Comparative Hepatology

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The aryl hydrocarbon receptor-mediated disruption of vitellogenin synthesis in the fish liver: Cross-talk between AHR- and ERα-signalling pathways

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Published: 02 May 2004

Comparative Hepatology 2004, **3**:2

Received: 05 September 2003

Accepted: 02 May 2004

This article is available from: http://www.comparative-hepatology.com/content/3/1/2

Abstract

Background: In the fish liver, the synthesis of egg yolk protein precursor vitellogenin (VTG) is under control of the estrogen receptor alpha (ERα). Environmental contaminants such as 2,3,7,8-tetrachloro-dibenzo-*p*-dioxin (TCDD) are suspected to have antiestrogenic effects. The aryl hydrocarbon receptor (AHR) is the initial cellular target for TCDD and related compounds. The AHR is a ligand-activated transcription factor that stimulates the expression of the genes encoding xenobiotic metabolizing enzymes, such as cytochrome P450 1A (CYP1A). In this study, the effects of activation of AHR on the hepatic expression of VTG and ERα genes, in primary cultured salmon hepatocytes, have been investigated.

Results: The expression of the genes encoding VTG and ERα were strongly induced by 17β-estradiol (E2). However, the expression of VTG was disrupted by exposure of the cells to TCDD while CYP1A expression was enhanced. The effect of TCDD on VTG and CYP1A expression was annulled by the AHR-inhibitor α-naphthoflavone. Furthermore, exposure of the cells to TCDD abolished E2-induced accumulation of ERα mRNA. The AHR-mediated inhibitory effects on the expression of the VTG and ERα genes may occur at transcriptional and/or post-transcriptional levels. Nuclear run-off experiments revealed that simultaneous exposure of the cells to E2 and TCDD strongly inhibited the initiation of transcription of the VTG and ERα genes. In addition, inhibition of RNA synthesis by actinomycin D treatment showed that post-transcriptional levels of VTG and ERα mRNAs were not significantly altered upon treatment of the cells with TCDD. These results suggested that activation of AHR may inhibit the transactivation capacity of the ERα. Further, electrophoretic mobility shift assays using nuclear extracts prepared from cells treated for one or two hours with E2, alone or in mixture with TCDD, showed a strong reduction in the DNA binding activities upon TCDD treatment. These results also suggested that activation of the AHR signalling pathway caused a marked decrease in the number of the nuclear ERα or that activated AHR blocked the ability of ERα to bind to its target DNA sequence. Finally, our results from Northern hybridizations indicated that E2 treatment of the cells did not cause any significant effect on the TCDD-induced levels of CYP1A mRNA.

Conclusion: In fish hepatocytes E2 induces ERα and VTG gene expression. The presence of dioxin (TCDD) abolishes this induction, probably through the action of AHR in complex with AHR nuclear translocator, and possibly by direct interference with the auto-regulatory transcriptional loop of ERα. Furthermore, E2 does not interfere with TCDD induced CYP1A gene expression, suggesting that cross-talk between the ERα- and AHR-signalling pathways is unidirectional.

Background

The liver is a central organ for sexual reproduction and development of the embryo in oviparous vertebrates. In teleost fish, much of the yolk is synthesized by liver cells in the form of a protein precursor: the vitellogenin (VTG). VTG is a large phosphoglycolipoprotein which is synthesized in the liver under hormonal control and secreted into the bloodstream [1,2]. The VTG is incorporated into the developing oocyte by receptor-mediated endocytosis [3,4] and processed into three smaller proteins: phosvitin, a phosphorus containing protein, and two lipid containing proteins, lipovitellins I and II [5-7]. These become the primary substances stored as yolk. In the female fish, the induction of VTG synthesis (vitellogenesis) is under control of the hepatic estrogen receptor α (ERα). The induction of vitellogenesis is triggered by environmental cues and is regulated by coordinated endocrine feedback loops between the hypothalamus, pituitary, gonad and liver (HPGL axis) [1]. Briefly, environmental signals induce the hypothalamus to release gonadotropin releasing hormones which stimulate the release of gonadotropins from the pituitary. The gonadotropin hormones, in turn, stimulate the follicle cells to synthesize 17β-estradiol (E2). The estradiol is released into the blood and transported into the liver where it enters the hepatocytes by diffusion and binds with high affinity to the ERα. The activated ERα triggers the expression of its own gene and subsequently that of the VTG.

Polycyclic aromatic hydrocarbons (PAHs) and polyhalogenated aromatic hydrocarbons (PHAHs) are suspected to have deleterious effects on fish vitellogenesis [8]. A number of these compounds including polychlorinated dibenzo-p-dioxins such as 2,3,7,8-tetrachlorodibenzo-*para*-dioxin (TCDD) exert their effects through the aryl hydrocarbon receptor (AHR). The AHR is a ligand-activated transcription factor that regulates the activation of several genes that encode phase I and phase II drug metabolism enzymes in the liver (reviewed in ref. [9]). The AHR belongs to the family of basic helix-loop-helix (bHLH)/Per-Arnt-Sim (PAS) proteins which are characterized by two conserved domains, the N-terminal bHLH and the PAS domain (reviewed in ref. [10]).

The cytochrome P4501A (CYP1A) is an enzyme involved in the metabolism of many drugs and xenobiotics which is regulated by the AHR. The molecular mechanism involved in the activation of the CYP1A have been extensively studied [11]. Prior to binding of the ligand the cytosolic form of the AHR is associated with a chaperoning complex consisting of heat shock protein 90 (HSP90) and several other co-chaperones [10]. Upon binding of the ligand, the AHR is released from the HSP90 complex and translocated into the nucleus where it dimerizes with a structurally related protein, the Ah Receptor Nuclear Translocator (ARNT). The AHR/ARNT complex binds with high affinity to specific DNA sequences termed dioxin response elements (DREs) located in the regulatory regions of the target genes leading to activation of their expression.

TCDD has been shown to inhibit several estrogen-induced responses in the rodent uterus, mammary gland and in the mammalian cell cultures (reviewed in [12,13]). The cross-talk between the ERα and AHR signalling pathway has not been extensively studied in the fish. However, recent studies using fish primary cultures of hepatocytes showed that AHR-ligands have an inhibitory effect on the estrogen-induced synthesis of VTG [14,15]. On the other hand, conflicting findings were observed in *in vivo* exposures of fish to xenoestrogens and Ah-receptor ligands [16].

The efforts of this study have been concentrated on the mechanism behind cross-talk between the AHR- and ERα-signalling pathways in the fish liver. Our results show that activation of the AHR pathway has contradictory effects on the molecular functions of the ERα in the liver cells. Activation of the AHR inhibits the ERα to initiate transcription of the VTG gene and blocks the auto-regulatory loop of the ERα gene expression. The cross-talk between the two receptors, however, appears to be unidirectional, i.e., activation of the ERα has no significant inhibitory effect on the AHR-mediated induction of the CYP1A.

Results

Effects of TCDD, β-naphthoflavone, and α-naphthoflavone on E2-induced vitellogenin gene expression

The expression of VTG gene in the fish liver is positively regulated by 17β-estradiol (E2). The first step in our studies was to examine how exposure of the liver cells to TCDD alters the E2-induced VTG gene expression. The primary cultured fish hepatocytes were exposed to a constant concentration of E2 and/or E2 combined with increasing concentrations of TCDD. As shown in Figure 1, exposure of the cells to 10 nM E2 resulted in a strong induction of the VTG gene expression. Exposure of the cells to a combination of E2 and TCDD, however, resulted in a strong reduction of VTG mRNA levels. The inhibitory effect of TCDD on the VTG gene expression was comparable to the negative effects of tamoxifen. Tamoxifen is able to interfere with binding of estrogen to its receptor and to prevent activation of the target genes by the receptor. Interestingly, the negative effects of TCDD on the VTG gene expression was diminished by the AHR antagonist α-NF, indicating that the antiestrogenic effects of TCDD was mediated through its interaction with the aryl hydrocarbon receptor. However, α-NF alone had no significant effect on the E2-induced expression of VTG gene (Figure

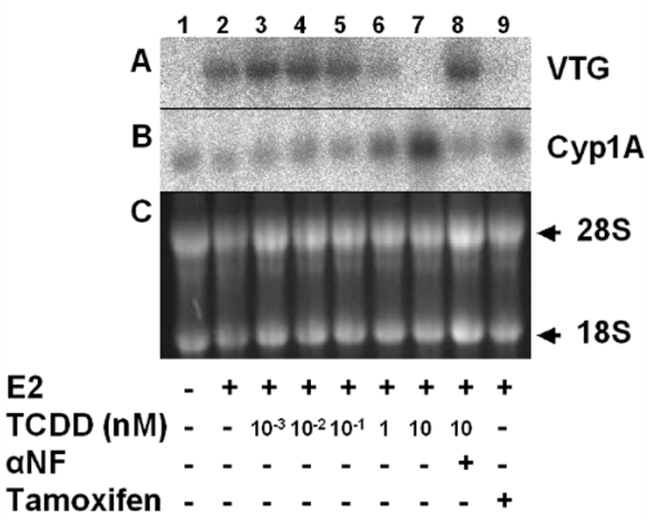

Figure 1
The inhibitory effects of TCDD on the vitellogenin mRNA levels. After 48 hrs of culture the fish hepatocytes were left untreated or were treated with a fixed concentration of 17β-estradiol (E2) or E2 and increasing concentrations of TCDD. Following a 12-h treatment period, total cellular RNA was isolated. Total RNA (20 μg per lane) was electrophoresed through a formaldehyde-containing agarose gel, transferred to a nylon membrane and sequentially hybridized to [α-^{32}P]dCTP labelled cDNAs specific for VTG (VTG) and cytochrome P4501A (CYP1A). The top panel (A) shows the VTG gene expression, the panel in the middle (B) shows the expression of the CYP1A gene and bottom panel (C) displays the ethidium bromide staining of the gel to demonstrate equal loading of the samples. The arrows indicate the position of 28S and 18S ribosomal RNAs. The numbers correspond to the kind of treatment of each sample as follows: #1: Control sample (cells treated with DMSO); #2: Cells treated with 10 nM E2; #3: Cells treated with E2 + 1 pM TCDD; #4: Cells treated with E2 + 10 pM TCDD; #5: Cells treated with E2 + 100 pM TCDD; #6: Cells treated with E2 + 1 nM TCDD; #7: Cells treated with E2 + 10 nM TCDD; #8: Cells treated with E2 + 10 nM TCDD + 1 μM α-NF; #9: Cells treated with E2 + 1 μM tamoxifen.

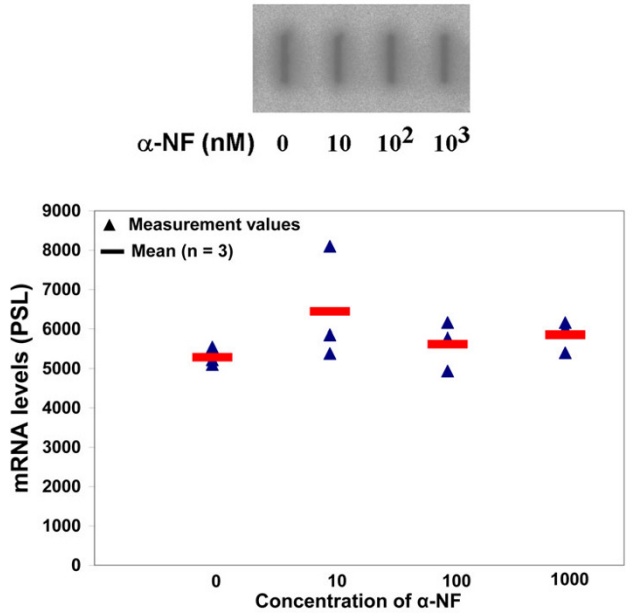

Figure 2
The effects of α-naphthoflavone on the E2-induced vitellogenin mRNA levels. The cultured fish hepatocytes were treated with 10 nM E2 or E2 plus increasing concentrations of α-naphthoflavone (α-NF) (0–1 μM) for12 hrs. Total cellular RNA was subsequently isolated and 7.5 μg per sample was analyzed by slot blot hybridization. The membrane was hybridized with a [α-^{32}P]dCTP labelled cDNA probe specific for VTG. The upper panel shows a representative slot blot membrane. The lower panel shows quantified radioactivity using phosphoimager as photo stimulated luminiscence (PSL). The PSL values of three independent experiments and their means are shown in the plot diagram.

2). To examine the capability of the AHR ligands to block expression of VTG gene at the high estradiol concentrations that may be reached during vitellogenesis [17], the dose of E2 was raised 10 times compared to the previous experiments. As shown in Figure 3a, TCDD showed similar inhibitory effects on the accumulation of VTG mRNA levels even when the cells were exposed to such high concentration of E2 (100 nM). Additional experimental evidence for involvement of AHR in this process was obtained by performing assays using combinations of estradiol with the AHR-ligand β-naphthoflavone (β-NF). β-NF is a weaker ligand of the AHR and its potency as an inducer of CYP1A protein and as an inhibitor of vitellogenesis is lower than TCDD [14]. Nevertheless, β-NF proved antiestrogenic at a concentration of 10 μM. Interestingly, α-NF was capable to invert the negative effects of β-NF on the expression of VTG gene at such low concentration as 1 nM (Figure 3b).

Down-regulation of the estradiol-induced ERα gene expression by TCDD

The expression of the ERα in the liver cells is auto-regulated. Therefore the antiestrogenic effects of TCDD might be mediated through inhibitory mechanisms influencing transcription of the ERα gene itself. This would result in decreased number of the activated receptor and thus reduced transcriptional activity of the VTG in response to estradiol. In order to investigate the effects of TCDD on

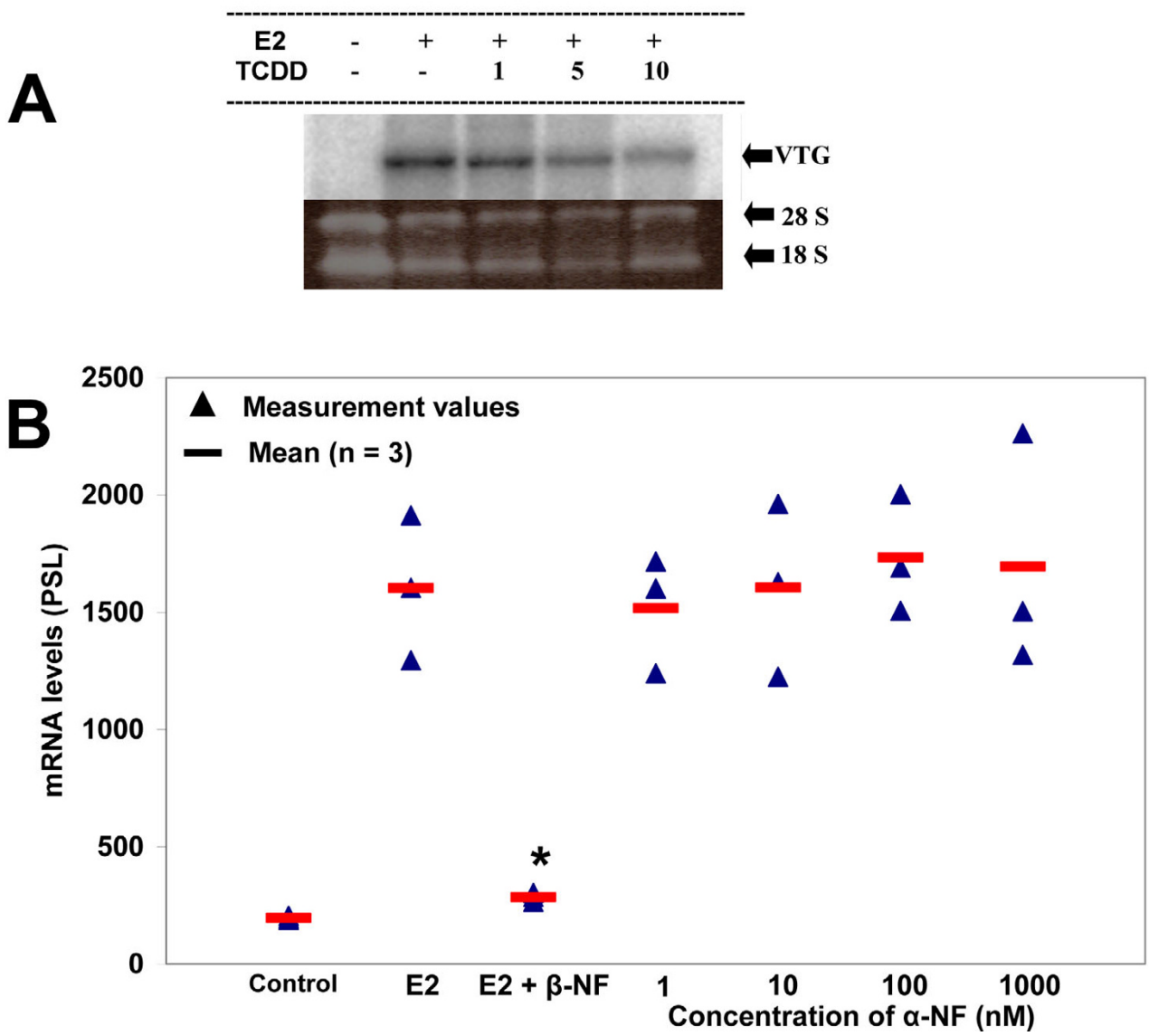

Figure 3

A) The negative effects of TCDD on the highly-induced vitellogenin gene expression. The salmon hepatocytes were treated with 100 nM E2 or E2 and increasing concentrations of TCDD (1–10 nM). Following a 12-h treatment period, total cellular RNA was isolated and the expression of the VTG gene was assessed by Northern blot analysis as described (Figure 1). The upper panel shows the expression of the VTG gene while the lower panel displays the ethidium bromide staining of the gel to demonstrate equal loading of the samples. The arrows indicate the position of 28S and 18S ribosomal RNAs. **B) Effects of α-NF on the antiestrogenic activity of β-NF** Slot blot hybridization analysis of VTG gene expression in cultured hepatocytes after treatment for 12 hrs with solvent (Control), fixed concentration of 17 β-estradiol (E2) (100 nM), E2 plus α-naphthoflavone (α-NF) (1 μM) or E2 plus increasing concentrations of α-naphthoflavone (from 1 to 10^3 nM). Total RNA (5 μg) was applied per slot and hybridized with a [α-^{32}P]dCTP labelled VTG cDNA probe. Radioactivity in each slot was quantified using phosphoimager as photo stimulated luminiscence (PSL). The PSL values of three independent experiments and their means are shown in the plot diagram. Asterisk indicates significant difference ($P < 0.05$) with respect to the E2-treated sample (Dunnett's test).

the expression of the ERα gene, the cells were exposed to a constant concentration of E2 and increasing concentrations of TCDD and the variations in the VTG and ERα mRNA levels were investigated. The results showed that exposure of the cells to TCDD markedly reduced the expression of both VTG and ERα genes (Figure 4). The expression of these genes was strongly inhibited by the two highest concentrations of TCDD (5 and 10 nM). A positive correlation between the patterns of down-regulation of the VTG and ERα genes indicated strongly that the expressions of these genes were inhibited by a similar mechanism.

Effects of TCDD on the transcription initiation and turnover rates of vitellogenin and estrogen receptor mRNAs

The AHR-mediated down-regulation of estrogen receptor and VTG gene expression may occur at transcriptional and/or post-transcriptional levels. In order to determine the respective contributions of each mechanism, two series of experiments were performed. In the first experiment, the ability of the activated AHR to influence the initiation rate of VTG and ERα gene expressions was investigated. The cells were either left untreated or treated with E2 or E2 plus TCDD. After 8 hours the nuclei were isolated and incubated with [α-^{32}P]UTP. During the period of incubation the radioactive label was incorporated into the nascent RNA chains which have been transcribing when the nuclei were isolated resulting in radioactive labelling of the activated genes. Our results showed that the transcriptional activities of the ERα and VTG genes strongly increased by E2 treatment, whereas TCDD treatment mediated a strong reduction in the transcriptional activities of these genes. At the same time, the transcriptional activity of the CYP1A1 gene was markedly increased suggesting that the inhibitory effects were mediated through activation of the AHR (Figure 5).

In the second experiment, expression of the ERα and VTG genes were induced by exposing the cells to E2 for 24 hours. At this point, the mRNA levels of the respective genes have reached their maxima. The synthesis of the cellular RNA was then blocked by exposing the cells to the transcription-inhibitor actinomycin D while the cells were either left untreated or exposed to 10 nM TCDD for a further period of 24 hrs. The cells were harvested at various time intervals and total cellular RNA from each sample was prepared and analyzed by slot blot hybridization using cDNA probes specific for ERα and VTG. The results, however, showed no significant differences in the VTG and ERα mRNA levels upon the treatment with TCDD (Figures 6a and 6b, respectively). These results indicated that activation of the AHR had no significant effect on the post-transcriptional levels of the VTG and ERα mRNAs.

Effects of TCDD on the DNA binding activities of the nuclear extracts

The results obtained from nuclear run-off experiments indicated strongly that activation of the AHR signalling pathway may interfere with the ERα auto-regulatory loop which might be caused by a significant decrease in the number of activated ERα in the nucleus. We investigated this possibility by performing an electrophoretic mobility shift assay. Nuclear extracts were prepared from the cells which were either left untreated or treated with E2 or E2 combined with increasing concentrations of TCDD. The DNA binding activities present in the nuclear extracts were detected using a biotin-labelled ERE oligonucleotide as probe. As indicated in Figure 7, E2 treatment of the cells increased formation of the ERα/ERE complex. The specificity of DNA binding was verified by incubation of the extract with a 100 times molar excess of the unlabelled ERE probe which resulted in significant weakening of the retarded band. Interestingly, the specific binding activity of the nuclear extracts prepared from the cells simultaneously treated with E2 and TCDD was markedly weakened.

Reciprocal inhibitory effects of TCDD and E2 on the induction of vitellogenin and cytochrome P4501A1 gene expression

When establishing the inhibitory effects of the AHR-ligands on the expression of the genes under control of the ERα, it was interesting to investigate whether estrogen exerts control over expression of the CYP1A, i.e., whether the cross-talk between the two signalling pathways was bidirectional. We performed assays where the cells were exposed to 10 nM TCDD or TCDD combined with increasing concentrations of E2. The results depicted in Figure 8 show that activation of ERα by E2 had no pronounced effect on the TCDD-stimulated levels of the CYP1A mRNA. The CYP1A mRNA levels appeared constant upon co-treatment with TCDD, E2 and ERα-inhibitor tamoxifen (Figure 8, lanes 8 and 9). However, treatment of the cells with TCDD plus testosterone did not affect induction of the CYP1A gene (Figure 8, lane 7).

Discussion

In the present work, the effects of TCDD on the estrogen-regulated gene expression in fish liver cells have been investigated. The results showed that TCDD can oppose E2-induced expression of the VTG gene in a concentration-dependent manner and exhibit a pronounced anti-estrogenic effect at higher doses. The inhibitory effects of TCDD on the E2-induced ERα signalling pathway were comparable to the effects of tamoxifen. The latter is an antagonistic ligand of ERα by competing with E2 in binding to the receptor and convert the receptor into an inactivated form [18]. Our results, however, indicate that the mechanism behind the anti-estrogenic action of TCDD is completely different from those of tamoxifen and similar

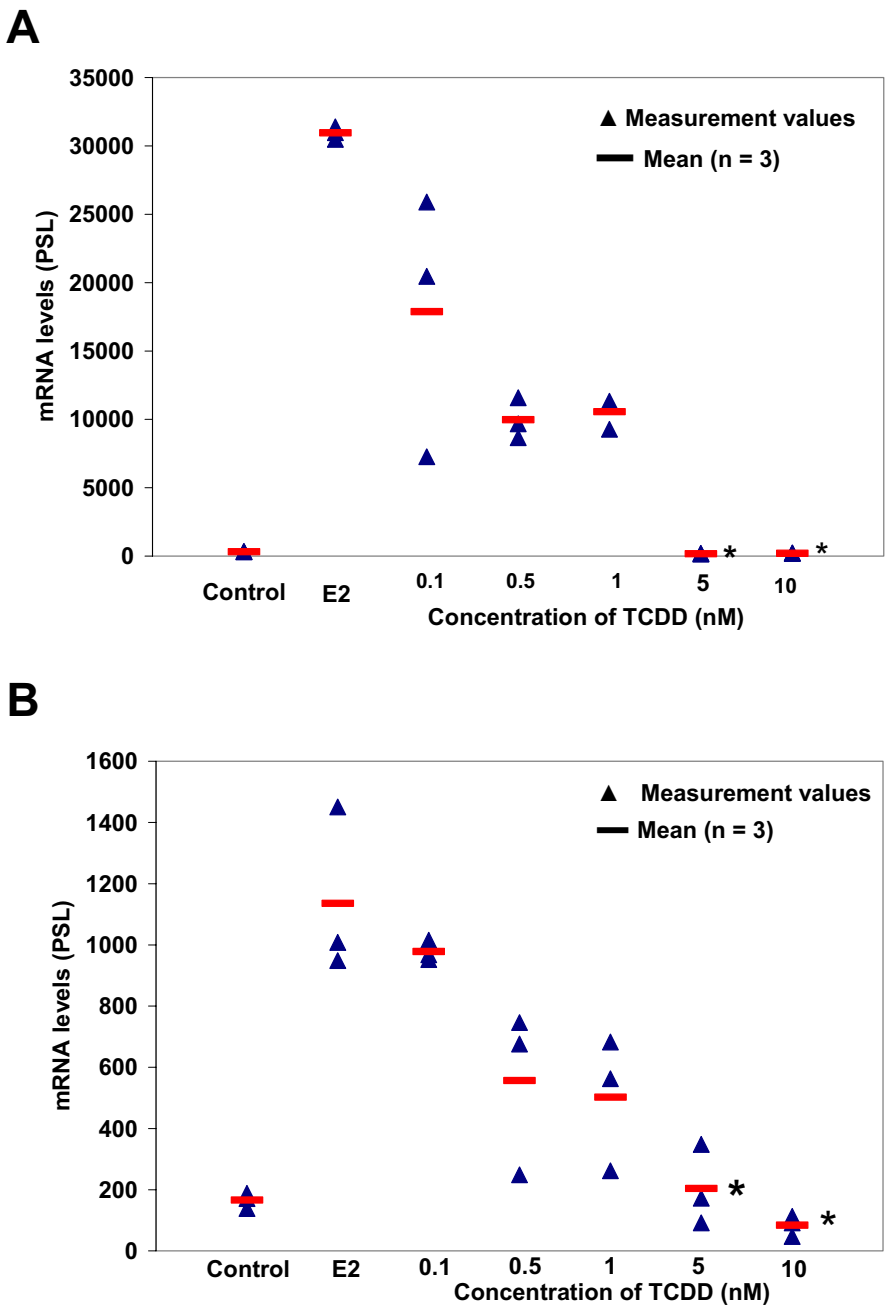

Figure 4

TCDD- mediated down-regulation of vitellogenin and estrogen receptor α mRNAs. Slot blot hybridization analysis of hepatic VTG (A) and estrogen receptor alpha (B) after treatment with 17 β-estradiol (E2) and increasing concentrations of TCDD. After 48 hrs of culture, the cells were co-treated with a constant concentration of E2 (10 nM) and increasing concentrations of TCDD for 12 hrs. Total RNA (7.5 μg) was applied per slot and sequentially hybridized with [α-^{32}P]dCTP labelled cDNA probes specific for VTG and ERα. Radioactivity in each slot was quantified using phosphoimager as photo stimulated luminiscence (PSL). The cells were treated as follows: Control: Cells treated with dimethylsulfoxid (DMSO) only. E2: Cells treated with 10 nM E2 for 12 hrs. Lane 3 – 7 labelled 0.1, 0.5, 1, 5, and 10: Cells co-treated with 10 nM E2 and increasing concentrations of TCDD (0.1, 0.5, 1, 5, and 10 nM, respectively) for 12 hrs. The PSL values of three independent experiments and their means are shown in the plot diagram. Asterisk indicates significant difference ($P < 0.05$) with respect to the E2-treated sample (Dunnett's test).

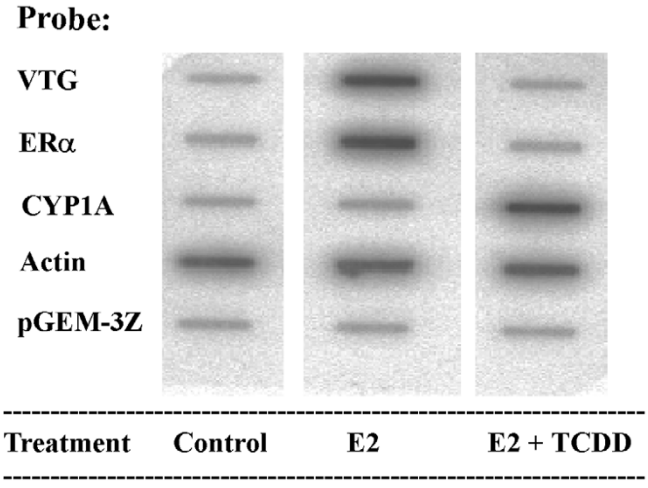

Figure 5
Effects of TCDD on the transcription rate of vitellogenin, estrogen receptor α and cytochrome P4501A mRNAs. Nuclear run-off experiments using primary cultured hepatocytes treated with E2 (10 nM) or E2 (10 nM) plus TCDD (10 nM) for 8 hours. The cells were harvested and the activated nuclei were prepared. The [α-^{32}P]UTP incorporated total RNA was prepared by *in vitro* transcription assay as described in the methods. The radioactive labelled RNA samples were hybridized to the specific cDNAs for vitellogenin (VTG), estrogen receptor alpha (ERα) and cytochrome P4501A (CYP1A) cross-linked to the nylon membranes. Non-specific hybridization is indicated by hybridization of the labelled RNA with cloning plasmid pGEM-3Zf. The results shown are from a representative experiment repeated three times.

classes of anti-estrogens. Our studies provide several lines of evidence to establish the role of the AhR in mediating the anti-estrogenic effects of TCDD: The Northern hybridizations depicted in Figure 1 show that there is a clear negative relationship between the concentration-dependent induction of the CYP1A1 gene and inhibition of the E2-induced VTG gene expression. Other *in vitro* studies, using primary cultured rainbow trout hepatocytes, showed that the potency of the different classes of the AHR-ligands to inhibit the VTG synthesis was directly related to their capability to induce CYP1A1 protein levels and enzymatic activities [14,15]. In addition, our results show that the AHR antagonist, α-NF, is capable of markedly inversing the inhibitory effects of the AHR ligands, TCDD and β-NF, on the E2-triggered expression of the VTG gene. These results supports previously reported studies using rat hepatoma cells [19] and rainbow trout hepatocytes [15].

Activation of AHR by TCDD resulted in a marked reduction of ERα mRNA levels. These results are interesting since ERα is the key regulator of the VTG gene expression. In vertebrate oviparous species the expression of ERα in the liver is induced by E2 [20-23]. Regulation of the ER gene expression is an important aspect of the vitellogenesis, since the sensitivity of the target gene is directly dependent on the cellular concentration of ER, i.e., the estrogen receptor number is a rate-limiting factor in the expression of the VTG gene [22]. There are several reports suggesting that the AHR-ligands exert their inhibitory effects on ER signalling by decreasing the levels of ER; for example, treatment of mice with TCDD induced a decrease in hepatic levels of ER mRNA levels [24]. Recent studies using human breast cancer cell line T47D showed that activation of the AHR by TCDD caused a specific proteasome-dependent degradation of ERα [25,26]. Nevertheless, the inhibitory effects of TCDD on the ERα gene expression appear to be cell-type specific. For example, treatment of the human breast cancer cell line MCF-7 with TCDD did not have any influence on the ERα mRNA levels while it significantly down-regulated the expression of the cathepsin D gene which is under the control of ERα [27]. In another report, the effect of TCDD on the expression of a reporter gene under the control of the *Xenopus* vitellogenin A2 regulatory sequences was studied. The study revealed that TCDD could prevent reporter-gene expression also when the cells transiently overexpressed ER. These results suggested that the mechanism did not involve downregulation of the ER by TCDD [28].

The negative effects of TCDD on the expression of ERα and VTG genes, as indicated by decreasing of the levels of their respective mRNAs, might be exerted at transcriptional or post-transcriptional stages. Our results indicate strongly that these effects are mediated through a mechanism which blocks the activation of those genes.

The nuclear run-off transcription assays showed that E2 treatment induced the transcriptional activities of the ERα and VTG genes while TCDD-treatment had a marked inhibitory effect on the activation of these genes. One important issue to be considered here is that the expression of the ERα and VTG genes in the fish liver are differentially regulated [23,29]. The nuclear run-off transcription studies showed that the expression of the ERα gene reaches a plateau in about 10–12 hours while the expression of the VTG gene continues to increase during the 24 hours after the E2 treatment. These results suggest that the auto-regulation loop of the ERα provides a quick response to estrogen stimuli and subsequently triggers the activation of the VTG gene. In this way, the ERα provides the proper hepatic function necessary to meet the challenging period of vitellogenesis. Our results, however, suggest that despite their different transcriptional rates, the activities of the ERα and VTG genes are both significantly down-regulated by TCDD.

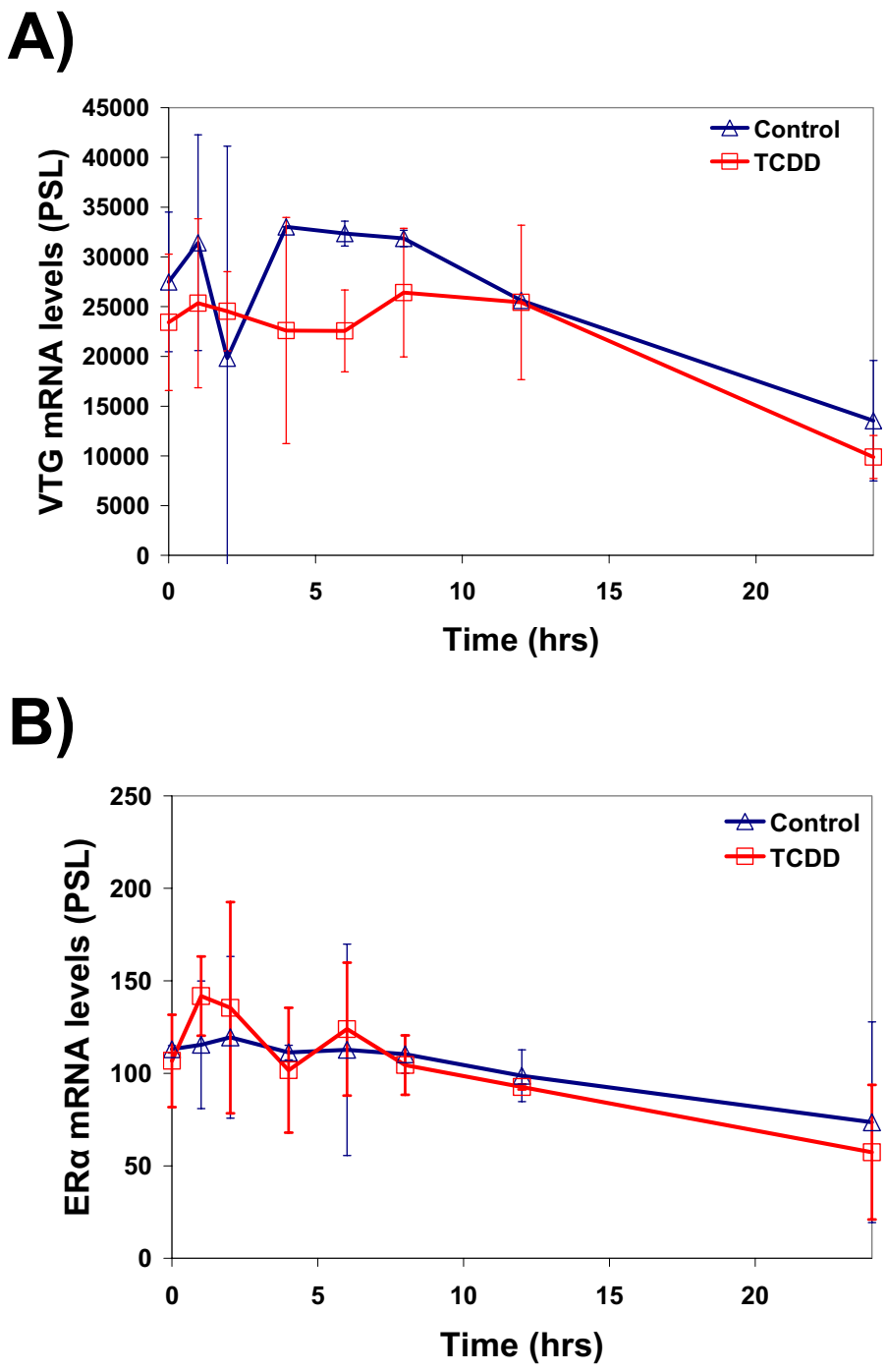

Figure 6
The effects of TCDD on the stability of vitellogenin (VTG) and estrogen receptor α (ERα) mRNAs. Primary cultured hepatocytes were treated with E2 (10 nM) for 24 hours. The synthesis of RNA was then inhibited using actinomycin D (0.5 mg/ml medium). The cells were incubated for 0 to 24 hours in the presence or absence of TCDD (10 nM). The total RNA was then prepared from samples and 7.5 μg per slot of each sample was applied to nylon membranes. The membranes were sequentially hybridized with the [α-^{32}P]dCTP labelled ERα and VTG probes. Radioactivity in each slot was quantified using phosphoimager as photo stimulated luminescence (PSL). The PSL values of two independent experiments are shown as mean ± 2SD in the plot diagram.

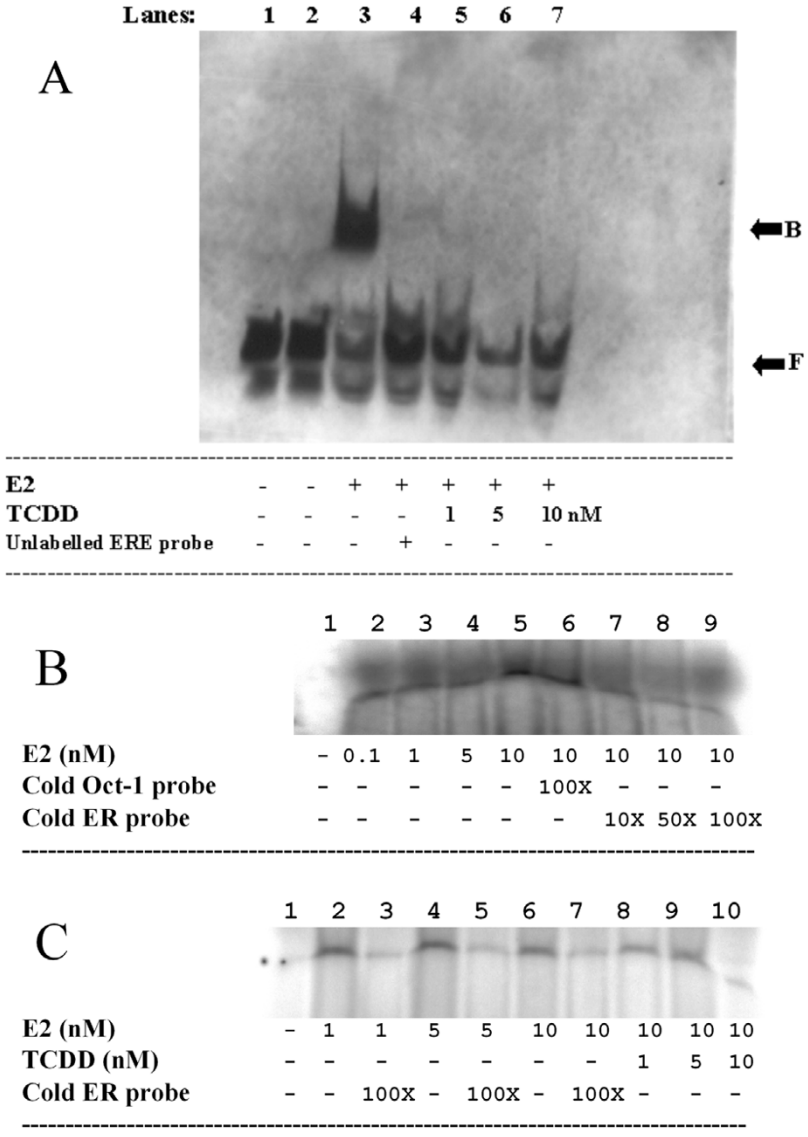

Figure 7

TCDD-mediated repression of DNA binding by ERα. Nuclear extracts prepared from primary hepatocytes, untreated or treated with estradiol (E2) or estradiol plus TCDD were used in the electrophoretic mobility shift assay. The oligonucleotide probe used contained the ERE (the underlined sequence) found in the 5'-regulatory region of the Atlantic salmon ERα gene (5'-TGTCATGTTGACC-3'). A) 3'-end biotin-labelled probe was mixed with nuclear extracts prepared from cells treated for 2 hrs as described below. The position of the retarded band (B) and the free probe (F) are indicated. Lane 1: Biotin-labelled ERE probe. Lane 2: nuclear extract from control cells (receiving DMSO only). Lane 3: cells treated with 10 nM E2. Lane 4: 10 nM E2 with 100 X excess of unlabelled-ERE probe. Lane 5: 10 nM E2 and 1 nM TCDD. Lane 6: 10 nM E2 and 5 nM TCDD. Lane 7: nuclear extracts from cells treated with 10 nM E2 and 10 nM TCDD. B) Radio labelled ERE-probe was mixed with nuclear extract from cells treated for 1 hour and analyzed 6% non-denaturating polyacrylamide gel electrophoresis as follows: Lane 1: free ERE probe; Lane 2: cells treated with 0.1 nM E2; Lane 3: cells treated with 5 nM E2; Lane 4: cells treated with 10 nM E2; Lanes 6–9: competition assays using nuclear extracts prepared from the cells treated with 10 nM E2 and receiving 100 X excess of cold Oct-1 probe (Lane 6), 10 X excess of cold ERE probe (Lane 7), 50 X excess of cold ERE probe (Lane 8) or 100 X excess of cold ERE probe (Lane 9). C) EMSA using radio labelled ERE-probe and nuclear extract from primary hepatocytes treated for 1 hour. Lane 1: free ERE probe. Lane 2: cells treated with 1 nM E2. Lane 3: extract from cells treated with 1 nM E2 and binding reaction supplemented with 100 fold excess cold ERE probe (100 X). Lane 4: cells treated with 5 nM E2. Lane 5: sample as Lane 4, with 100 X cold ERE probe. Lane 6: cells treated with 10 nM E2. Lane 7: sample as Lane 6 with 100 X cold ERE. Lane 8: cells treated with 10 nM E2 and 1 nM TCDD. Lane 9: cells treated with 10 nM E2 and 5 nM TCDD. Lane 10: cells treated with 10 nM E2 and 10 nM TCDD.

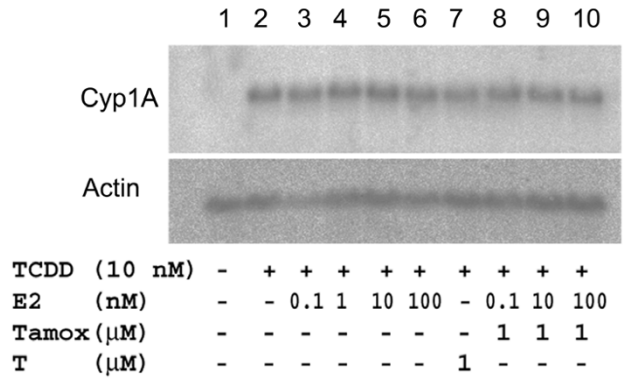

Figure 8

The effects of E2 on the TCDD-stimulated expression of the cytochrome P4501A (CYP1A) gene. After 48 hrs of culture the fish hepatocytes were left untreated or were treated with a fixed concentration of TCDD or TCDD plus increasing concentration of E2. Following a 12-h treatment period, total cellular RNA was isolated. Total RNA (20 μg per lane) was electrophoresed through a formaldehyde-containing agarose gel, transferred to a nylon membrane and sequentially hybridized to [α-^{32}P]dCTP labelled cDNAs specific for cytochrome P4501A (CYP1A) and β-actin. The results shown are from a representative experiment repeated three times. The numbers correspond to treatments as follows: #1: Control sample (cells treated with DMSO); #2: Cells treated with 10 nM TCDD; #3, 4, 5 and 6: Cells treated with 10 nM TCDD plus increasing concentrations of E2 (0.1, 1, 10 and 100 nM respectively); #7: Cells treated with 10 nM TCDD+ 1 μM testosterone; #8: Cells treated with 10 nM TCDD + 10 nM E2 + 1 μM tamoxifen; #9: Cells treated with 10 nM TCDD + 100 nM E2 + 1 μM tamoxifen; #10: Cells treated with 10 nM TCDD + 1 μM tamoxifen.

Post-transcriptional events including degradation and/or stabilization of mRNA species are important mechanisms for gene expression. Flouriot and co-workers showed that, in fish liver, the E2-mediated induction of VTG mRNA was brought about by an increase in the rate of VTG gene transcription and by stabilization of cytoplasmic VTG mRNA [23]. Further, these studies indicated that the process of ERα mRNA stabilization was dependent on the presence of an uncharacterized E2-induced protein factor. In general, the stability of the mRNA has been determined by site-specific mRNA endonucleases. Endonuclease-catalyzed mRNA decay is regulated by RNA-binding proteins which specifically bind to the target mRNAs and block their cleavage by endonucleases (reviewed in [30]). For example, stability of the hepatic VTG mRNA in *Xenopus laevis* is regulated by an E2-induced RNA-binding protein, vigilin, which binds specifically to a segment of the 3'-UTR of the VTG mRNA and protects it from degradation [31-33]. The results depicted in Figure 4 showed that treatment of hepatocytes with 10 nM TCDD caused a significant reduction the VTG and ERα mRNA levels. Therefore, we performed an assay to investigate whether activation of AHR could increase the turnover rate of VTG and ERα mRNAs, i.e., whether activation of the AHR signalling pathway would trigger any VTG and/or ERα specific mRNA endonuclease activity. As the results presented in Figure 6 show, exposure of the cells to TCDD had no significant effect on the destabilization of VTG and ERα mRNAs.

Electrophoretic mobility shift assays showed that the nuclear extracts prepared from hepatocytes treated with E2 specifically bound to the ERE, i.e., the nuclei were enriched in the activated estrogen receptor. On the other hand, the DNA binding activity of the nuclear extracts from cells co-treated with estradiol and TCDD was markedly decreased. The nature of this pronounced reduction remains unknown. Nonetheless, our results indicate that TCDD treatment may cause a marked depletion of the nuclear ERα. Recently, Wormke et al. suggested a mechanism for the inhibitory AHR-ERα cross-talk in breast cancer cells [26]. These authors showed that ligand activated AHR recruits both ERα and proteasomes which results in the degradation of both AHR and ERα. Due to lack of specific antibodies raised against salmon ERα protein, we were not able to examine this mechanism in the salmon liver cells.

Another interesting issue is whether cross-talk between the two receptors is bidirectional. A bidirectional inhibitory mechanism could arise from competition between the two receptors for a common co-activator. For instance, squelching of the nuclear factor-1 has been described [34]; in addition, it has been shown that both AHR/ARNT complex [35] and ERα are able to interact with general transcription factor Sp1 [36,37]. Our experimental data indicate that squelching would not primarily account for cross-talk between the AHR- and ERα-signalling pathways, as E2 did not exert any inhibitory effect on the expression of the CYP1A gene (Figure 8). These results confirm data from experiments using primary cultured rainbow trout hepatocytes [38]. However, Ricci et al. [34] using several E2-sensitive human cells lines showed that E2 treatment decreased the TCDD-induced CYP1A1 mRNA levels and transcriptional activities. A similar effect has been reported in the human breast cancer cell line, MCF-7 [27]; however, conflicting results has also appeared [39]. Thus, the mutual interactions between the ERα- and AHR-mediated signalling pathways appears to be cell-type specific and to be regulated by specific protein factors (co-activators or co-repressors) restricted to each cell type. In our own laboratory, *in vivo* experiments with

salmon have indicated that AHR agonists, such as PCB-77, can act both antagonistic and synergistic to xenoestrogen (nonylphenol) stimulation on vitellogenesis and zonagenesis, whereas no effect of the xenoestrogen was observed on CYP1A levels [2,16]. Nevertheless, we have also observed that prolonged treatment with high doses of E2 or nonylphenol will provoke reduced CYP1A mRNA levels in the liver of juvenile Atlantic salmon [40]. Activation of the mammalian ER-signalling pathway by the AHR/ARNT complex or ARNT alone has been described recently [41,42]. It has been shown that upon ligand binding and nuclear translocation the AHR heterodimerize with nuclear ARNT, and then associates with the unliganded ERα or ERβ. The resulting complex recruits the coactivator p300 to the promoter of the E2-responsive genes leading to an E2-independent activation of the genes [41]. ERα or ERβ can also interact with the ARNT via their ligand-binding domains and activate the target genes. This kind of interaction is E2-dependent and involves physical interaction of the ERs with C-terminal trans-activation domain of ARNT [42].

Conclusions

TCDD and other AH receptor ligands are capable to disrupt E2-induced expression of the VTG gene. The negative effect of TCDD on the expression of VTG gene is accompanied by a reduction in ERα levels. Our results provide evidence that this effect is mediated through a direct interference with the auto-regulatory transcriptional loop of ERα. Additionally, E2 does not interfere with TCDD induced CYP1A gene expression, suggesting that cross-talk between the ERα- and AHR-signalling pathways is unidirectional.

Methods

Chemicals

Collagenase, 17β-estradiol, and testosterone were purchased from Sigma (USA). 2,3,7,8-tetrachloro-dibenzo-*p*-dioxin was purchased from Cambridge Isotope Laboratories, Inc. (UK).

Preparation and culture of hepatocytes

Juvenile Atlantic salmon (*Salmo salar*), with approximately 500–700 g in weight, were kept in running seawater at a constant temperature of 12°C, at the Industrial Laboratory (ILAB), HIB, Bergen, Norway. Hepatocytes were isolated by a two-step perfusion described by Berry and Friend [43], and modified by Andersson et al. [44]. Briefly, the liver was first perfused *in situ* with a calcium-free solution containing NaCl (7.14 g/l); KCl (0.36 g/l), $MgSO_4$ (0.15 g/l); Na_2HPO_4 (1.6 g/l); NaH_2PO_4 (0.4 g/l); $NaHCO_3$ (0.31 g/l) and EGTA (20 mg/l), at approximately 20°C. The liver was then perfused for about 10 min with the same buffer, but with calcium (0.22 g/l) instead of EGTA, and with collagenase A (EC 3.4.24.3, Sigma) (80 mg/150 ml). The cell suspension was filtered through a 150 μm nylon monofilament filter and centrifuged at 50 g for 5 min. Cells were washed with serum-free medium three times and finally resuspended in the complete medium. Viability of the cells was determined by Trypan blue exclusion. The cells were plated on 35 mm Primaria plates (Becto Dickinson Labware, USA) at a density of about 5×10^6 per plate, in Minimum Essential Medium with Earle's salts (EMEM), without phenol red (Gibco, Invitrogen Corp. USA), and containing 2.5% (v/v) foetal bovine serum, glutamine (0.3 g/l medium) and 1% (v/v) antibiotic mixture (Penicillin, Streptomycin, Amphotericin B). The cells were kept at 12°C in a humid atmosphere containing 5% CO_2 and 95% air. The cells were kept in culture for, at least, 48 hours prior to treatment with chemicals. The viability of the cells after each treatment was always over 90%, as determined by the Trypan blue exclusion assay.

RNA purification, Northern and slot blot analysis

Total cellular RNA was isolated using Trizol reagent, according to the manufacturer's protocol (Invitrogen). For Northern blot analysis, the samples were separated on a 1% formaldehyde-containing agarose gel, and transferred to a nylon membrane by vacuum blotting. The RNA samples were immobilized on the membranes by UV-cross-linking. The membranes were prehybridized at 42°C, for about 12 hrs, in prehybridization solution containing 50% formamide, 5 X Denhardt's solution, 6 X SSPE (1xSSPE equals 0.15 M NaCl, 10 mM sodium dihydrophosphate and 5 mM EDTA pH 7.2), 0.5% SDS, and 100 μg denatured herring sperm DNA, and hybridized with the respective probe at 42°C for about 12 hrs in the same hybridization solution. The membranes were washed 2 times (20 min/wash) in 1X SSPE/0.1% SDS at 42°C and 2 times (15 min/wash) at high stringency in 2 X SSPE/0.1% SDS at 55°C. For slot blot analysis, total RNA was applied to a nylon membrane using a slot blot apparatus, immobilized, hybridized and washed by the same conditions as described for Northern blot membranes.

Probes and labelling

The ERα probe was prepared by PCR amplification of the hinge region of the ERα cDNA (accession number X89959) obtained from Dr S.A. Rogers, School of Molecular and Medical Biosciences, University of Wales, UK. The primers used for amplification were ELaA (5'-AGG-CAC-TTT-GTT-CTT-ACA-TTT-3') and ELaS (5'-TGG-TGC-CTT-CTC-CTT-CTG-TT-3'). The PCR reaction was run using AmpliTaq (Applied Biosystems) by the following conditions: 1 cycle at 94°C for 5 min, 30 cycles at 94°C/30 sec, 55°C/30 sec, 72°C/45 sec., 1 cycle at 72°C for 7 min. The VTG probe was derived from a VTG cDNA isolated in our laboratory (Yadetie F., Goksøyr A. and Male R., unpublished). The CYP1A1 probe was a NheI

restriction fragment from the plasmid pSG-15 containing 2553 base pairs of the rainbow trout CYP1A1 (accession number M21310). The plasmid was a gift from Dr. D.W. Nebert, Laboratory of Developmental Pharmacology, NICHD; Bethesda, MD, USA. The β-actin probe was prepared by PCR amplification of a region spanning nucleotides 225–724 of the Atlantic salmon β-actin cDNA (accession number AF012125). The primers used for amplification were ActinF (5'-CGT-CAC-CAA-CTG-GGA-CGA-CA-3') and ActinR (5'-GCT-CGT-AGC-TCT-TCT-CCA-G-3'). The PCR conditions were as described for preparation of ERα probe. All probes were labelled by random priming method using [α-^{32}P]dCTP (3000 Ci/mmol)(Amersham) according to Ausubel et al. [45]. The images were scanned using Phosphoimager FLA-2000 (Fuji Photo Film CO. Ltd, Japan). Radioactivity was quantified using Phosphoimager FLA-2000 as photo stimulated luminescence (PSL).

Nuclear run-off transcription assay

Preparation of the active nuclei and performance of the run-off transcription assay was performed following an established protocol [45], with some modifications.

Preparation of the active nuclei

The cells were treated with E2, TCDD or a combination of these chemicals for 8 hrs. The medium was removed and the cells were washed two times with 2 ml of ice-cold phosphate-buffered saline (PBS). The cells were gently dislodged from the plastic surface by scraping with a rubber policeman. The cells were collected by centrifugation at 500 g for 5 min, at 4°C, and lysed using 1% Nonidet-40 buffer (10 mM Tris-HCl pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, 1% V/V NP-40). The nuclei were isolated by centrifugation at 2500 rpm in an Eppendorf centrifuge and resuspended in 100 μl of glycerol storage buffer (50 mM Tris-HCl pH 8.3, 40% glycerol, 5 mM $MgCl_2$, 0.1 mM EDTA) by gently vortexing. The prepared nuclei were immediately frozen and kept at -80°C.

Binding of plasmid DNA to nylon membrane

200 ng of each the respective plasmid DNA was linearized with appropriate restriction enzyme, and denatured by adding 1 M NaOH to a final concentration 0.1 M and by incubation at room temperature, for 30 min. 10 X vol of 6 X SSC (1 X SSC contains 0.15 M NaCl and 0.015 M trisodium citrate pH 7.0) per sample was added and the plasmid DNA was spotted onto the membrane using a slot blot apparatus (Schleicher & Schuell, Germany).

In vitro transcription assay

An aliquot of 100 μl of frozen nuclei was thawed at room temperature and mixed with 100 μl of freshly prepared 2 X reaction buffer containing nucleotides (10 mM Tris-HCl, pH 8.0, 5 mM $MgCl_2$, 300 mM KCl, 5 mM dithiothreitol, and 0.5 nM of GTP, CTP, ATP and [α-^{32}P]UTP (800 μCi/mmol)) and incubated for 30 min at 30°C. Following *in vitro* transcription 1 X vol. of Trizol reagent was added and the total RNA was isolated following the instructions of the manufacturer. The yield of labelled RNA was determined using a scintillation counter. The membranes were hybridized to 1 × 10^6 cpm labelled RNA. Prehybridization, hybridization and wash of the membranes were performed by the same conditions as described for Northern blot membranes.

Electrophoretic mobility shift assay (EMSA)

Preparation of nuclear extracts

The nuclear extracts were prepared by a previously described protocol [46], with some modifications. A triplet of cells cultured on 35 mm dishes was used in each assay. After exposure of the cultured cells to the appropriate chemicals the dishes were washed two times with ice-cold PBS and the cells were harvested by trypsinization, collected in 15-ml Falcon tubes by centrifuge at 500 g at 4°C, for 5 min, and washed 3 X with ice-cold PBS. After the final wash, the cells were resuspended in 5 ml of homogenization buffer (10 mM HEPES, pH 7.6, 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM DTT) and incubated on ice for 10 min. The cell suspensions were transferred to a precooled 7 ml tissue homogenizer and homogenized by 10 strokes. The homogenized cell suspensions were centrifuged at 500 g for 5 min. The pellet (enriched nuclei) was resuspended in 3 ml of S1 solution (0.25 M sucrose, 10 mM $MgCl_2$). The resuspended pellet was carefully layered over 3 ml of S2 solution (0.35 M sucrose, 0.5 mM $MgCl_2$) and centrifuged at 4000 g, for 5 min, at 4°C. This step resulted in a cleaner preparation of the nuclei. The nuclear pellet was resuspended in 50 μl of nuclear lysis buffer (10 mM HEPES, pH 7.6, 100 mM KCl, 0.1 mM EDTA pH 8.0, 3 mM $MgCl_2$, 10% glycerol and 1% NP-40 including a protease inhibitor cocktail, 1 mM phenylmethylsulfonyl fluoride (PMSF), pepstatin A, leupeptin, antipain, and aprotinin) and transferred to an Eppendorf tube. The resuspended pellet was incubated, on ice, for 10 min and then centrifuged at 13000 rpm (12000 g), at 4°C, for 10 min. The supernatant was dialyzed against 1000 X volume of the same buffer without NP-40, for 30 min, using a Slide-A-Lyzer MINI dialysis unit (Pierce, USA) at 4°C. The protein concentration in the samples were determined by the method of Bradford [47] and the extracts were kept at -80°C until use.

Performance of the EMSA

The complementary oligonucleotides (Medprobe, Oslo, Norway) were mixed and annealed by incubation at 100°C, for 10 min, and slowly cooling to the room temperature (for 1 hr). The probes used were a 3'-end biotin-labelled DNA duplex of the sequence 5'-CATTCTGTTT-GCTGTGTCATGTTGACCTGCTCTAGA-3', containing a

putative ERE derived from the proximal promoter region of the Atlantic salmon ERα gene (Hanne Ravneberg and Rune Male, unpublished), or a radioactive labelled ERE oligo (ERE-sense oligo: 5'-CGGGGATCCTAGTAGGTCACAGTGACCTCATGGATCC-3', ERE-antisense oligo: 5'-GGGGATCCATGAGGTCAGTGTGACCTACTAGGATCC-3'). The radioactive probe was labelled by end-filling reaction using DNA polymerase (Klenow fragment), and [α-^{32}P]dCTP (3000 Ci/mmol) according to the standard protocols [45]. The Oct probe was composed of a sense oligonucleotide Oct-1: 5'-CGGGGATCCTGATCCATGCAAATCGACGACT-3' and antisense oligonucleotide Oct-1: 5'-GGGGATCCAGTCGTCGATTTGCATGGATCAG-3'. The binding reaction with the biotinylated probe was performed according to the instructions of the manufacturer, using the LightShift Chemiluminescent EMSA kit (Pierce) and 1 μg of nuclear extracts per reaction. The DNA-protein complexes were electrophoresed through a 6% native polyacrylamide gel, and transferred to a nylon membrane electrophoretically. The samples were fixed to the membrane by UV-cross-linking. The chemiluminescent detection was performed as described by the manufacturer. The membranes were exposed to an enhanced chemiluminescence film (Kodak), for 5 seconds, before developing or scanned using Phosphoimager FLA-2000 (Fuji Photo Film CO. Ltd, Japan). Alternatively, 3 μl of nuclear extract was mixed with 1 μg poly (dI-dC) in binding buffer (10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM DTT, 1 mM EDTA and 4% glycerol), and incubated at room temperature for 15 min. The radio-labelled probe was subsequently added to the mixture, and the binding reaction was further incubated, at room temperature, for an additional 20 min period, before fractionation by electrophoresis. The gels were subsequently dried and exposed to phosphoimager plates, overnight, before scanning.

Statistical analysis

Statistical analysis was performed by one-way ANOVA (n = 3 in each group, unless otherwise indicated) using JMP (version 5.0), SAS Institute Inc., USA. Data were tested for normal distribution (Shapiro-Wilk W test) and for homogeneity of variance. Sets with homogeneous variances were analysed by Dunnett's test to determine which means were significantly different from a reference group ($P < 0.05$).

Authors' contributions

VB carried out the experiments and drafted the manuscript. RM initiated the study and participated in its design and coordination. AG participated in the design and coordination of the study.

Acknowledgements

This study was supported by the Norwegian Research Council (NFR grants 125692/720 and 156166/130) and Biosense Laboratories AS (Bergen, Norway). The authors would like to thank Wenche Telle, for technical assistance. We are also most grateful to Dr. B.E. Grøsvik, for his advise about statistical analysis of data.

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