3-methylcholanthrene displays dual effects on ERα and ERβ signaling in a cell-type specific fashion.

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Running Title: Dual effects of PAH's on ER signaling.

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ABSTRACT

The biological effects of 17β-estradiol (E_2) are mediated by the two estrogen receptor isoforms ERα and ERβ. These receptors are ligand-inducible transcription factors that belong to the nuclear receptor superfamily. These receptors are also target for a broad range of natural and synthetic compounds that induce ER activity, including dietary compounds, pharmaceuticals and various types of environmental pollutants such as bisphenols and polychlorinated hydroxy-biphenyls. Here, we study the effect of the combustion byproduct 3-methylcholanthrene (3-MC) on ERα and ERβ. 3-MC is a compound previously identified as an activator of the aryl hydrocarbon receptor (AhR). Activation of AhR is classically associated with an inhibition of the E_2 signaling network. In this study we demonstrate that 3-MC is a cell-specific activator or inhibitor of E_2 signaling pathways. We show that 3-MC acts as a repressor in some cells, presumably via the AhR, whereas it is a potent activator of ER activity in other cells. Interestingly, we demonstrate that the estrogenic effects of 3-MC are dependent on the ability of cells to metabolize parental 3-MC to alternative compounds. In summary our results suggest that exposure to AhR ligands like 3-MC can lead to either activation or repression of E_2 signaling depending on the cellular context.

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INTRODUCTION

Eukaryotic cells respond and adapt to changes in their environment by altering their enzymatic activities. This can be accomplished in part by increasing or decreasing the transcription rate of genes encoding relevant proteins. A critical point for cells is to correctly decipher the environmental changes that occur. To meet this challenge, eukaryotic cells have developed receptor proteins with the ability to distinguish between different environmental cues. However, the intensive use of chemicals in today's modern society has introduced a plethora of man-made compounds into the environment that possess abilities to interfere with receptor-mediated signaling pathways, a phenomenon known as endocrine disruption.

The two estrogen receptor isoforms, ERα and ERβ, regulate the cellular response to estrogens which are involved in the regulation of a wide range of physiological functions, including cell growth and proliferation, regulation of the cardiovascular system and maintaining of bone homeostasis. Besides the endogenous hormones, a broad range of natural and synthetic compounds induces ER activity. For example, dietary substances like isoflavonoids and coumestans activate the ERs, and so do also various types of environmental pollutants such as bisphenol A and polychlorinated hydroxy-biphenyls.

The ERs belong to the nuclear receptor (NRs) superfamily and share a conserved structural arrangement with other members of the family. NRs carry a centrally located, highly conserved, DNA-binding domain (DBD) that mediates both dimerization and specific DNA binding. The DBD is flanked by an N-terminal A/B domain, comprising a transcriptional activation function known as AF-1. In the C-terminus resides the ligand-binding domain (LBD), which harbors the ligand-binding pocket, dimerization interfaces and a second transcriptional function called AF-2 (Gronemeyer et al., 2004).

The NRs share a common mechanism of action where binding of an agonistic ligand induces a complex transition, where the receptor undergoes a conformational change and goes from

inactive to the active state. To attain full transcriptional activity, the receptors also need to recruit auxiliary proteins such as co-activators.

In addition to endogenously produced estrogens, the activity of the ERs can be modulated by a wide array of compounds. Some examples are dietary substances such as isoflavonoids and coumestans, industrial chemicals and chemical pollutants such as bisphenols and polyaromatic hydrocarbons (Nilsson et al., 2001). Many of these exogenous substances act by occupying the ER ligand-binding pocket and thus function as bona fide ligands to one or both ER isoforms, whereas others trigger alternative signaling pathways, which in turn interfere with ER function. An example of the latter mechanism is the effect of a group of environmental pollutants known as dioxins. They interfere with ERα and ERβ signaling but do not bind to the LBD of the ERs. The most potent dioxin, TCDD (2,3,7,8-tetrachlorodibenzo-*p*-dioxin) is formed through incomplete combustion of waste material, or as a side product in certain industrial processes. The biological responses to dioxin include toxic and teratogenic effects and a marked up-regulation of drug-metabolizing enzymes such as cytochrome P450 1A1 (Poland and Knutson, 1982). In addition dioxins and related compounds interfere with several nuclear receptor pathways, including estrogen, androgen and thyroid hormone signaling (Chen et al., 2001; Kharat and Saatcioglu, 1996; Safe et al., 2000) although the molecular mechanism behind this interference is not well-characterized. The mediator of the biological effects of dioxin is the AhR (Aryl hydrocarbon Receptor). The AhR is a member of the bHLH-PAS (basic helix-loop-helix-Per-ARNT-Sim) family of proteins that includes transcription factors such as the hypoxia-inducible factors HIF-1α and EPAS, the circadian regulatory proteins CLOCK and PER, the general dimerization partner protein ARNT to mention a few (Gu et al., 2000).

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3-methylcholanthrene is an environmental pollutant mainly formed by incomplete combustion processes, with a potent ability to induce AhR-dependent transcription. Recently it has been suggested that the AhR-ARNT complex, upon activation by the AhR agonist 3-methylcholanthrene (3-MC), interacts with the ERs and induce transcription of E_2-target genes in the absence of E_2 (Ohtake et al., 2003). Given that other AhR agonists, such as TCDD, have a documented inhibitory effect on E_2-transcriptional pathways, these are somewhat surprising experimental findings. In addition, another study challenges this viewpoint, and suggests that 3-MC rather acts as a direct activator of ERα and that the AhR is not involved in this activation process (Abdelrahim et al., 2006). These discrepancies are puzzling, in light of that both studies utilized similar cellular backgrounds (MCF-7 cells) and experimental conditions. It is also noteworthy that numerous previous studies on AhR-ER crosstalk demonstrate exclusively negative, not positive, effects on ER signaling by AhR agonists. It may therefore be of particular importance to note the difference in choice of AhR ligand in the study by Ohtake *et al*, in which 3-MC was found to induce ligand-independent activation of the ERs, where other studies have shown inhibitory effects of TCDD on estrogen signaling (Astroff et al., 1990; Kharat and Saatcioglu, 1996; Safe, 2001; Safe et al., 2000).

In light of these data, we decided to compare the effects of TCDD and 3-MC, respectively, on the transcriptional activity of the ERs in different cellular model systems. TCDD, as other dioxins, is highly resistant to biotransformation and subsequent clearing by cellular drug-metabolizing enzymes, whereas polyaromatic hydrocarbons such 3-MC are more readily metabolized (Myers and Flesher, 1990; Shou and Yang, 1990). Xenobiotic biotransformation can lead to the generation of metabolites whose biological properties can differ considerably from those of the parent compound. To enable tracking of putative metabolites displaying differing biological properties, additional studies were carried out using radio-labeled 3-MC in cell lines derived from

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different tissues. In summary, our results indicate that 3-MC can either repress or activate the ERs depending on the cellular background.

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MATERIALS AND METHODS

Plasmids and Reagents.

2,3,7,8-tetrachlorodibenzo-*p*-dioxin, 3-methylcholanthrene and benzo(a)pyrene were purchased from AccuStandard (New Haven, CT). Diethylstilbestrol, 17β-Estradiol and 4-OH-Tamoxifen were from Sigma (St Louis, MO). ICI 182,780 was from AstraZeneca. Radio-labeled [^{3}H]-3-methylcholanthrene was supplied by Moravek, Brea, U.S.A. (radiochemical purity: 98.2%, checked by HPLC; specific activity 44.4 GBq/mmol). Flo-Scint II and Ultima Gold liquid scintillation cocktails were from Perkin Elmer Life Sciences (Courtabœuf, France). HPLC grade solvents were purchased from Scharlau (Barcelona, Spain). Water for HPLC was purified with a Milli-Q system (Millipore, Saint-Quentin-en-Yveline, France).

The plasmids pSG5-mERα, pSG5-mERβ, 3xERE-TATA-Luc, Gal4-Luc, pCMV5-βGal, Gal4-ERα-LBD and Gal4-ERβ-LBD have been described elsewhere. Details can be obtained from the authors upon request.

Polyclonal anti-ERα H-184, polyclonal anti-ARNT1 H-172, polyclonal anti-AhR H-211, and actin antibody sc-8432 (all Santa Cruz Biotechnology, Inc.) and ERβ GTX14021 (GeneTex) were utilized at the conditions suggested by the manufacturers.

ERα and ERβ ligand binding domain (LBD) scintillation proximity assay (SPA).

The binding of TCDD and 3-MC to ERα and ERβ-LBD were analyzed by SPA on the same test occasion. Briefly, the compounds, dissolved in DMSO at a concentration of 10mM and reference compound (17β-estradiol, E_2), were diluted 13 times in a microtiter plate and further serially diluted in 1:4 steps to twelve different concentrations. 18 µl of each dilution was added to a 384 well assay plate. Next, 35 µl of ERα-LBD- or ERβ-LBD streptavidine-coated polyvinyltoluene

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SPA beads was added to the plates, followed by 35 µl of tracer (^{3}H-E_2, 2.8nM). The plates were centrifuged for one minute at 1000rpm and then incubated at room temperature over night with constant shaking. A second centrifugation was made at 2000 rpm for 5 minutes the next morning, before the plates were measured in the Trilux Microbeta.

The resulting curves were plotted by using the XLfit Version 2.0.11.70, and the IC50 values were calculated using a four-parameter logistic equation y=A+((B-A)/(1+((C/x)^D))).

Cell culture and transient transfection assays.

HC11 cells and stably transfected 3xERE HC11 cells (H-ERE; previously described in Faulds et al., 2004) were maintained in RPMI 1640 medium (Invitrogen) supplemented with 8% fetal calf serum (FCS; Invitrogen), L-glutamine (Gibco), 50 µg/ml gentamycin (Gibco), 10 ng/ml EGF (Sigma) and 5 µg/ml insulin (Sigma, Inc.). HepG2 and CV-1 cells were routinely maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS and penicillin (100U/ml) and streptomycin (100 µg/ml).

HepG2 or HC11 cells were transfected as described earlier (Brunnberg et al., 2003). Typically, cells were seeded in 12-well plates in phenol red-free medium 24h prior to transfection. Cells were transfected using Lipofectamine reagent (Invitrogen) according to the manufacturer's recommendations. We used 200ng of appropriate reporter, 3xERE-TATA-Luc or Gal4- plasmids. Following transfection, the medium was exchanged with phenol red-free medium supplemented with 5% dextran-coated charcoal (DCC)- treated FCS and the cells were allowed to grow for an additional 24h. At this point, cells were harvested and luciferase and β-galactosidase activities were determined. Data is presented either as % activity ± SD, where reporter activity obtained at 10nM E_2 was arbitrarily set to 100%, or as fold induction ± SD (Gal4 assay), where activity of

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reporter plasmid alone without hormone treatment was arbitrarily set to one. Each bar represents the mean of at least three independent transfections performed in duplicates or triplicates. Stably transfected H-ERE cells were grown, treated and lysed similarly, before luciferase activity was determined and protein concentrations measured by the Bradford method (Bradford, 1976) using bovine serum albumin (BSA) as standard. The luciferase values are correlated to the protein concentration of each sample.

Statistical methods.

Differences in reporter gene activity and gene expression were analyzed by two-tailed Student's t-test. A p-value of 0.05 or less was considered statistically significant.

Incubation of HepG2 and HC11 cells with radio-labeled 3-MC.

24 h HepG2 and HC11 cells incubations were carried out, in triplicate, in the presence of 0.1 µM or 10 µM 3-MC / [^{3}H]-3-MC (4.4 10^3 Bq/well). At 24 h, media were removed from the wells and the cells were recovered in a Tris buffer (50mM/pH 7.8, 10mM EDTA, 150mM NaCl).

Real-time PCR to detect transcription of pS2 and CYP1A1.

Wild type HepG2 or cells stably transfected with ERα expression vector were seeded out into 60 mm dishes and grown in phenol red-free medium with 5% DCC-treated FCS for 48 h. After treatment with E_2, 3-MC or TCDD for 6 h, RNA was isolated using Trizol (Invitrogen) according to the manufacturer's recommendations. 1 µg of total RNA was treated with DNAseI and reverse transcribed using random hexamer primers (InVitrogen). 1 µl of the resulting cDNA was then used for real-time PCR with SYBR green (InVitrogen). For pS2 the primers were 5'-CCTCCCAGTGTGCAAATAAGG-3' and 5'-TGGAGGGACGTCGATGGTAT-3', and for CYP1A1 5'-TGGTCTCCCTTCTCTACACTCTTGT-3' and 5'-

ATTTTCCCTATTACATTAAATCAATGGTTCT-3'. All gene transcripts were normalized to the 18s rRNA content and to the untreated samples.

SiRNA-mediated down regulation of AhR intracellular levels.

Expression vectors for siRNA: siRNA oligonucleotides for AhR, ARNT, and a scrambled sequence were cloned into the pSuperior plasmid (Oligoengine) according to the manufacturer's instructions. The siRNA was directed against the following sequences: AhR; TACTTCCACCTCAGTTGGC, ARNT; CCATCTTACGCATGGCAGT, scramble; ACTCTATCTGCACGCTGAG. Hep-ERα cells were seeded and grown in 6-well plates in complete medium until 50-60% confluency. The cells were then transfected with INTERFERin siRNA transfection reagent according to manufacturer's instructions. In short, 50 nM siAhR or scrambled plasmid per well, in serum-free medium was mixed with INTERFERin reagent, vortexed briefly and incubated in RT for 10 minutes. Then the mix was added to the cells and left for 48h before treated with 10 nM TCDD, 10 μM 3-MC or 50 nM diethylstilbestrol （DES) for an additional 24h. The cells were then harvested using Trizol and analyses were performed using qRT-PCR.

Radio-HPLC analyses of incubation media ([^{3}H]-3-MC experiments) and HPLC fractionation.

Incubation media were purified using Chromabond C_{18} glass cartridges (Macherey Nagel, Hoerdt, France). Cartridges were activated with 5ml methanol and were equilibrated with 5ml H_2O/0.5% acetic acid. Incubation media were applied on the cartridges, which were washed with 5 ml of H_2O/0.5% acetic acid. Target analytes were eluted with 20 ml of methanol followed by 20 ml of acetonitrile. Eluates were evaporated to dryness under vacuum, then dissolved in

acetonitrile:ammonium acetate (20mM, pH 3.5) 50:50 v/v and injected into the HPLC system for analysis or fraction collection.

Reverse-phase HPLC was performed using a Lachrom L7100 HPLC system (VWR International, Fontenay sous bois, France) connected to a Prontosil C_{18} column (5 µm, 250 x 4.6 mm; ICS, Lapeyrouse-Fossat, France) protected by a Kromasil C_{18} precolumn (5 µm, 10 x 4.6 mm). Mobile phases consisted of ammonium acetate buffer (20 mM, pH 3.5) and acetonitrile 90:10 v/v in A, and acetonitrile 100% in B, respectively. Solvents were delivered at a flow rate of 1 ml/min at 35°C. A three-step gradient was used as follows: 0-10 min linear gradient from 100% A to A:B 75:25 v/v; 10-20min A:B 75:25 v/v; 20-30 min linear gradient from A:B 75:25 v/v to A:B 50:50 v/v; 30-40min A:B 50:50 v/v; 40-60 min linear gradient from A:B 50:50 v/v to 100% B; 60-70 min 100% B. Radioactivity was monitored using an on-line radioactivity detector (Radiomatic flow scintillation analyser Flo-One/ß A500, Perkin Elmer Life Sciences, Courtabœuf, France) using Flo-Scint II as the scintillation cocktail, to establish metabolic profiles. The separation of 5 min HPLC fractions for activity testing was performed using a Gilson model 202 fraction collector (Gilson France, Villiers-Le-Bel, France), which was performed on two thirds of the incubation media, the rest being used for radio-HPLC profiling and radioactivity counting.

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RESULTS

3-MC modulates ERα- and ERβ-dependent transcription in a cell type-specific manner.

The adverse/inhibitory effects of dioxin on estrogen signaling activity is a well-characterized phenomenon and extensive studies have shown that these effects are dependent on the presence of a functional AhR (Fernandez-Salguero et al., 1996; Kohle et al., 2002; Mimura et al., 1997). In light of the results presented recently, which suggest that 3-MC activates ER signaling through activation of the AhR/ARNT complex, we decided to compare the effects of 3-MC to the well-characterized AhR agonist, TCDD, in different cell types to evaluate the possibility of cell-type specific effects. HepG2, CV-1 and HC11 cells were used since these cell lines have previously been shown able to support ER and AhR signaling pathways. HepG2 and CV-1 cells are derived from human liver and kidney, respectively, whereas HC11 are epithelial cells derived from mouse mammary gland, with endogenous expression of ERα and ERβ. In addition, an HC11 reporter cell line, H-ERE, containing a stably integrated luciferase reporter construct under the control of a 3xERE TATA element and a HepG2-derived sub-clone that expresses a stably integrated ERα (Hep-ERα; Barkhem et al., 1997) were used.

HepG2 cells were transiently transfected with a 3xERE-TATA-regulated luciferase reporter gene construct and treated with E_2 or different concentrations of TCDD or 3-MC for 24h. Following treatment, cells were harvested and luciferase activity was determined. Interestingly, 3-MC efficiently activated the ERα-induced reporter gene activity in a dose-dependent manner (Figure 1A, lanes 5-7). However, exposure of HepG2 cells to TCDD did not cause ERE reporter activity (Figure 1A lanes 3-4). This suggests that the specific effect of 3-MC on ERα is not a general feature among AhR ligands. Since the effect was not reproduced by the other AhR agonist TCDD, it rather suggests inherent differences between TCDD and 3-MC on ERα-dependent activity in HepG2 cells.

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Next, we investigated the capacity of 3-MC to activate the ERα and ERβ isoforms separately in CV-1 cells, which do not express either ER isoform endogenously. CV-1 cells were transiently transfected with the ERE-regulated luciferase reporter construct, together with expression vectors for ERα or ERβ (Figure 1B). Following transfection, the cells were treated with 0.1, 1 or 10 μM 3-MC, or 10nM E_2 for 48h. The cells were harvested and luciferase activity was measured. As shown in Figure 1B, both ERα (lanes 3-5, black bars) and ERβ (white bars) were dose-dependently activated by 3-MC. However, ERα appeared to respond to 3-MC both more strongly and at lower concentrations (Figure 1B, compare white bars to black bars). In agreement with the results obtained in HepG2 cells, TCDD treatment had no effect on either ERα or ERβ-dependent transcriptional activity in the CV-1 cells (data not shown).

Next, similar experiments were performed in HC11 cells. The cells were exposed to 10nM E_2, 10nM TCDD or 10μM 3-MC (Figure 1C). Following 24h treatment, the cells were harvested, whole cell extracts were prepared and luciferase activity was determined. As expected, ERE activity was significantly induced in the presence of 10nM E_2 (Figure 1C, lanes 1 and 2). However, in contrast to our observations in HepG2 and CV-1 cells, no activation of the 3xERE-regulated luciferase reporter gene construct upon exposure to 3-MC, nor TCDD (lanes 3-4 and 5-7) was observed. In addition, we considered the possibility that the AhR activated by a short time (6h) exposure of 3-MC may acquire a specific conformation that is able to activate ERα. To investigate this, experiments were performed in HepG2 cells transiently transfected with ERα expression vectors and treated with 3-MC for 6h. Following this treatment, the transcriptional activity of ERα and AhR was investigated. Under these conditions an XRE-regulated luciferase reporter gene was efficiently activated by both TCDD and 3-MC (see Supplemental Figure S1B, lanes 3-4 and 7-8). In contrast, ERE-dependent transcription was not significantly induced by 3-MC (Figure S1A, lane 7) whereas 50 nM DES efficiently induced the ERE-regulated expression

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of the luciferase reporter (Figure S1A; compare lanes 6 and 7). Taken together, these results suggest that the ability of 3-MC to activate ERα-dependent transcription is dependent both on the cellular background and on the time of exposure to 3-MC.

To examine the levels of the estrogen receptors, AhR and ARNT in the different cell lines after treatment, we performed western blot analysis. The treatment with DES, TCDD, and/or 3-MC had no significant impact on the AhR, ARNT and ER protein levels. These experiments show that under our experimental conditions, the levels of the different transcription factors, with the exception of ERβ which is not expressed in HepG2 cells, remain relatively stable ruling out protein degradation as a possible explanation for the inability of TCDD and 3-MC to induce ER-dependent transcription.

In summary, these observations suggest that selected AhR ligands may exert distinct agonistic effects on ER activity, depending on the cellular background.

3-MC inhibits E_2-induced transcriptional activity in HC11 cells.

The experiments presented above indicate that 3-MC can act as an ERα and ERβ agonist in certain cell types (HepG2 and CV-1) but not in others, exemplified by HC11. We decided to investigate whether the previously documented inhibitory actions of 3-MC and TCDD on E_2-induced activity were present in HC11 cells. For this purpose, HC11 cells were treated with 10nM E_2 in combination with 10 nM TCDD or 10 μM 3-MC and the activity of the stably incorporated 3xERE-regulated luciferase was determined. In the presence of 10 nM E_2 a strong increase in ERE-dependent transcription was observed (Figure 2, compare lanes 1 and 4). However, co-exposure to 3-MC or TCDD resulted in reduced ERE-dependent expression (Figure 2, compare lanes 4, 5 and 6), showing that both TCDD and 3-MC are able to interfere with the

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transcriptional response induced by the estrogen receptors in HC11 cells, but neither compound induces ER activity as ER ligands.

3-MC interacts with the LBD of the ERs in a ligand-like manner.

The results presented above show that, depending on the cellular background, AhR agonists can either activate or repress ER-dependent transcription. To investigate whether 3-MC induced transcriptional activity through occupation of the ligand-binding pocket of ERα and ERβ, the inhibitory effects of the classical ER antagonists ICI 182,780 (ICI) and 4-OH-Tamoxifen (4-OHT) was examined in transfection assays. To enable evaluation of both ER isoforms, CV-1 cells were chosen for these investigations. The cells were transiently co-transfected with the 3xERE-Luciferase reporter construct together with expression vectors for either ERα or ERβ and incubated in the presence of 10 μM 3-MC separately or in combination with ICI and 4-OHT. As before, 3-MC induced a robust transcriptional response of ERα, and to a lesser extent of ERβ(Figure 3A, lane 3 and 8, respectively). In the presence of ICI or 4-OHT, the response was severely blunted (Figure 3A, lanes 4 and 5, respectively). This observation may indicate that ICI and 4-OHT antagonize the 3-MC-induced activity of ERα and ERβ by competing for binding to the ligand-binding pocket of the receptors, and that 3-MC mediates its effect through a bona fide agonist-like mechanism.

To verify these observations, 3-MC treatment was tested on the isolated LBDs of the ERs, fused in chimeric constructs to the DNA binding domain of Gal4, which would also exclude ER isoform-specific effects on DNA-binding. CV-1 cells were transiently transfected with a Gal4-regulated luciferase reporter construct together with expression vectors for either Gal4-ERα or Gal4-ERβ. The cells were exposed to 10 nM E_2, 0.1, 1 and 10 μM 3-MC or 10 nM TCDD for 48h. Following transfection, cells were harvested and luciferase activity was determined.

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3-MC induced a transcriptional response of both Gal4-ERα-LBD and ERβ-LBD, albeit to a lesser extent compared to the intact receptors (Figure 3B, lanes 3-5 and 9-11). The ERβ-LBD displayed a weaker response to 3-MC compared to ERα, consistent with the results obtained with the full-length receptors (see Figure 1B for comparison). None of the receptors were activated by TCDD, consistent with the previous experiments. These results support our previous notion that 3-MC mediates ER agonistic effects through a mechanism similar to that of a bona fide ligand, but do not explain the observed lack of agonistic activity of 3-MC in HC11 cells. This discrepancy indicated to us that the chemical properties of 3-MC were influenced by the different cellular contexts. Ligand binding experiments to determine the affinity of the pure 3-MC compound were therefore carried out using the ERα and ERβ-LBD scintillation proximity assay (SPA). We found that neither TCDD (Figure 3E) or 3-MC (Figure 3D) caused any major displacement of the tracer (tritiated E_2), demonstrating that both compounds were very weak binders of ERαand ERβIn control experiments however, non-labeled E_2 efficiently competed with the tracer demonstrating the validity of the method. The system did not allow us to calculate the IC_{50} values for TCDD to any of the ERs because of its extremely low affinity, while the binding affinity of 3-MC was calculated to 37μM and 22 μM for ERα and ERβ, respectively (not shown), inconsistent with the ability of 3-MC to induce ER-dependent transcription at significantly lower doses in HepG2 and CV-1 cells.

3-MC induces transcriptional activation of E_2- and TCDD-inducible genes.

The experiments presented above suggest that 3-MC is able to activate expression of E_2 target genes in HepG2 and CV-1 cells. In addition, 3-MC is a well-characterized inducer of AhR target genes such as the CYP1A1. We thus compared the effects of both TCDD and 3-MC on natural E_2- and TCDD-inducible genes in wild type or ERα-expressing HepG2 cells. Real Time PCR

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(RT-PCR) analysis of the dioxin-inducible CYP1A1 gene and the E_2-regulated pS2 gene was carried out. The cells were treated with E_2, TCDD or 3-MC for 6h, where the exposure time was chosen in order to reflect direct transcriptional effects of ERα and AhR and not potential downstream events. In addition, assessing the effects on mRNA expression of an endogenous target gene would reflect the ability of 3-MC and TCDD to affect ERα transcriptional activation in a biologically relevant context. The effects would not involve the requirement to synthesize luciferase protein as presented in Figure 1. Cells were treated with E_2, TCDD or 3-MC for 6h and following incubation, the cells were harvested and mRNA was extracted as described in Materials and methods.

As expected, TCDD and 3-MC exposure induced the dioxin-responsive CYP1A1 gene expression in both HepG2 wild type and Hep-ERα cells (Figure 4 A and B, lanes 7 and 8). In contrast, exposure of wild type HepG2 to E_2 did not alter either pS2 or CYP1A1 gene expression, which was maintained at background levels (Figure 4A, lane 2). Interestingly, pS2 gene expression was induced by 3-MC to a similar extent as that of E_2 only in the ERα-expressing cells and not in wild type cells, confirming that ERα is required for the observed pS2 gene up-regulation (compare Figure 4 A and B, lanes 4). TCDD treatment, in contrast, had no effect on pS2 expression in either cell type.

Since TCDD and 3-MC are well known AhR ligands we decided to assess if AhR-mediated signaling is required for the ability of 3-MC to activate ERα-mediated transcription using SiRNA (short inhibitory RNA) against AhR and analyze the effects on pS2 and CYP1A1 expression. A SiRNA construct against AhR was transiently introduced into Hep-ERα cells and the down-regulation of the AhR was monitored to identify the optimal timepoint to perform expression analysis. We were able to efficiently lower the intracellular levels of the AhR as shown in Figure

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4 C (upper panel, compare lanes 1-2), without affecting the levels of the AhR partner factor ARNT or actin (Figure 4 C middle and lower panels; compare lanes 1-2).

Next we treated Hep-ERα cells with 10 nM TCDD, 10 nM DES, 10 μM 3-MC or vehicle in the presence of either a SiRNA against the AhR or a scrambled control sequence and monitored CYP1A1 and pS2 expression. In cells treated with the scrambled sequence, both TCDD and 3-MC efficiently induced the expression of the CYP1A1 gene, whereas, as shown previously, 3-MC, but not TCDD, was able to induce expression of the pS2 gene (Figure 4D, top panel, compare lanes 6 and 7). In the presence of SiRNA against the AhR, the expression of the CYP1A1 gene was severely blunted both in the presence of TCDD and 3-MC, as expected (see Figure 4D, lower panel, lanes 2 and 3). Importantly however, the ability of the cells to activate pS2 expression in response to 3-MC was also greatly diminished (Figure 4D top and lower panel, compare lanes 7), suggesting that induction of AhR-activated genes is an important prerequisite for 3-MC-induced activation of ERα target genes. In control experiments, the ability of the ERα agonist DES to activate pS2 expression was unaffected by the SiRNA against the AhR, showing that ERα is not dependent *per se* on the presence of AhR for its ability to induce transcription of pS2 (Figure 4 D, compare lanes 8).

In conclusion, these results demonstrate that a subset of chemicals that interact with the AhR may cause distinct effects on E_2-mediated transcription. 3-MC is able to either induce or repress ER activity in a cell type-specific manner, whereas TCDD displays purely negative effects on E_2 signaling. These findings highlight the critical importance of taking into account the characteristics of individual cell types, when predicting the outcome of exposure to a given xenobiotic compound.

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HepG2, but not HC11 cells, generate metabolites with ER-activating properties following exposure to 3-MC.

The striking difference between the effects of 3-MC on ER-dependent transcriptional activity in HC11 and HepG2 cells, and the low affinity of the ERs for 3-MC in the ligand-binding assay, prompted us to investigate the reasons behind these discrepancies. Furthermore, our observations that down-regulation of the protein levels of AhR (using SiRNA against the AhR) severely reduces the ability of 3-MC to act as a agonist for ERα suggest that downstream events are necessary to activate pS2 expression by 3-MC. Given the respective structures of TCDD and 3-MC and the available literature concerning their biological effects, we speculated that, depending on cell-type, the alternative effects of 3-MC could be determined by the cellular ability to convert the parental 3-MC to biologically active metabolites. Therefore, a comparison of the ability of HC11 and HepG2 cells, respectively, to generate metabolites following exposure to 3-MC was performed. HC11 or HepG2 cells were exposed to low (0.1μM) or high (10 μM) concentrations of [^{3}H]-3-MC for 24h. Following exposure, the growth media was collected and fractionated using an HPLC system developed for the study. In this system, the retention time (Rt) of 3-MC was *ca.* 63 minutes. When 3-MC was incubated with HC11 cells, the parental compound did not display any conversion to alternative metabolites. Besides unchanged 3-MC, only a minor radioactive peak was detected with a Rt of 52 min, without any major difference observed between the analysis of media from 10 μM incubations (Figure 5A) or from 0.1 μM incubations (data not shown), respectively. Similar radio-chromatograms were obtained from control experiments (24 h, no cells) indicating that the formation of the compound eluted at 52 min could be (at least partly) attributed to a chemical degradation rather than a biotransformation of 3-MC by cells. In contrast, when HepG2 cells were exposed to 3-MC, an extensive conversion of the parental compound was observed. A representative 10 μM media analysis is shown in Figure 5B.

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For these incubations, unchanged 3-MC accounted for only 1.3 ± 0.3 % of the radioactivity after 24 h. Radio-chromatograms obtained after a 24 h exposure of HepG2 cells to 0.1 μM 3-MC were qualitatively similar, unchanged 3-MC accounting for 0.6 ± 0.3 % of the detected radioactivity.

Metabolic conversion of 3-MC is required for its ability to induce ER-dependent transcription.

The ability of 3-MC to induce ERα-mediated transcription in HepG2 cells seemed to be coupled to the capacity of these cells to generate alternative compounds from original 3-MC. Therefore, we decided to test whether the active compounds present in HepG2 cell medium could induce ERE transcriptional activity in the HC11 cells. Fractions from media of HepG2 cells that had been exposed to 10 μM 3-MC for 24h were collected. Separation was achieved by collecting the eluted material each 5 min, resulting in 13 fractions in total. Each fraction was pooled (3 incubations), concentrated and dissolved in 1ml DMSO. The ability of the individual fractions to induce ER activity was assessed in the H-ERE cells and compared to parental 3-MC. Consistent with our previous results, 3-MC did not induce expression of the integrated luciferase reporter. However, exposure of the cells to fractions 2 and 3 induced noticeable reporter activity (Figure 6 A). A similar fractionation was carried out for incubation media from HepG2 cells incubated with 0.1 μM 3-MC. Neither of these fractions was able to active transcription (data not shown). Given the fact that radio-HPLC profiles were qualitatively the same for 0.1 and 10 μM incubation media, it was concluded that the amount of active compounds present in fractions 2 and 3 of 0.1 μM incubations media was too small to induce reporter activity. However, the negative results obtained for 0.1 μM 3-MC incubations rules out the possibility that the observed ER activity induction was due to endogenous compounds present in HepG2 cells incubates. These experiments suggest that metabolic conversion of 3-MC generates compounds that can activate ERα, and that HC11 cells in contrast to HepG2 cells, appear to lack the capacity to perform such

conversion. To further assess the 3-MC conversion products as ERα ligands, H-ERE cells were co-treated with the ER-activating fractions and the full ER antagonist ICI 182,780. Again, we observed that fractions 2 and 3 were able to activate expression of the integrated luciferase reporter construct while the original 3-MC did not (Figure 6B). Interestingly, co-treatment with ICI resulted in a clear reduction of ERα transcriptional activity (Figure 6B). These observations strengthen the hypothesis that the fractions from 10 μM 3-MC HepG2 cells incubation media contain compounds that may act as ligands for the ERs.

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DISCUSSION

The ability of industrial chemicals and environmental pollutants to interfere with hormonal signaling pathways, a phenomenon known as endocrine disruption, has caused attention and concern during the last decades. Among these compounds, the potency of dioxins as endocrine disruptors is well documented, in particular with regard to their negative effects on E_2 signaling pathways (Safe and Krishnan, 1995). A recent publication however, suggested that in the presence of the AhR agonist 3-MC, the AhR/ARNT complex activated the ERs in an E_2-independent fashion, proposing a pro-estrogenic function of the activated AhR/ARNT complex (Ohtake et al., 2003). This study is inconsistent with numerous epidemiological studies as well as biological and experimental data demonstrating an anti-estrogenic effect of AhR ligands (Chen et al., 2001; Kharat and Saatcioglu, 1996; Safe, 2001; Safe et al., 2000; Zacharewski et al., 1994). In addition, another recently published study suggests that 3-MC does not require the AhR to activate ERα-dependent transcription (Abdelrahim et al., 2006).

In this study, we show that the ability of 3-MC to activate the ERs and in particular ERα is cell-specific. In HC11 cells, 3-MC fails to activate E_2 signaling. In fact, 3-MC in HC11 displays an inhibitory activity on the ERs.

Also in transient transfection assays in HepG2 cells treated for 6 hours, we fail to observe any activation of an ERE-regulated reporter gene construct. In contrast, using Hep-ERα cells and RT-PCR analysis, induction of the pS2 gene can be observed already after 6 hours exposure. We believe that in contrast to the sensitive RT-PCR assay, it may not be possible to detect small changes in reporter gene expression in transient transfection assays. In addition, transient transfections require both transcription and translation of detectable amounts of the luciferase

protein whereas RT-PCR assays detect the mRNA levels and thus do not require protein translation.

Moreover, in HepG2 or CV-1 cells, longer (at least 12 h) exposure to 3-MC leads to considerable activation of the transcriptional activity of both ER isoforms, in particular ERα. In Hep-ERα cells, 3-MC also increased the expression of the pS2 gene, a well-known estrogen-responsive gene. TCDD on the other hand did not activate either ERα or ERβ transcriptional activity, and co-exposure of cells to E_2 and TCDD resulted in a decline in ER transcriptional response, demonstrating the anti-estrogenic effects. Clearly, depending on the cellular system, different ligands of the AhR can either repress, or activate the transcriptional activity of ERα and ERβ. We speculate that cell-type specific differences in the ability of the AhR to activate drug-metabolizing enzymes may account for these differences.

The ability of 3-MC to activate dual pathways seems to be coupled to the capacity of certain cells to convert parental 3-MC into alternative compounds with ER agonistic properties. In ligand binding experiments, we show that 3-MC has an extremely low affinity for the ERs, ruling out the possibility that 3-MC itself can directly activate or repress either ERα or ERβ. It is well known that 3-MC, through the AhR, induces a battery of xenobiotic metabolizing enzymes that convert 3-MC into alternative compounds. This is part of the clearing process aimed to dispose of 3-MC. By reducing the cells' capacity to activate this battery of drug-metabolizing enzymes, by siAhR, the cellular ability to induce ERα-mediated pS2 expression in response to 3-MC, but not DES, was blunted. Interestingly, exposure of HC11 cells to fractions of medium from HepG2 cells exposed to 3-MC resulted in activation of ERE-dependent transcriptional activation, suggesting that HepG2 cells convert 3-MC into a compound that acts as a ligand for the ERs. This has been shown to occur also for instance with benzo-(a)-pyrene (BaP), a compound related to 3-MC, and also an AhR agonist. Interestingly both 3-MC and BaP are so-called pro-

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carcinogens, which require metabolic activation to reactive intermediates in order to cause toxic effects. In the case of BaP, the parent compound displays little affinity for the ERs. However, following P450 activation, monohydroxylated derivatives with estrogenic activity are formed (Arcaro et al., 1999). These derivatives of BaP have also been reported to have ER isoform-specific interactions, with a preference for ERβ (Fertuck et al., 2001). In this study, we demonstrate that 3-MC and BaP have similar properties.

Our experiments demonstrate that TCDD and 3-MC do not bind to the LBD of either ERα or ERβ, challenging the concept that 3-MC itself would be a ligand for the ERs. In addition, we find that while TCDD impaired E_2-induced activity in all tested cell systems, the effect of 3-MC on ER activity varied profoundly depending on the cellular background. 3-MC had an inhibitory effect on E_2-dependent transcription in HC11, derived from mouse mammary epithelial cells, whereas it was, in itself, a strong inducer of ER activity in liver HepG2 or kidney CV-1 cells in reporter assays as well as on the endogenous E_2-inducible pS2 gene. The agonistic effects of 3-MC were found to operate through the LBD of the ERs, and were inhibited by simultaneous treatment with conventional anti-estrogens. Such inhibition caused by antagonists like tamoxifen and ICI is explained as a ligand effect, however, one cannot rule out the possibility of other explanations for the effects on transcription.

Intriguingly, the capacity of 3-MC as an ER activator required a cellular system that could support conversion of the parent compound to reactive intermediates. We were able to demonstrate this through comparison of fractionated cell media collected after incubation of HepG2 cells and HC11 cells, respectively. The radio-chromatographic profiles of the collected media from HepG2 cells incubated with [^{3}H]-3-MC demonstrated that 3-MC undergoes extensive metabolism in this cell system. In contrast, very little metabolism (if any) occurred in HC11 cells. Metabolic capabilities have been shown to vary widely among different cell models (Brandon et

al., 2006). HepG2 cells express several phase I and phase II enzymes, enzymes which activity depends on the origin of the cell line and on culture conditions (Hewitt and Hewitt, 2004). Although the expression of these enzymes is low compared to, for instance, primary hepatocytes (Wilkening et al., 2003), our experiments show that contrary to HC11 cells, HepG2 cells are able to extensive biotransformation of 3-MC. After 24 h of incubation, there was near complete loss of detectable parental compound. This biotransformation resulted in a complex mixture of a multitude of different compounds. Among the most polar metabolites formed, several fractions of 3-MC-derived metabolites (or mixtures of metabolites) were shown to activate a stably integrated ERE luciferase reporter gene activity in HC11 cells. Using this approach we show that, while 3-MC itself does not activate ERE-regulated gene expression, alternative metabolites function as strong activators of ER. We are currently trying to identify and structurally characterize the compound or compounds that are able to bind to and activate the ERs.

Recently, a study suggested that the AhR is able to activate ERα in the absence of E_2. Furthermore, a different study suggested that 3-MC is able to displace E_2 and thus act as ligand to ERα. 3-MC induced ERα transcriptional activation at far lower concentration compared to its ligand binding ability. Our study presented here provides a mechanistic explanation to these data. Using 3-MC as an AhR agonist, it is likely that metabolites were formed that would activate ERα-dependent transcription. In addition, our experiments show that while the parent 3-MC has very limited ligand binding activity, several metabolites of 3-MC are ERα ligands.

In summary, our experiments show that the AhR ligands 3-MC and TCDD display distinct abilities to either activate or to repress ERα and ERβ transcriptional activity. While TCDD in all systems tested acted as a repressor of ERα and ERβ, 3-MC is able to induce transcriptional activation independent of AhR, mainly through ERα. These differences are depending on the

cellular context and in particular, the cellular ability to transform the parent 3-MC compound to alternative compounds.

Interestingly, in cells able to transform 3-MC into ER ligands, exposure to 3-MC leads to activation of both ER and AhR target genes. These observations accentuate the need to take cellular transformation of compounds into account when assessing the potential endocrine disrupting activity of xenobiotics.

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FIGURE LEGENDS.

Figure 1.

The effect of 3-MC on ER-mediated transcription is dependent on cellular context. A) Hepatoma-derived HepG2 cells, stably transfected with ERα (Hep-ERα), were transiently transfected with 3xERE-TATA-Luc and β-gal reporter gene constructs. The cells were treated with 100nM E_2, 10 and 20nM TCDD and 0.1, 1 and 10μM 3-MC, as indicated. After 24 hours, the cells were harvested and reporter gene activity was measured as described in Materials and methods. **B)** Kidney CV-1 cells were transiently transfected with expression vectors for ERα (black bars) or ERβ (white bars) together with 3xERE-TATA-Luc and β-gal reporter gene constructs as described in materials and methods. **C)** HC11 cells transiently transfected with 3xERE-TATA-Luc and β-gal reporter gene constructs were treated with 10nM E_2, 10nM and 20nM TCDD, 0.1, 1 and 10μM 3-MC, as indicated. After 24 hours, luciferase activity was determined as described in Materials and methods. All results shown are means and S.E. for three independent experiments with triplicate samples; *, p <0.05 compared to control.
D) Hep-ERα and H-ERE cells were treated with 1) solvent, 2) E_2, 3) TCDD, 4) TCDD+E_2, 5) 3-MC or 6) 3-MC+E_2 for 24 hours. Equal amounts of nuclear extracts of the different samples were used for western blot analysis.

Figure 2.

Both TCDD and 3-MC repress E_2-signaling in HC11 cells. HC11 cells with a stably integrated 3xERE luciferase reporter gene construct (H-ERE) were treated with 10nM E_2 alone or in combination with 10 nM TCDD or 10μM 3-MC. After 48h, the cells were lysed and reporter gene activity was determined. Luciferase activity in each sample was adjusted to protein

concentration, determined by the Bradford method. Results are means and S.E. for three independent experiments, *, significantly different from E_2-treated samples at $p < 0.05$.

Figure 3.

The effects of 3-MC are mediated by the LBDs of the ERs. A) CV-1 cells transiently co-transfected with either pSG5-ERα or pSG5-ERβ together with 3xERE-TATA-Luc and β-gal reporter gene constructs. The cells were treated with 10nM E_2 and 3-MC, separately or in combination with ER antagonists ICI 182,780 (10nM) or 4-OHT (1μM). After 48 hours, cells were harvested and reporter gene activity measured *, significantly different from 3-MC-treated samples at $p < 0.05$. **B)** CV-1 cells were co-transfected with Gal4-ERα LBD or Gal4-ERβ LBD together with a Gal4-regulated luciferase reporter gene construct. After 48 hours, reporter gene activity was determined. **C-E)** The recombinant LBD of ERα and ERβ was incubated with labeled E_2 in the presence or absence of the non-labeled **C)** E_2, **D)** 3-MC or **E)** TCDD. The ability of the non-radioactive compounds to displace E_2 was assessed as described under Materials and methods.

Figure 4.

Expression of the E_2-regulated gene pS2 is induced by 3-MC but not by TCDD. A) Wildtype HepG2 or **B)** Hep-ERα cells were starved for 48 h and treated with 10nM E_2, 10nM TCDD, or 10μM 3-MC for 6 h. Total RNA was isolated, reverse-transcribed, and analyzed by Real-Time PCR with primers for pS2 or CYP1A1. The results were normalized with 18s rRNA and untreated samples. Shown are means and S.E. from three independent experiments. Values of untreated cells were arbitrarily set to 1. *, differ significantly from vehicle control at $p < 0.05$.

C) Hep-ERα cells were starved for 48 h and transfected with SiRNA expressing vectors against the AhR or scrambled sequence as control. The cells were then grown for 48h before the effect of the SiRNA expressing vector was determined. **D)** SiRNA-treated Hep-ERα cells were exposed to DES, TCDD or 3-MC for 24h and RT-PCR was performed to measure the levels of pS2 or CYP1A1 gene expression. *, differ significantly from vehicle control at $p < 0.05$.

Figure 5.

Radio-chromatographic profiles of 3-MC collected from medium from HepG2 and HC11 cells. A) HC11 or **B)** HepG2 cells were treated with 10μM 3-MC for 24h and the medium was collected and fractionated as described under Materials and methods. The chromatograms were run in triplicates. **C)** Medium incubated without any cells serves as a control. Representative radio-chromatograms are presented.

Figure 6.

Metabolic conversion of 3-MC is required to activate ER transcriptional activity. A) HepG2 cells were treated with 10μM 3-MC for 24h and the medium was collected and fractionated as described in Materials and methods. Following fractionation, fractions were collected, pooled, evaporated and resuspended in 200μl methanol. 5μl of the different fractions were subsequently used to treat HC11 3xERE-Luc reporter cells. 10nM E_2, 10μM 3-MC and 5μl of methanol were included as controls. After 24h, the cells were harvested and luciferase activity was determined. * indicates significant changes compared to control. **B)** H-ERE reporter cells were treated with 5, 7.5 and 10μl of fraction 2 and 3 in combination with 10nM ICI for 24h. 10nM E_2, 10nM ICI, 10μM 3-MC and 10μl of methanol were included as controls. Following this incubation, the cells were harvested and luciferase activity was determined. Luciferase activity was adjusted to protein

concentration in each sample, determined by the Bradford method. Shown are means and S.E. from three independent experiments and * indicates significant decrease in the presence of ICI at $p < 0.05$.

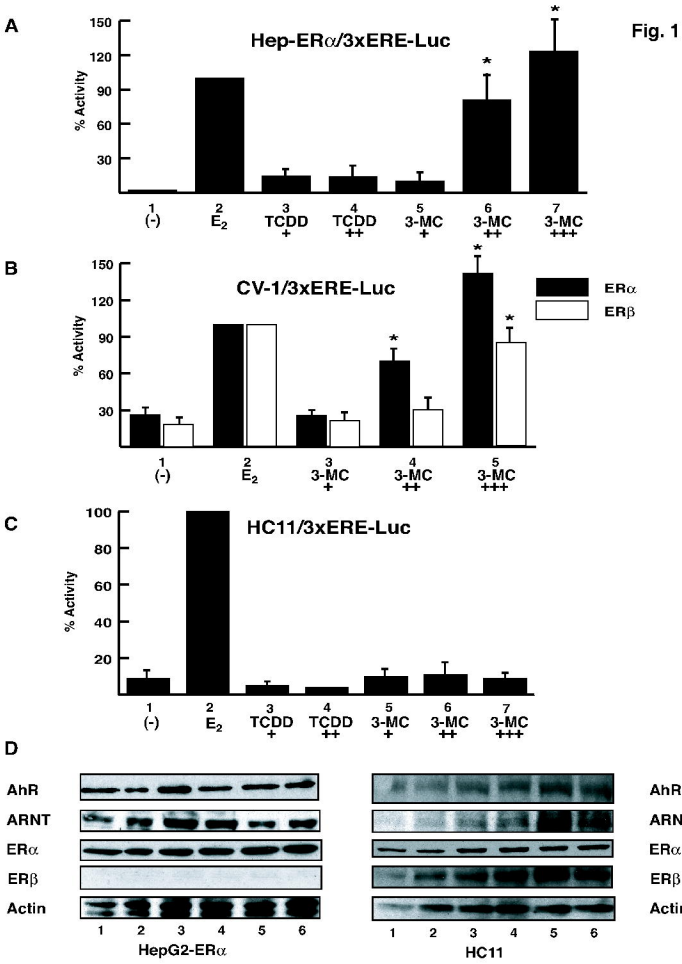

A
Hep-ERα/3xERE-Luc
% Activity
150
120
90
60
30
1 (-)
2 E2
3 TCDD +
4 TCDD ++
5 3-MC +
6 3-MC ++
7 3-MC +++
B
CV-1/3xERE-Luc
ERα
ERβ
1 (-)
2 E2
3 3-MC +
4 3-MC ++
5 3-MC +++
C
HC11/3xERE-Luc
100
80
60
40
20
D
AhR
ARNT
ERα
ERβ
Actin
1 2 3 4 5 6
HepG2-ERα
HC11

Fig. 1

Fig. 2

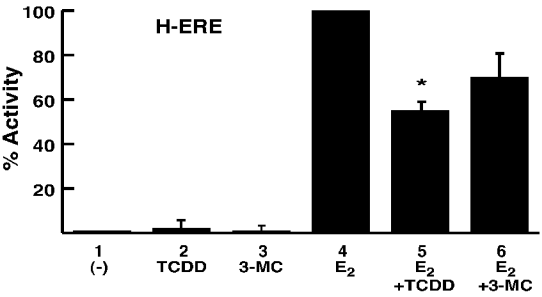
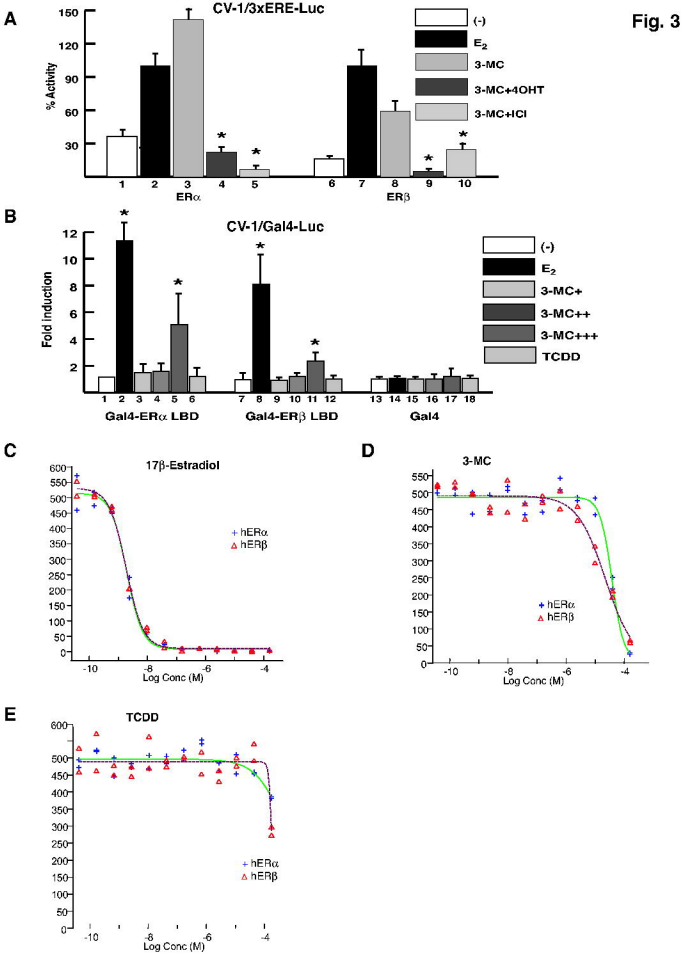


Fig. 3


A
CV-1/3xERE-Luc
% Activity
(-)
E2
3-MC
3-MC+4OHT
3-MC+ICI
ERα
ERβ
B
CV-1/Gal4-Luc
Fold induction
(-)
E2
3-MC+
3-MC++
3-MC+++
TCDD
Gal4-ERα LBD
Gal4-ERβ LBD
Gal4
C
17β-Estradiol
hERα
hERβ
Log Conc (M)
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hERβ
Log Conc (M)

Fig. 4

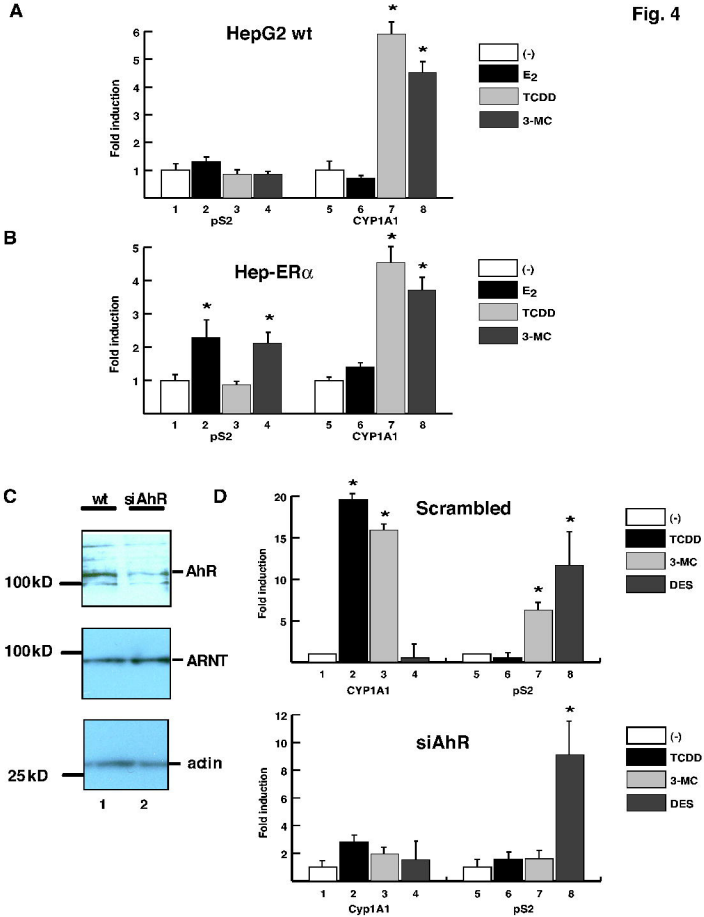

A
HepG2 wt
Fold induction
(-)
E2
TCDD
3-MC
pS2
CYP1A1
B
Hep-ERα
C
wt
siAhR
100kD
AhR
ARNT
25kD
actin
D
Scrambled
siAhR
DES
Cyp1A1

Fig. 5

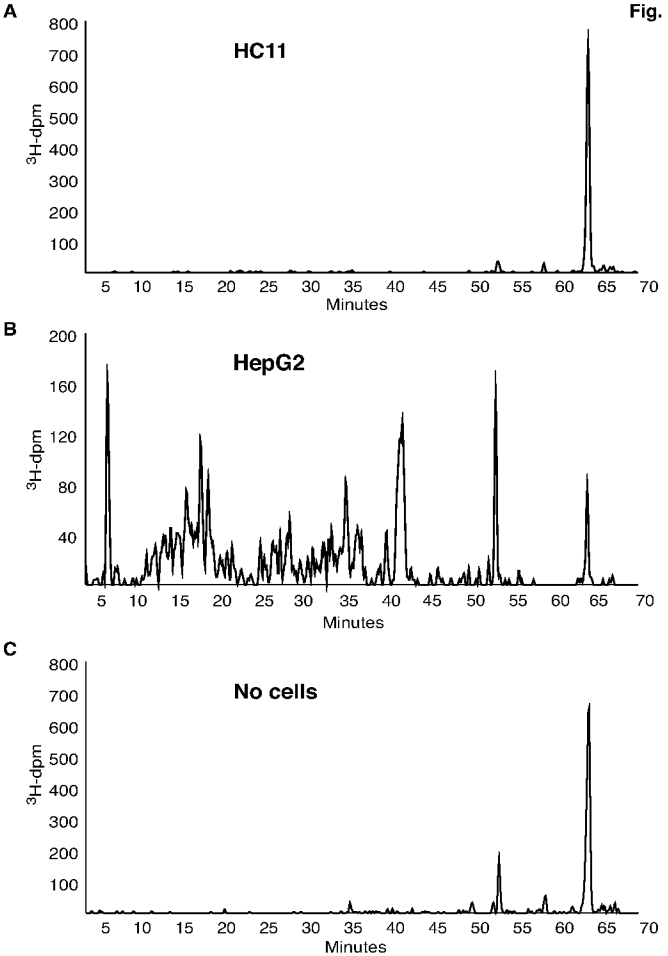
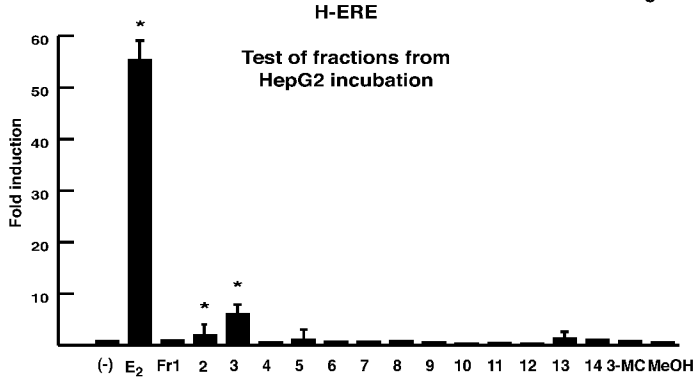
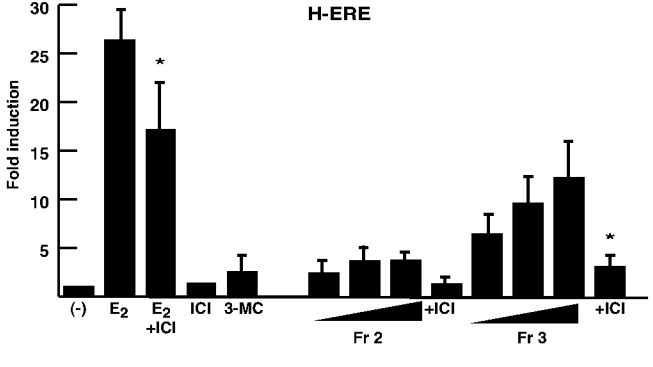

A
HC11
^{3}H-dpm
800
700
600
500
400
300
200
100
5 10 15 20 25 30 35 40 45 50 55 60 65 70
Minutes
B
HepG2
^{3}H-dpm
200
160
120
80
40
5 10 15 20 25 30 35 40 45 50 55 60 65 70
Minutes
C
No cells
^{3}H-dpm
800
700
600
500
400
300
200
100
5 10 15 20 25 30 35 40 45 50 55 60 65 70
Minutes

Fig. 6

A


H-ERE
Test of fractions from HepG2 incubation
Fold induction
60
50
40
30
20
10
*
*
*
(-) E2 Fr1 2 3 4 5 6 7 8 9 10 11 12 13 14 3-MC MeOH

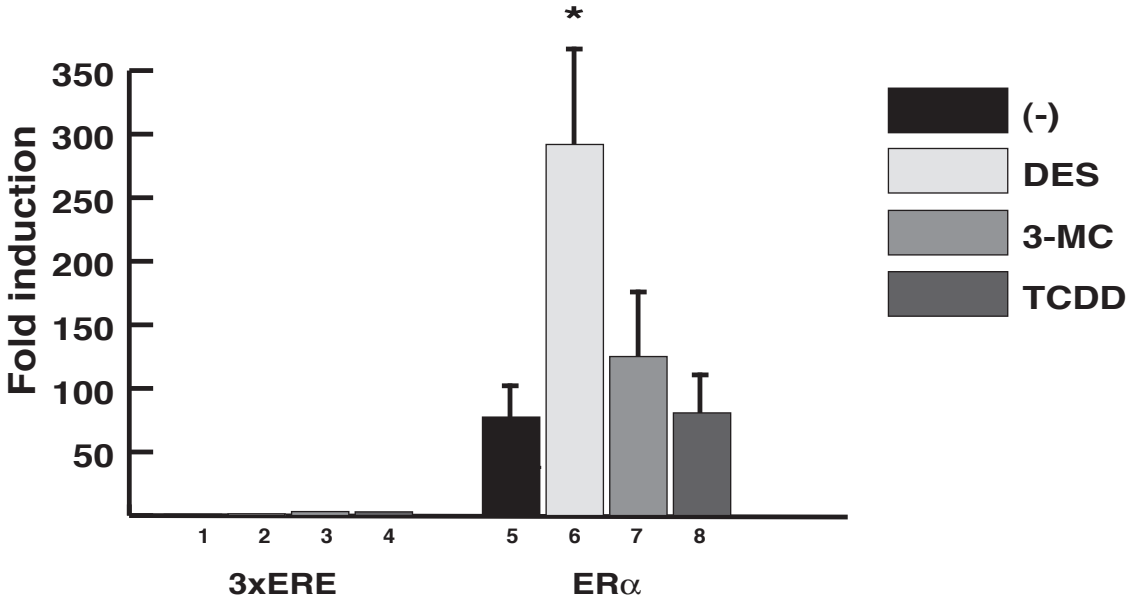
B


H-ERE
Fold induction
30
25
20
15
10
5
*
*
(-) E2 E2 +ICI ICI 3-MC +ICI +ICI
Fr 2
Fr 3

A

HepG2/3xERE-Luc

Fig. S1



B