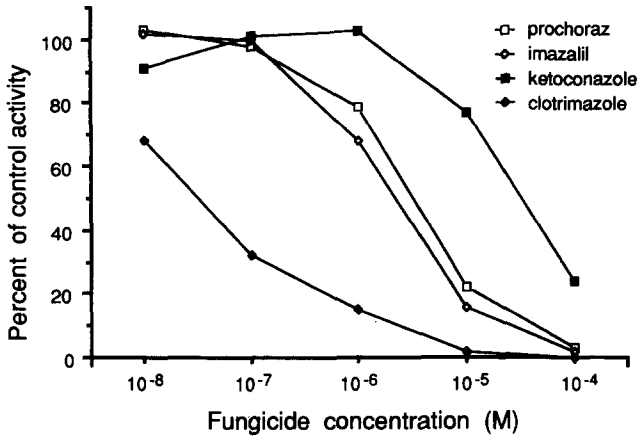

Fig. 1. Effect of imidazole fungicides on ovarian microsomal aromatase. Each assay was performed in duplicate, mean is indicated. Blank (TCA added prior [^{3}H]androstenedione; performed in triplicate) and androstatrienedione assays were 0·5% and 0·6% of control assay (assay without pesticide), respectively. Each value was obtained by subtracting the blank from the assay. In control assay (100% activity), 8·8% of initial radioactivity was converted to [^{3}H]H_2O (0·0117 pmole [^{3}H]androstenedione/min × mg protein, if equimolar reaction).

out as previously described.[11] Each assay corresponded to 25 follicles maintained in 2 ml of culture medium at 12°C with or without the addition of fungicides in 10 μl ethanol. After 42 h of incubation, the culture medium was collected for oestradiol radioimmunoassay.[11]

Figure 1 shows a dose-dependent inhibition of rainbow trout aromatase by imidazole-based antifungals. The order of decreasing inhibitory effect was: clotrimazole > prochloraz and imazalil > ketoconazole. The concentrations of antimycotic drugs inhibiting 50% of rainbow trout aromatase activity were within the same range as in mammals[12] (suggesting similar sensitivity of aromatase to chemical inhibitors in fish and mammals), the molecular basis of inhibition being the presence of an imidazole moiety that interacts strongly with the iron atom of cytochrome P450[3] and competitive inhibition at the substrate-binding site by the non-imidazole moiety.[4]

In vitro exposure of ovarian follicles to prochloraz decreased oestradiol secretion (Fig. 2). A similar result was obtained with ketoconazole-exposed rat ovaries *in vitro*.[13] We can hypothesize that at least aromatase is inhibited by prochloraz, but several steps of steroid synthesis are cytochrome P450-dependent[14] and most of them were demonstrated as taking place in fish ovary.[15] Consequently, it may be possible that the inhibiting effect of prochloraz on follicular-oestradiol secretion resulted from interaction with several steroid-synthesizing cytochrome P450s catalyzing androgen synthesis. Further experiments are necessary to substantiate this hypothesis.

This study supports the hypothesis that reproductive failure in fish could

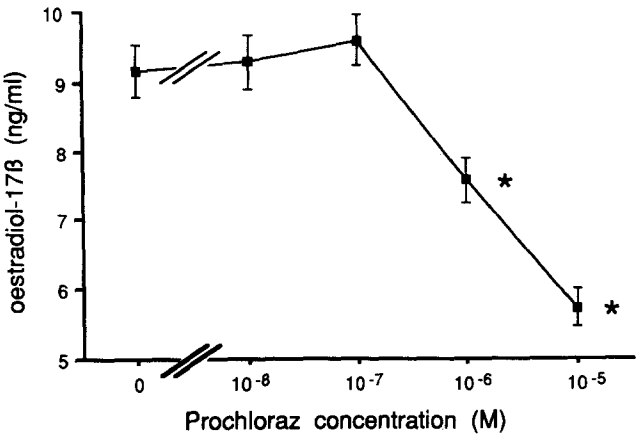

Fig. 2. Effect of prochloraz on oestradiol secretion by cultured follicles (vitellogenic ovary, gonadosomatic index = 3·5%, ovocyte diameter = 3·6 mm). Mean ± SEM of 10 assays. *Significantly different from control ($p < 0{\cdot}05$, Dunnett's t-test).

originate from interaction between pollutants and cytochrome P450-dependent enzymes of steroid synthesis, particularly aromatase.

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