Modulation of Cytochrome P450 1A and Vitellogenin Gene Expressions in the Fish Liver by Suggested Natural Ligands for the Aryl Hydrocarbon Receptor

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Introduction

The aryl hydrocarbon receptor (AHR) is a ligand-activated transcription factor that stimulates the expression of the genes encoding xenobiotic metabolizing enzymes such as cytochrome P450 1A (CYP1A). AHR resides in the cytosolic compartment as a multiprotein chaperone complex including the 90 kDa heat shock protein. AHR ligands probably diffuse across the plasma membrane and bind to the receptor, wherby AHR undergoes conformational changes resulting in release of the chaperone complex and the receptor enters the nucleus. Here it interacts with aryl hydrocarbon receptor nuclear translocator (ARNT), forming the AHR/ARNT complex, which binds to specific DNA sequences called dioxin response elements (DREs) [1]. The most characteristic AHR ligands are environmental contaminants of industrial origin which comprise several classes of chemical compounds: halogenated aromatic hydrocarbons such as polychlorinated dibenzo-*p*-dioxins, polychlorinated dibenzofurans, and polycyclic aromatic hydrocarbons such as benzo(a)pyrene and 3-methylchloroantrene. However, several structurally diverse dietary AHR ligands have been described recently [2]. Our efforts in this study have been concentrated on the activation of the AHR signaling pathway by the two indole derived compounds indolo[3,2-b]carbazole (ICZ) and 6-fomylindolo[3,2-b]carbazole (FICZ). ICZ is a highly potent ligand for AHR formed by acidic condensation of indole-3-carbinol in the stomach [3]. FICZ is produced by photo-oxidation of tryptophan and is able to induce the expression of CYP1A in mammalian cells at pM concentrations [4, 5]. While activation of the AHR by these ligands has been extensively studied in the mammalian cells, very little is known about the actions of these substances in lower vertebrates such as fish. In the fish liver the synthesis of egg yolk protein precursor vitellogenin (VTG) is under the control of the estrogen receptor alpha (ERα) and is induced by 17 β-estradiol (E2). VTG is secreted into the bloodstream, transported, and incorporated into oocytes as yolk [6]. TCDD and other AHR ligands are able to disrupt the E2-induced responses in the mammalian cells [7, 8]. In addition recent studies indicated that AHR ligands are capable to elicit an inhibitory effect on the estrogen-induced synthesis of VTG [9]. In this study, the effects of ICZ and FICZ on the expression of two physiologically important hepatic proteins, CYP1A and VTG, have been investigated.

Materials and Methods

Chemicals: Indolo[3,2-b]carbazole (ICZ) and 6-fomylindolo[3,2-b]carbazole (FICZ) were obtained from NOVUM, Karolinska Inst. Huddinge, Sweden. 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) was from Cambridge Isotope Lab. Inc. and 17 β-estradiol (E2) from Sigma Inc. The chemicals were disolved in dimethylsolfoxid (DMSO) from Merck. *AHR-ligand binding assay:* The assay was performed using the AHR-immunoassay kit (Paracelsian, USA) according to the protocols provided by the manufacture. *Cell culture and treatments:* Juvenile Atlantic salmon (*Salmo salar*) approximately 500-700 g were kept in sea water at a constant temperature of 12°C. The hepatocytes were isolated by two-step perfusion as described [10, 11]. The cells were cultured in EMEM without phenol red containing 2.5% (v/v) newborn calf serum (Invitrogen, USA); glutamine (0.3 g/l medium),

1% (v/v) antibiotic mixture (Penicilin, Streptomycin, Amphotericin B) and incubated at 12°C. The cells were kept in culture for at least 48 hours prior to treatment with. *RNA purification and Northern blot analysis:* Total RNA was isolated using Trizol reagent (Life Technologies, USA). 20 µg of total cellular RNA per sample was analyzed by Northern blot using standard protocols [12]. The salmon vitellogenin [13] (accession number AY04992) and trout cytochrome P450 1A [14] probes were labeled by random priming method using [a-^{32}P]-dCTP (3000 Ci/mmol)(Amersham, UK) according to Ausubel et al. [12]. *Western blot analysis:* Total cellular proteins were prepared by lysis of the cells in 1% Nonidet P-40 buffer (150 mM Tris-HCl pH 7.8, 150 mM NaCl,1 mM PMSF, 1 mg/ml Pepstatin, 1 mg/ml Leupeptin, and 1% Nonidet P-40). The cellular debris was removed by centrifugation and the supernatant (cytosol) was used in the Western blotting. 16 µg of total cellular proteins per sample was separated in 8% SDS polyacrylamide gels and blotted into the nitrocellulose membranes. The membranes were probed with an anti-fish CYP1A peptide monoclonal antibody (C10-7; Biosense Laboratories, Norway) according to the instructions provided by the manufacture.

Results and Discussion

The first step in our studies was to examine that ICZ and FICZ were capable to transform the AhR to an active form using the AHR-immunoassay. The assay is based on the binding of the ligands to an inactive cytosolic AHR and formation of a AHR/ARNT complex. The AHR/ARNT heterodimer binds to the DRE which is subsequently immobilized into the matrix of a ELISA plate. The presence of the ternary complex is detected using an antibody capable of binding to ARNT associated with the complex. The DRE bound complex is detected colourometrically where the intensity of the colour development is proportional to the amount of the liganded AhR. As shown in Figure 1, both ICZ and FICZ were capable to bind and activate AHR with binding affinities similar to that of TCDD.

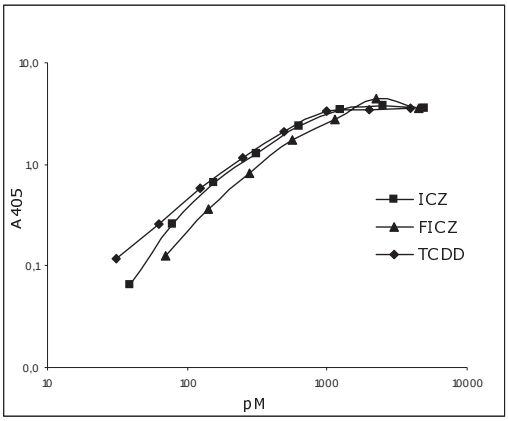

Figure 1: Binding and activation of the AhR by ICZ and FICZ
Binding of ICZ (5 pM to 5 nM), FICZ (4 pM to 4.5 nM) and TCDD (3.88 pM to 3.98 nM) to AHR and detected as formation of DRE/AHR/ARNT complex using ARNT specific immunoassay (AHR-Immunoassay, Paracelsian Inc.). Each point in the plot represents the mean of the values obtained by three independent experiments.

Establishing that ICZ and FICZ were capable to bind and activate the AHR, we were interested to study how exposure of the fish liver cells to these substances can affect the expression of CYP1A. The results depicted in Figure 2 show that exposure of the primary cultured fish hepatocytes to ICZ and/or FICZ resulted in a marked induction of the CYP1A gene transcriptional and translational activities.

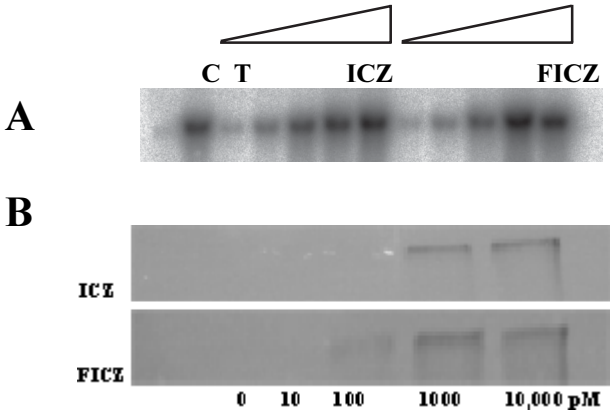

Figure 2: ICZ and FICZ induce expression of the CYP1A in primary cultured Atlantic salmon hepatocytes.
A. Fish hepatocytes treated with ICZ or FICZ (1 pM, 10 pM, 0.1 nM, 1 nM and 10 nM) for 12 hours analyzed by Northern hybridization using 20 µg RNA/lane and a radio-labeled CYP1A specific probe. C; untreated (control) cells, T; cells treated with 100 pM TCDD. **B.** Hepatocytes treated with ICZ or FICZ (10 pM - 10 nM) for 24 hours analyzed by Western blotting (16 µg protein/lane) using a CYP1A spesific antibody.

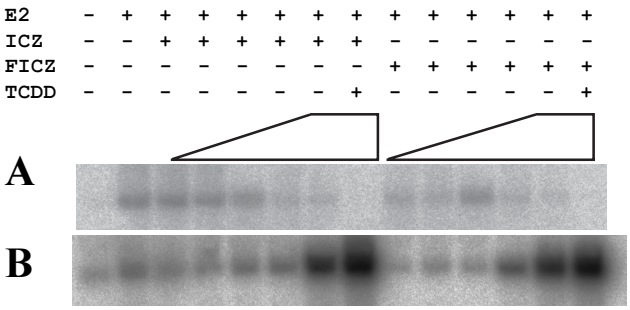

Figure 3: ICZ and FICZ inhibit vitellogenin expression in primary cultured Atlantic salmon hepatocytes.
Fish hepatocytes untreated or treated with 10 nM E2 alone or in combination with TCDD (10 nM), ICZ or FICZ (1 pM, 10 pM, 0.1 nM, 1 nM and 10 nM) for 12 hours before isolation of total cellular RNA and analysis by Northern hybridization using [^{32}P]-dCTP labeled cDNAs specific for VTG (panel A) and CYP1A (panel B).

Next we investigated how activation of AhR by these ligands affects the expression of Vtg in fish liver cells. Cultured liver cells were exposed to E2 (10 nM) alone or in combination with increasing concentrations of each of the test compounds ICZ or FICZ. As depicted in Figure 3, ICZ and FICZ reduced the VTG mRNA levels in a concentration-dependent manner, while CYP1A mRNA levels revealed an opposite pattern of expression. Combination of ICZ or FICZ with TCDD demonstrated an additive effect on the inhibition of VTG and the stimulation of CYP1A gene expression.

Conclusions:

In this study, we investigated how activation of the AHR in the fish liver can affect AHR- and ERα-mediated signaling pathways. Our results indicated that ICZ and FICZ are very potent ligands of the AHR, as has been reported previously using competitive binding assays [7, 15]. Both ligands posses an anti-estrogenic activity in fish hepatocytes. Several investigations indicated that environmental contaminating agents such as TCDD exert an inhibitory effect on the vitellogenine synthesis [9, 16]. Anti-estrogenic activities of the indole derived AHR ligands in a lower vertebrate such as fish indicate strongly that the AHR may have an important physiological role, wich includes an inhibitory effect on the ERα signaling.

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