Estrogen receptors as modulators of aryl hydrocarbon receptor mediated transcription.
Summary

The aryl hydrocarbon receptor is a member of the basic-helix-loop-helix - Per (Period) - ARNT (aryl hydrocarbon nuclear translocator) - SIM (single minded) (bHLH/PAS) family. 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) induce gene transcription of AhR target genes cytochrome P450 (CYP) 1A1 and CYP1A2. The transcriptional activity of AhR induced by TCDD in cancer cell lines has been reported to be in link with 17β-estradiol (E2) and estrogen receptor (ER). TCDD inhibits E2 dependent cell proliferation, secretion of tissue plasminogen activator and E2 induced proteins, such as pS2 and cathepsin-D in ER positive human breast cancer cell lines.

TCDD do not bind ER but it inhibits ERα mediated signalling. The effect that ER have on the AhR activity is not clear. To study the role of ER in AhR signalling; HC11 (mouse mammary epithelia cells) wild type and small inhibitory RNA (siRNA) stable cell lines were treated with ER subtype specific ligands alone or in combination with TCDD. HC11 is known to express both ER subtypes, AhR and Aryl Hydrocarbon Receptor Translocator (ARNT). Using RNA isolation and Real-Time Polymerase Chain Reaction the level of TCDD induced CYP1A1 mRNA was determined. To detect binding of specific proteins to DNA segments in vivo chromatin immunoprecipitation (ChIP) assay was used.

In the HC11 cells with siRNAs for the ERα subtype the dioxin response was almost depleted, whereas HC11 stable cells transfected with empty vector and ERβ containing vector showed a 15-20-fold induction of CYP 1A1 expression for both TCDD treatment alone and cotreatments. No significant difference was seen for the ER ligands. Our data suggest that ERα and not ERβ is an important modulator of AhR induced gene expression. Further experiments are needed to confirm these results.

Background

The Aryl Hydrocarbon Receptor

The aryl hydrocarbon receptor is a member of the basic-helix-loop-helix - Per (Period) - ARNT (aryl hydrocarbon nuclear translocator) - SIM (single minded) (bHLH/PAS) family. Other members in this family include hypoxia factor 1 α (HIF1 α ), SIM, p160 coactivator proteins and Per. The receptor is a ligand-activated transcription factor that interacts with several nuclear cofactors (1, 2, 3).

AhR is found in the cytosol in a complex with heat shock protein 90 and other proteins. Interaction with ligands causes the receptor to translocate into the nucleus and form a heterodimer with the ARNT. The complex interacts with dioxin/xenobiotic responsive elements (DREs/XREs) in the genome. Toxic halogenated aromatics, such as TCDD and polycyclic aromatic hydrocarbons (PAHs) act as ligands for the AhR (2, 4). Known target genes of AhR target genes include cytochrome P450 (CYP) 1A1, CYP1A2 and aldehyde dehydrogenase 3A (2).
Estrogen Receptor α and β
ERα and ERβ mediate the biological actions of estrogen. ERs are members of the nuclear transcription superfamily. These receptors are ligand-regulated transcription factors (5). Receptors included in the nuclear receptor superfamily consist of a common domain structure (Fig.1). Domain C and E is highly conserved among the family. The DNA-binding domain (DBD) is located in the centre, and the ligand binding domain (LBD), (AF-2) is in the C-terminal end of the receptor. The N-terminal A/B domain contains the constitutive ligand-independent transactivation domain, AF-1. Transactivation of AF-2, located in the LBD, is dependent on ligand binding (6).

Fig 1 Common domain structure for the nuclear receptor superfamily.

Agonists and antagonists of ERα cause different conformational changes of the receptors AF-2 domain. When bound to the agonist estradiol, helix H12 of the receptor moves and causes the formation of a hydrophobic groove involving helix H3, H5, H6 and H12. This groove is important for the interaction with the NR box in p160 coactivator proteins. Raloxifene a steroid receptor antagonist dislocates H12 into a position were it blocks the hydrophobic coactivator binding groove (5, 7).

Under identical circumstances the AF-1 domain is highly active on a range of estrogen responsive promotors in ERα but not ERβ (5). The amino acid sequences of the DNA binding domain (DBD) of ERα and β show 96 % similarity but the ligand binding domain (LBD) is only 59 % identical.

Ligand bound ER regulate gene transcription by binding to consensus estrogen response elements (ERE), non-consensus ERE and ERE halfsites. The receptor is able to interact with other transcription factors for example Sp-1, AP-1 and NFκB. Through this pathway ER affect transcription without direct DNA-interaction (8, 9).

The expression of the two ER receptor subtypes is tissue and cell type specific and the receptors are transcribed from different genes. ER α and β can form functional heterodimers when co expressed. The receptors can be found in the same tissue but not necessarily in the same cell type. ER α is expressed mainly in the uterus, liver, heart and kidney, while the β subtype is expressed primarily in the ovary, prostate, lung, bladder, gastrointestinal tract, hemic, immune and central nervous system. The two specific ER’s differ in transcriptional activities in context to ligand, cell-type and promotors. ERβ can antagonize ERα induced gene expression.

On naked DNA the two receptor subtypes display similar transcription activity but not on chromatin template. ERβ is not able to activate transcription to the same extent as ERα a possible mechanism is that ERβ is incapable to recruit a stable preinitiation complex to chromatin. The inability of ERβ maybe due to the difference in the N-terminal transactivation
region, since after exchanging the AF-1 region of the ERβ with that of ERα, strongly improved the ability of ERβ to induce transcription at chromatin templates (5).

AhR-ER Cross-talk
The transcriptional activity of AhR induced by TCDD in cancer cell lines has been reported to be in link with E2 and ER. TCDD inhibits E2 dependent cell proliferation, secretion of tissue plasminogen activator and E2 induced proteins, such as pS2 and cathepsin-D in ER positive human breast cancer cell lines. These effects were not observed in ER negative cell lines (2). Inhibitory AhR-ER cross-talk was detected in rodent uterus, rodent mammary tumors, breast, ovarian and endometrial cancer cell lines (4). Rats and mice treated with E2 plus TCDD showed inhibition of E2 induced uterine responses (i.e. uterine wet weight), evidently supporting the inhibitory action of TCDD. The incidence of spontaneous uterine and mammary tumours in female Sprague-Dawley rats decreased in rats on a TCDD diet for up to two years. Uterine and mammary tumours are closely linked to E2 and ER (2, 4).

The effect that ER have on the AhR activity is less clear and not much is known about the effects of AhR on ERβ or vice versa. Recent studies indicate possible modulation effects of ER on AhR induced gene expression. ER-negative MDA-MB-231 and Hs578 T (11) human breast cancer cells express AhR but CYP1A1 expression is not induced by TCDD treatment. In MDA-MB-231 and Hs578 T cells cotransfected with pRNH11c or pMCAT5.12 (two Ah responsive plasmids) and human ER (hER) expression plasmid and treated with 10 nM TCDD the Ah responsiveness is restored. (12). In MCF-7 cells long time exposure of E2 is needed for maintenance of AhR expression and induction of TCDD induced CYP1A1 and CYP1B1 (13). TCDD induced cytochrome P-450 1A1 expression in intact and ovariectomized rats was significantly enhanced by E2 treatment. E2 alone or in cotreatment with TCDD showed an increase in nuclear AhR in immunoblot analysis in female Long-Evans rats (14). These studies implicate a possible role for ER in AhR activation.

In this project ER subtype specific ligands, propyl-pyrazole-triol (PPT) ERα specific agonist, diarylpropionitrile (DPN) ERβ specific agonist, E2 (Fig. 2) alone or in combination with TCDD were used to study the role of ERs in the AhR signalling in mouse mammary epithelia cell line (HC11). HC11 are known to express both ER subtypes, AhR and ARNT. HC11 cell lines stably transfected with small inhibitory RNAs for the two estrogen receptor subtypes were used to further determine ERα and ERβ effect on AhR signalling. Using RNA isolation and Real-Time Polymerase Chain Reaction (RT-PCR) the relative abundance of TCDD induced CYP1A1 mRNA levels were determined. The HC11 cells stably transfected with siRNA for ERα and treated with TCDD showed a significant decreased AhR response compared to HC11 cells transfected with empty vector and with siRNA for ERβ. Our results support the theory that ERα is important for AhR function.
Material and Methods

Cell culture
HC11 wild type cells were cultured in RPMI 1640 without L-glutamine (Invitrogen Corp.) supplemented with 10% fetal calf serum (FCS) (Invitrogen Corp.), 5 mM L-glutamine, (1% = 5 nM) penicillin/streptomycin, 0.5 nM insulin, Epidermal growth factor (EGF).
The media used for culturing of the HC11 siRNA cells were supplemented with additional 10 ng/L blastocidin to select for the stables. Cells were maintained at 37°C containing 5% CO2.

RNA isolation, small inhibitory RNA and real-time PCR
Wild type HC11 cells and HC11 cells stably transfected with either empty vector, vector harboring short hairpin oligo directed against ERα, or vector harboring short hairpin oligo directed against ERβ cells (received from Lars-Arne lab CBT) (15) were seeded in 6-well plates and grown in phenol red and L-glutamine free RPMI 1640 (Invitrogen Corp.) supplemented with 5% DCC-FCS, EGF, insulin, penicillin/streptomycin and L-glutamine for 1-day before 24 h ligand-treatment. RNA was isolated using RNeasy spin columns (Qiagen). 1μg RNA was treated with DNaseI for 15 min at room temperature. Using random hexamer primers and SuperScriptII (Invitrogen) the extracted RNA was reverse transcribed. 1μl of the synthesized cDNA was used with SYBR green (Invitrogen) for real-time PCR. Primers used for CYP 1A1 mRNA expression, were forward primer 5´ CGT TAT GAC CAT GAT GAC CAA GA 3´and reverse primer 5´ TCC CCA AAC TCA TTG CTC AGA T 3´ (DNA Technology A/S, Science Park Aarhus, Denmark). All target gene transcript were normalized to the 18S rRNA (PE Applied biosystems)(1).

Chromatin immunoprecipitation assay (ChIP)
Chromatin immunoprecipitation assay (ChIP) has become a well used technique for experimental studies of transcriptional regulation. ChIP technique is a breakthrough for in vivo analysis of transcription factor binding to endogenous promoters (5). This method preserves the higher chromatin structure by crosslinking cells in vivo with formaldehyde. The fixed cells are resistant to restriction enzymes and DNase I therefore the cells are sonified to shear the chromatin. The chromosomal proteins are identified via immunoprecipitation with specific antibodies targeted to the DNA-binding proteins. After the precipitation the cross-linking is reversed and DNA purified. RT-PCR can be used to analyse the immunoprecipitated DNA (16).
HC11 wild type cells were cultured in 15 cm dishes for two days in phenol red-free RPMI 1640 without L-glutamine supplemented with 10% DCC-FBS. The cells were then treated with ligands dissolved in DMSO for one hour in 37°C incubator containing 5% CO2. The ChIP assay was performed as previously described (1).
Results

To determine the effect of ERs on dioxin signalling HC11 siRNA cells were treated with TCDD alone or in combination with ER subtype specific ligands 10 nM respectively. We used ER natural ligand E2, DPN (7) and PPT (8). Levels of CYP1A1 mRNA were measured with RT-PCR and normalized to the 18S rRNA.

Effect of transfecting process on CYP1A1 responsiveness.

A comparison between wild-type HC11 cells and cells stably transfected with empty siRNA vector showed that the transfection and selection process mildly affected AhR responsiveness (Fig 3). The vector containing cells show a lower overall induction of AhR mediated CYP1A1 mRNA expression, but the same trend in CYP1A1 expression was observed. This is possibly an effect of the incorporation of the vector in the mouse genome. The incorporation is random and may affect other functions in the cell.

Figure 3. Comparison of the effects of HC11 cells stably transfected with empty vector and wild type HC11 cells treated with 10 nM TCDD alone or in cotreatment with 10 nM E2, PPT or DPN for 24 h as described in Materials and Methods. RNA was isolated and the CYP1A1 mRNA levels were determined using real-time PCR. The data are representative of two independent experiments.

Figure 4. Effect of ERα (Fig. 4a) and ERβ (Fig. 4b) on AhR mediated activity. Effects of ER subtypes on AhR–dependent gene expression was assessed in HC11 siRNA stables treated with 10 nM TCDD alone or in cotreatment with 10 nM E2, PPT or DPN for 24 h as described in Materials and Methods. RNA was isolated and the CYP1A1 mRNA levels were determined using real-time PCR. The data are representative of two independent experiments.
Role for ERs in AhR mediated gene expression.

When ERα expression is knocked down by siRNA the dioxin response in HC11 cells is depleted with fold induction merging between 1.5-3 fold (Fig 4a). The high expression level for CYP1A1 mRNA in HC11 siERα cells treated with 0.1 nM TCDD is an error due to RNA isolation. The HC11 cells harboring the vector containing short hairpin oligo for ERβ and HC11 cells transfected with the empty vector showed a 15-20-fold induction of CYP 1A1 expression for both TCDD treatment alone and cotreatments (Fig 4b and Fig 5). No significant difference or effect was seen for the ER ligands.

The low expression of CYP1A1 mRNA for HC11 siERβ cells seen in the treatment with TCDD + DPN was not shown in all data sets (Fig 5b). Although these data are representative of two independent experiments, more replicates are needed to confirm this result. A possible mechanism for this phenomenon may be that in absence of ERβ the potency selective agonist for the receptor, DPNs agonism for ERα is increased. DPN was a 30-fold more potent ER-beta agonist compared to the receptors α isoform in various ER inducible gene expression sites in human endometrial cancer cells (HEC-1) (8).

The summary graph (Fig 5) clearly demonstrates that ERα has an affect on AhR responsiveness compared to ERβ. When ERα expression is knocked down by siRNA the dioxin response in HC11 cells is decreased with fold induction merging between 1.5-3 fold.

No results are presented from the ChIP assay in this report due to non functional antibodies. The sensitivity of this method relies on the quality of the antibodies and their ability to bind their antigen and within the time limit of this course no such was found for the HC11 cells and the estrogen receptors.

Further replicates are needed to confirm these results since there was variability in the data. The variability might be due to pipeting since the RT-PCR is an extremely sensitive technique, the same plating may give slightly different results from day to day. Differences in cell density may also be a contributing factor.
Discussion

It has been shown that there is interaction between the AhR and the ER. The E2 induced ER mediated signalling is inhibited by TCDD induced AhR (4, 10). The mechanism behind this repeatedly proven interplay is not known and many different mechanisms have been proposed. Possible cross-talk mechanisms proposed are: rapid metabolism of estrogen, increased ER degradation, inhibitory dioxin responsive elements located in the estrogen-responsive genes, squelching of common cofactors and induction of inhibitory factors (1). Blocking of proteolysis in cells treated with TCDD and E2 results in loss of TCDD inhibited ER gene expression implicating the role of a TCDD induced protein, not yet found (10).

Recently conducted studies indicate that ERα play a positive role in modulating AhR signalling (1, 12). Estrogen negative breast cancer cells expressing AhR, without inducing CYP1A1 gene expression upon TCDD treatment, had their Ah response restored when transiently transfected with human ER (12). Little is known of the modulating effects of Eβ on AhR gene expression.

In our study we used HC11 cells stably transfected with vectors containing short hairpin oligos fixed on the different ER subtypes to demonstrate the different receptors affect on the TCDD induced AhR signalling.

Our data show subtype selectivity for the modulating effects of the ER receptors on AhR mediated transcription. When ERα is abolished by siRNA the dioxin response in HC11 cells is almost completely depleted, suggesting that ERα plays a positive modulating role in AhR signalling in mouse mammary epithelia cells. This effect was also seen in studies were T47D cells were transfected with plasmids containing siRNA directed against ERα and treated with TCDD (1). The siRNA ERβ and mock HC11 cell line showed a 15-20-fold induction of CYP 1A1 expression for both TCDD treatment alone and cotreatments with ER ligands. The collected data propose a positive modulating function of the ERα subtype but not the ER β in transcriptional activation mediated by AhR. Conflicting results has been published on the effect of ER subtype specific ligand activation of dioxin response genes. Our data show no significant effect of the ER subtype ligands. This indicates that it is the protein, the receptor that is important and that the ligands play a different role.

Tissues express different levels of the ERs. ER α is expressed mainly in the uterus, liver, heart and kidney and ERβ is expressed primarily in the ovary, prostate, lung, bladder, gastrointestinal tract, hemic, immune and central nervous system. Our results indicate that the dioxin response may differ among tissues depending on the relative expression level of ER subtypes.

In future studies we would like to use ChIP assays for different AhR responsive genes such as CYP1A1, CYP1A2 and ALDH 3 to further investigate at the level of recruitment the modulating effects of ERα and ERβ on AhR mediated transcription. ChIP assay have recently showed TCDD induced temporal recruitment of ERα to AhR target genes. TCDD and E2 cotreatments resulted in a significant recruitment increase (1). Stable cell lines with ERα and ERβ can be used to further show the modulating role of the receptors. ERα stable cells should have a lower dioxin response then ERβ stable cells to confirm our results. To further investigate the modulating effects of the two subtypes on AhR activity and determine the biological role of this modulation, ERα and ERβ knock out mice treated with TCDD will be done to evaluate the in vivo affect of ERs.
References


