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# 3-Methylcholanthrene Displays Dual Effects on Estrogen Receptor (ER) $\alpha$ and ER$\beta$ Signaling in a Cell-Type Specific Fashion[S]

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## ABSTRACT

The biological effects of 17$\beta$-estradiol ($E_2$) are mediated by the two estrogen receptor (ER) isoforms ER$\alpha$ and ER$\beta$. These receptors are ligand-inducible transcription factors that belong to the nuclear receptor superfamily. These receptors are also targets 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 ER$\alpha$ and ER$\beta$. 3-MC is a compound identified previously as an activator of the aryl hydrocarbon receptor (AhR). Activation of AhR is traditionally 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. It is interesting that 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.

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 manmade 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, ER$\alpha$ and ER$\beta$, 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 various types of environmental pollutants such as bisphenol A and polychlorinated hydroxy-biphenyls.

The ERs belong to the nuclear receptor (NR) superfamily and share a conserved structural arrangement with other members of the family. NRs carry a centrally located, highly conserved DNA binding domain that mediates both dimerization and specific DNA binding. The DNA binding domain

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**ABBREVIATIONS:** ER, estrogen receptor; 3-MC, 3-methylcholanthrene; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; LBD, ligand binding domain; AhR, aryl hydrocarbon receptor; $E_2$, 17$\beta$-estradiol; NR, nuclear receptor; SPA, scintillation proximity assay; FCS, fetal calf serum; PCR, polymerase chain reaction; siRNA, short inhibitory RNA; DES, diethylstilbestrol; RT-PCR, real-time polymerase chain reaction; BaP, benzo(*a*)pyrene; 4-OHT, 4-OH-tamoxifen; ARNT, aryl hydrocarbon receptor nuclear translocator; HPLC, high-performance liquid chromatography; ERE, estrogen-response element; ICI, ICI 182,780, fulvestrant; $\beta$-gal, $\beta$-galactoside.

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 in which binding of an agonistic ligand induces a complex transition, in which 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 coactivators.

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 ER$\alpha$ and ER$\beta$ signaling but do not bind to the LBD of the ERs. The most potent dioxin, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) 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 (Kharat and Saatcioglu, 1996; Safe et al., 2000; Chen et al., 2001), although the molecular mechanism behind this interference is not well characterized. The mediator of the biological effects of dioxin is the aryl hydrocarbon receptor (AhR). The AhR is a member of the basic helix-loop-helix-Per-ARNT-Sim family of proteins that includes transcription factors such as the hypoxia-inducible factors HIF-1$\alpha$ and EPAS, the circadian regulatory proteins CLOCK and periodicity, and the general dimerization partner protein ARNT, to mention a few (Gu et al., 2000).

3-Methylcholanthrene is an environmental pollutant mainly formed by incomplete combustion processes with a potent ability to induce AhR-dependent transcription. It has been suggested that the AhR-ARNT complex, upon activation by the AhR agonist 3-methylcholanthrene (3-MC), interacts with the ERs and induces 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 ER$\alpha$ and that the AhR is not involved in this activation process (Abdelrahim et al., 2006). These discrepancies are puzzling, in light of the fact that both studies used similar cellular backgrounds (MCF-7 cells) and experimental conditions. It is also noteworthy that numerous previous studies on AhR-ER cross-talk 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. (2003), in which 3-MC was found to induce ligand-independent activation of the ERs, whereas other studies have shown inhibitory effects of TCDD on estrogen signaling (Astroff et al., 1990; Kharat and Saatcioglu, 1996; Safe et al., 2000; Safe, 2001).

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, like 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 radiolabeled 3-MC in cell lines derived from different tissues. In summary, our results indicate that 3-MC can either repress or activate the ERs, depending on the cellular background.

## 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, 17$\beta$-estradiol, and 4-OH-tamoxifen were from Sigma (St. Louis, MO). ICI 182,780 was from AstraZeneca (Wilmington, DE). Radiolabeled [$^3$H]3-methylcholanthrene was supplied by Moravek (Brea, CA) (radiochemical purity: 98.2%, checked by HPLC; specific activity 44.4 GBq/mmol). Flo-Scint II and Ultima Gold liquid scintillation cocktails were from PerkinElmer Life and Analytical 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-mER$\alpha$, pSG5-mER$\beta$, 3xERE-TATA-Luc, Gal4-Luc, pCMV5-$\beta$Gal, Gal4-ER$\alpha$-LBD, and Gal4-ER$\beta$-LBD have been described elsewhere. Details can be obtained from the authors upon request. Polyclonal anti-ER$\alpha$ H-184, polyclonal anti-ARNT1 H-172, polyclonal anti-AhR H-211, and actin antibody sc-8432 (all from Santa Cruz Biotechnology, Inc., Santa Cruz, CA), and ER$\beta$ GTX14021 (GeneTex, San Antonio, TX) were used at the conditions suggested by the manufacturers.

**ER$\alpha$ and ER$\beta$ LBD Scintillation Proximity Assay.** The binding of TCDD and 3-MC to ER$\alpha$ and ER$\beta$-LBD were analyzed by scintillation proximity assay (SPA) on the same test occasion. In brief, the compounds, dissolved in dimethyl sulfoxide at a concentration of 10 mM and reference compound (17$\beta$-estradiol, $E_2$), were diluted 13 times in a microtiter plate and further serially diluted in 1:4 steps to 12 different concentrations. An 18-$\mu$l sample of each dilution was added to a 384-well assay plate. Next, 35 $\mu$l of ER$\alpha$-LBD- or ER$\beta$-LBD streptavidin-coated polyvinyl toluene SPA beads was added to the plates, followed by 35 $\mu$l of tracer ($^3$H[$E_2$], 2.8 nM). The plates were centrifuged for 1 min at 1000 rpm and then incubated at room temperature overnight with constant shaking. A second centrifugation was made at 2000 rpm for 5 min the next morning before the plates were measured in the Microbeta TriLux (PerkinElmer). The resulting curves were plotted by using the XLfit version 2.0.11.70, and the $IC_{50}$ values were calculated using a four-parameter logistic equation: $y = A + [(B - A)/(1 + ((C/x)^D))]$. $A$ represents zero inhibition; $B$ represents 100% inhibition, $C$ represents the $IC_{50}$, and $D$ represents the slope.

**Cell Culture and Transient Transfection Assays.** HC11 cells and stably transfected 3xERE HC11 cells (H-ERE; described previously in Faulds et al., 2004) were maintained in RPMI 1640 medium (Invitrogen, Carlsbad, CA) supplemented with 8% fetal calf serum (FCS; Invitrogen), L-glutamine (Invitrogen), 50 $\mu$g/ml gentamicin (Invitrogen), 10 ng/ml epidermal growth factor (Sigma), and 5 $\mu$g/ml insulin (Sigma). HepG2 and CV-1 cells were routinely maintained in Dulbecco's modified Eagle's medium supplemented with 10% FCS and penicillin (100U/ml) and streptomycin (100 $\mu$g/ml).

HepG2 or HC11 cells were transfected as described previously (Brunnberg et al., 2003). In general, cells were seeded in 12-well plates in phenol red-free medium 24 h before transfection. Cells were transfected using Lipofectamine reagent (Invitrogen) according to the manufacturer's recommendations. We used 200 ng of appropriate reporter, 3xERE-TATA-Luc or Gal4 plasmids. After transfection, the medium was exchanged with phenol red-free medium supplemented with 5% dextran-coated charcoal-treated FCS, and the cells were allowed to grow for an additional 24 h. At this point, cells were harvested, and luciferase and $\beta$-galactosidase activities were determined. Data are presented either as the percentage of activity $\pm$ S.D., in which reporter activity obtained at 10 nM $E_2$ was arbitrarily set to 100%, or as fold induction $\pm$ S.D. (Gal4 assay), in which activity of reporter plasmid alone without hormone treatment was arbitrarily set to 1. Each bar represents the mean of at least three independent transfections performed in duplicate or triplicate.

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 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 Radiolabeled 3-MC.** Twenty-four-hour HepG2 and HC11 cell incubations were carried out, in triplicate, in the presence of 0.1 or 10 $\mu$M 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 (50 mM, pH 7.8, 10 mM EDTA, and 150 mM NaCl).

**Real-Time PCR to Detect Transcription of pS2 and CYP1A1.** Wild-type HepG2 or cells stably transfected with ER$\alpha$ expression vector were seeded out into 60-mm dishes and grown in phenol red-free medium with 5% dextran-coated charcoal-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. Total RNA (1 $\mu$g) was treated with DNaseI and reverse-transcribed using random hexamer primers (Invitrogen). The resulting cDNA (1 $\mu$l) 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.

**Short Inhibitory RNA-Mediated Down-Regulation of AhR Intracellular Levels.** Expression vectors for short inhibitory RNA (siRNA) were as follows: siRNA oligonucleotides for AhR, ARNT, and a scrambled sequence were cloned into the pSuperior plasmid (Oligoengine, Seattle, WA) according to the manufacturer's instructions. The siRNA was directed against the following sequences: AhR, TACTTCCACCTCAGTTGGC; ARNT, CCATCTTACGCATGGCAGT; and scramble, ACTCTATCTGCACGCTGAG. Hep-ER$\alpha$ cells were seeded and grown in six-well plates in complete medium until it reached 50 to 60% confluence. The cells were then transfected with INTERFERin siRNA transfection reagent according to the 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 at room temperature for 10 min. Then the mix was added to the cells and left for 48 h before treatment with 10 nM TCDD, 10 $\mu$M 3-MC, or 50 nM diethylstilbestrol (DES) for an additional 24 h. The cells were then harvested using TRIzol, and analyses were performed using quantitative RT-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 5 ml of methanol and were equilibrated with 5 ml of $H_2O$/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 (20 mM, 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 $\mu$m, 250 $\times$ 4.6 mm; ICS, Lapeyrouse-Fossat, France) protected by a Kromasil $C_{18}$ precolumn (5 $\mu$m, 10 $\times$ 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- to 10-min linear gradient from 100% A to A/B 75:25 (v/v); 10- to 20-min A/B 75:25 (v/v); 20- to 30-min linear gradient from A/B 75:25 (v/v) to A/B 50:50 (v/v); 30- to 40-min A/B 50:50 (v/v); 40- to 60-min linear gradient from A/B 50:50 (v/v) to 100% B; 60- to 70-min 100% B. Radioactivity was monitored using an online radioactivity detector (Radiomatic flow scintillation analyzer Flo-One/$\beta$ A500; PerkinElmer) 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.

## Results

**3-MC Modulated ER$\alpha$- and ER$\beta$-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; Mimura et al., 1997; Köhle et al., 2002). 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 because these cell lines have been shown previously 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 ER$\alpha$ and ER$\beta$. 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 subclone that expresses a stably integrated ER$\alpha$ (Hep-ER$\alpha$) (Barkhem et al., 1997) were used.

HepER$\alpha$ 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 24 h. After treatment, cells were harvested, and luciferase activity was determined. It is interesting that 3-MC effi-

ciently activated the ERα-induced reporter gene activity in a dose-dependent manner (Fig. 1A, lanes 5–7). However, exposure of HepG2 cells to TCDD did not cause ERE reporter activity (Fig. 1A, lanes 3–4). This suggests that the specific effect of 3-MC on ERα is not a general feature among AhR ligands. Because 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.

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 (Fig. 1B). After transfection, the cells were treated with 0.1, 1, or 10 μM 3-MC or with 10 nM $E_2$ for 48h. The cells were harvested, and luciferase activity was measured. As shown in Fig. 1B, both ERα (lanes 3–5, ■) and ERβ (□) were dose-dependently activated by 3-MC. However, ERα seemed to respond to 3-MC both more strongly and at lower concentrations (Fig. 1B, compare □ with ■). 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 10 nM $E_2$, 10 nM TCDD, or 10 μM 3-MC (Fig. 1C). After 24-h 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 10 nM $E_2$ (Fig. 1C, lanes 1

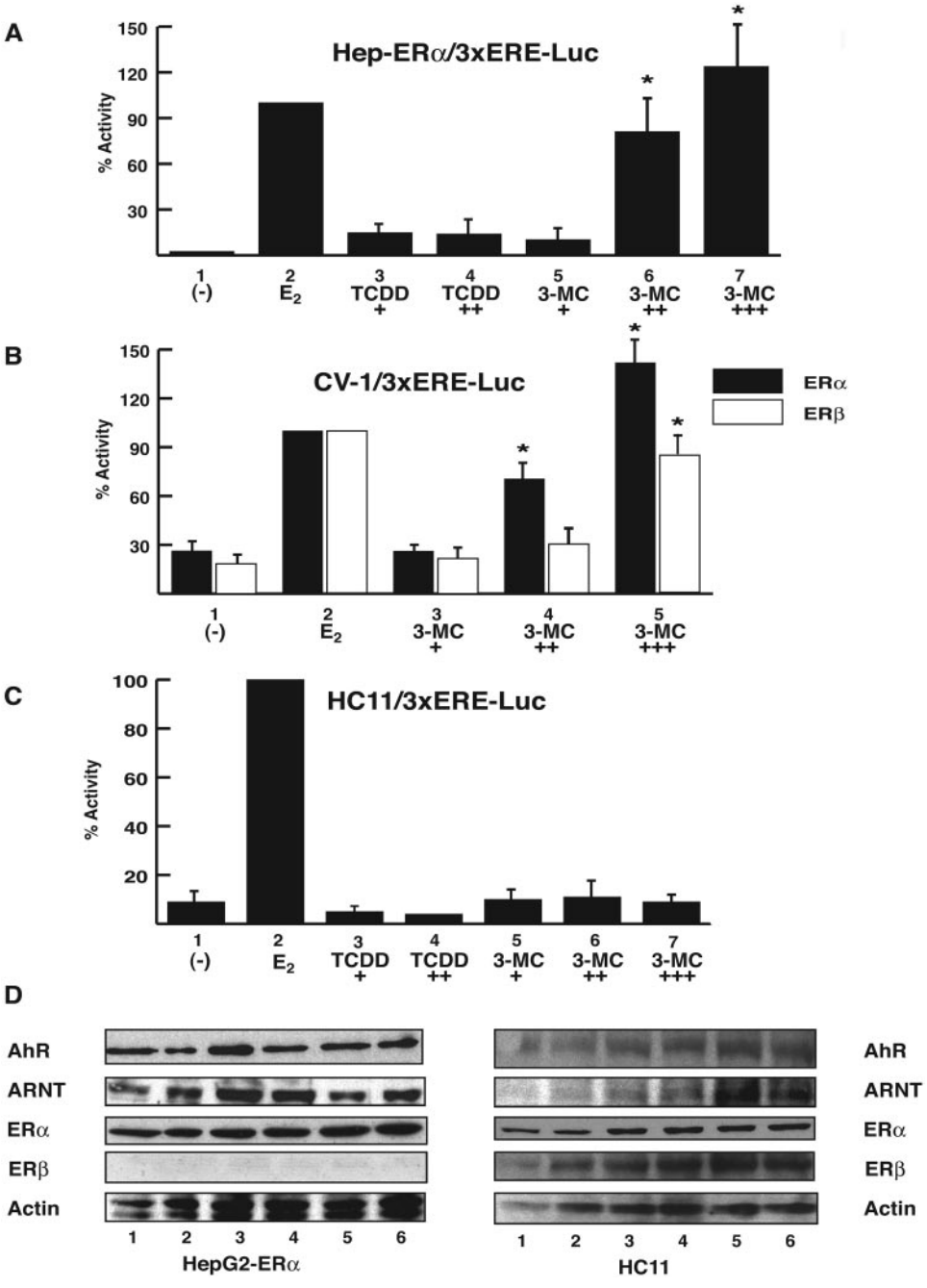


**Fig. 1.** The effect of 3-MC on ER-mediated transcription is dependent on cellular context. A, hepatoma-derived HepG2 cells, stably transfected with ERα (Hep-ERα) were transiently transfected with 3xERE-TATA-Luc and β-gal reporter gene constructs. The cells were treated with 100 nM $E_2$, 10 and 20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

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 or TCDD (lanes 3–4 and 5–7) was observed. In addition, we considered the possibility that the AhR activated by a short time (6 h) exposure of 3-MC may acquire a specific conformation that is able to activate ER$\alpha$. To investigate this, experiments were performed in HepG2 cells transiently transfected with ER$\alpha$ expression vectors and treated with 3-MC for 6 h. After this treatment, the transcriptional activity of ER$\alpha$ and AhR was investigated. Under these conditions a xenobiotic response element-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 (Fig. S1A, lane 7), whereas 50 nM DES efficiently induced the ERE-regulated expression of the luciferase reporter (Fig. S1A; compare lanes 6 and 7). Taken together, these results suggest that the ability of 3-MC to activate ER$\alpha$-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 ER$\beta$, 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 Inhibited $E_2$-Induced Transcriptional Activity in HC11 Cells.** The experiments presented above indicate that 3-MC can act as an ER$\alpha$ and ER$\beta$ agonist in certain cell types (HepG2 and CV-1) but not in others, exemplified by HC11. We decided to investigate whether the inhibitory actions documented previously of 3-MC and TCDD on $E_2$-induced activity were present in HC11 cells. For this purpose, HC11 cells were treated with 10 nM $E_2$ in combination with 10 nM TCDD or 10 $\mu$M 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 (Fig. 2, compare lanes 1 and 4). However, coexposure to 3-MC or TCDD resulted in reduced ERE-dependent expression (Fig. 2, compare lanes 4, 5, and 6), showing that both TCDD and 3-MC are able to interfere with the transcriptional response induced by the estrogen receptors in HC11 cells, but neither compound induces ER activity as ER ligands.

**3-MC Interacted 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 ER$\alpha$ and ER$\beta$, the inhibitory effects of the classic ER antagonists ICI 182,780 (ICI) and 4-OH-tamoxifen (4-OHT) was examined in transfection assays. To enable the evaluation of both ER isoforms, CV-1 cells were chosen for these investigations. The cells were transiently cotransfected with the 3xERE-Luciferase reporter construct together with expression vectors for either ER$\alpha$ or ER$\beta$ and incubated in the presence of 10 $\mu$M 3-MC separately or in combination with ICI and 4-OHT. As before, 3-MC induced a robust transcriptional response of ER$\alpha$ and, to a lesser extent, of ER$\beta$ (Fig. 3A, lanes 3 and 8, respectively). In the presence of ICI or 4-OHT, the response was severely blunted (Fig. 3A, lanes 4 and 5, respectively). This observation may indicate that ICI and 4-OHT antagonize the 3-MC-induced activity of ER$\alpha$ and ER$\beta$ 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-ER$\alpha$ or Gal4-ER$\beta$. The cells were exposed to 10 nM $E_2$, 0.1, 1, and 10 $\mu$M 3-MC, or 10 nM TCDD for 48 h. After transfection, cells were harvested, and luciferase activity was determined.

3-MC induced a transcriptional response of both Gal4-ER$\alpha$-LBD and ER$\beta$-LBD, albeit to a lesser extent compared with the intact receptors (Fig. 3B, lanes 3–5 and 9–11). The ER$\beta$-LBD displayed a weaker response to 3-MC compared with ER$\alpha$, consistent with the results obtained with the full-length receptors (see Fig. 1B for comparison). None of the receptors was 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 ER$\alpha$ and ER$\beta$-LBD SPA. We found that neither TCDD (Fig. 3E) or 3-MC (Fig. 3D) caused any major displacement of the tracer (tritiated $E_2$), demonstrating that both compounds

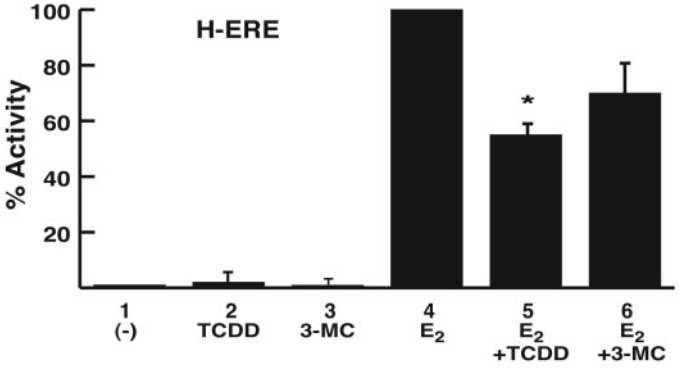


**Fig. 2.** Both TCDD and 3-MC repress $E_2$-signaling in HC11 cells. HC11 cells with a stably integrated 3xERE luciferase reporter gene construct (H-ERE) were treated with 10 nM $E_2$ alone or in combination with 10 nM TCDD or 10 $\mu$M 3-MC. After 48 h, the cells were lysed, and reporter gene activity 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 $p < 0.05$.

were very weak binders of ER$\alpha$ and ER$\beta$. In control experiments, however, nonlabeled $E_2$ 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, whereas the binding affinity of 3-MC was calculated to 37 and 22 $\mu$M for ER$\alpha$ and ER$\beta$, respectively (data 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 Induced Transcriptional Activation of $E_2$- and TCDD-Inducible Genes.** The experiments presented above suggest that 3-MC is able to activate the 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 ER$\alpha$-expressing HepG2 cells. Real-time PCR (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 6 h, where the exposure time was chosen to reflect direct transcriptional effects of ER$\alpha$ 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

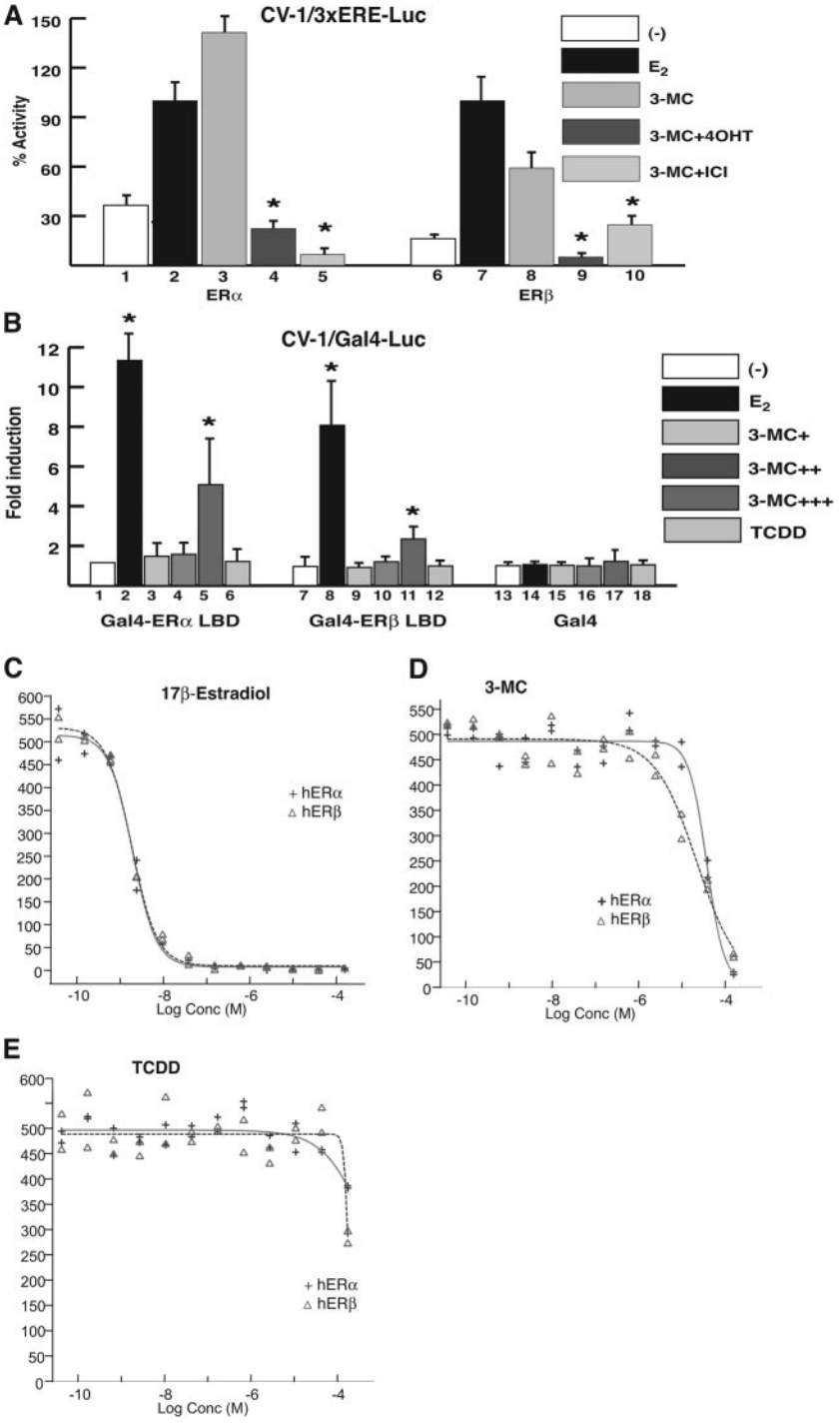


**Fig. 3.** The effects of 3-MC are mediated by the LBDs of the ERs. A, CV-1 cells transiently cotransfected with either pSG5-ER$\alpha$ or pSG5-ER$\beta$ together with 3xERE-TATA-Luc and $\beta$-gal reporter gene constructs. The cells were treated with 10 nM $E_2$ and 3-MC, separately or in combination with ER antagonists ICI 182,780 (10 nM) or 4-OHT (1 $\mu$M). After 48 h, cells were harvested and reporter gene activity measured; $*$, significantly different from 3-MC-treated samples at $p < 0.05$. B, CV-1 cells were cotransfected with Gal4-ER$\alpha$ LBD or Gal4-ER$\beta$ LBD together with a Gal4-regulated luciferase reporter gene construct. After 48 h, reporter gene activity was determined. C through E, the recombinant LBD of ER$\alpha$ and ER$\beta$ was incubated with labeled $E_2$ in the presence or absence of the nonlabeled $E_2$ (C), 3-MC (D), or TCDD (E). The ability of the nonradioactive compounds to displace $E_2$ was assessed as described under *Materials and Methods*.

affect ERα transcriptional activation in a biologically relevant context. The effects would not involve the requirement to synthesize luciferase protein as presented in Fig. 1. Cells were treated with $E_2$, TCDD, or 3-MC for 6 h and after incubation, the cells were harvested and mRNA was extracted as described under *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 (Fig. 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 (Fig. 4A, lane 2). It is interesting that pS2 gene expression was induced by 3-MC to an extent similar to 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 Fig. 4, A and B, lane 4). TCDD treatment, in contrast, had no effect on pS2 expression in either cell type.

Because TCDD and 3-MC are well known AhR ligands, we decided to assess whether AhR-mediated signaling is required for the ability of 3-MC to activate ERα-mediated transcription using siRNA against AhR and analyze the effects on pS2 and CYP1A1 expression. An siRNA construct against AhR was transiently introduced into Hep-ERα cells, and the down-regulation of the AhR was monitored to identify the optimal time point to perform expression analysis. We were able to efficiently lower the intracellular levels of the AhR as shown in Fig. 4C (top, compare lanes 1 and 2) without affecting the levels of the AhR partner factor ARNT or actin (Fig. 4C, middle and bottom; compare lanes 1 and 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 an

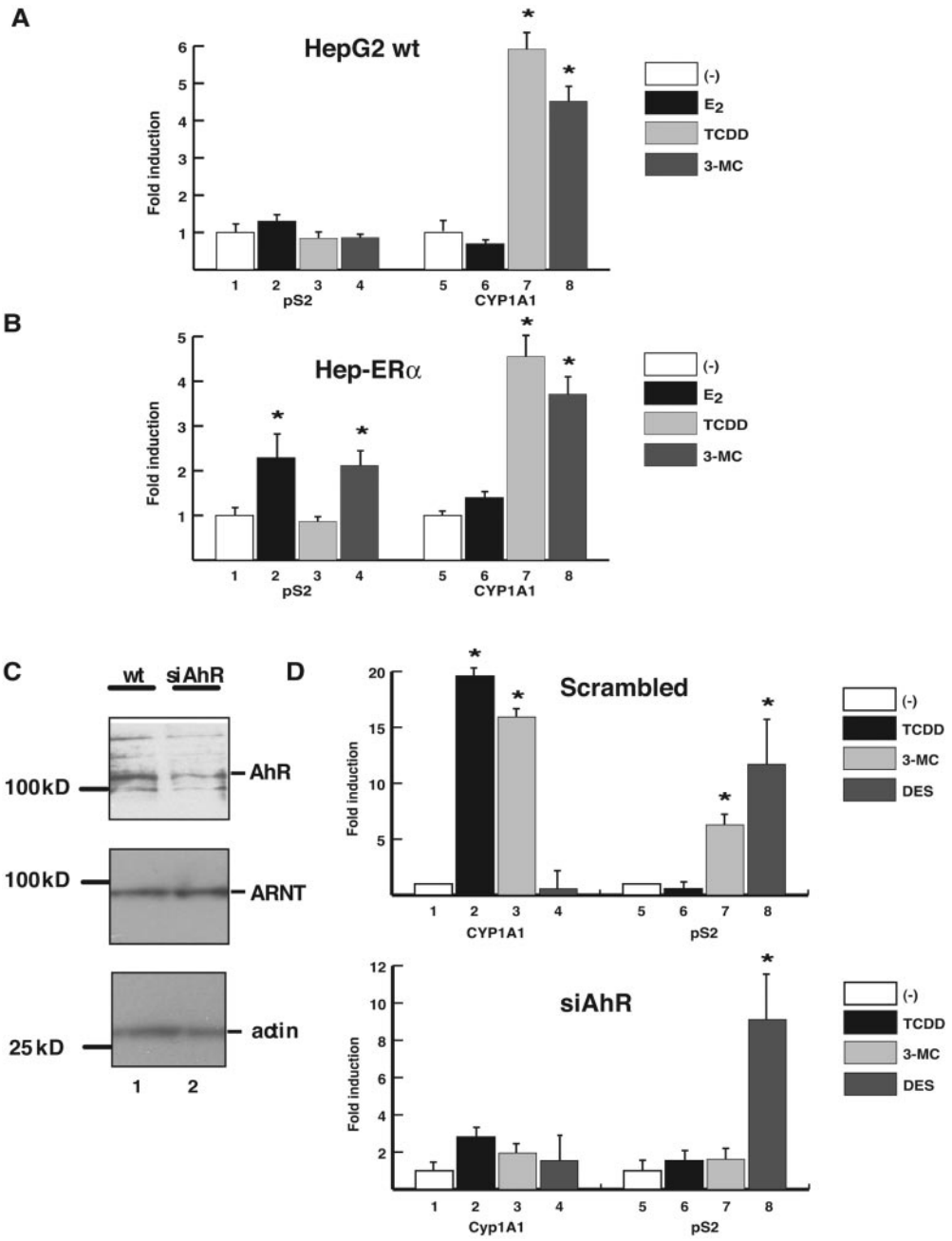


**Fig. 4.** Expression of the $E_2$-regulated gene pS2 is induced by 3-MC but not by TCDD. Wild-type HepG2 (A) or Hep-ERα (B) cells were starved for 48 h and treated with 10 nM $E_2$, 10 nM TCDD, or 10 μM 3-MC for 6 h. Total RNA was isolated, reverse-transcribed, and analyzed by real-time PCR with primers 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 48 h 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 24 h, and RT-PCR was performed to measure the levels of pS2 or CYP1A1 gene expression. *, differ significantly from vehicle control at $p < 0.05$.

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 the expression of the pS2 gene (Fig. 4D, top, 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 (Fig. 4D, bottom, lanes 2 and 3). It is important to note, however, that the ability of the cells to activate pS2 expression in response to 3-MC was also greatly diminished (Fig. 4D, top and bottom, compare lanes 7), suggesting that the induction of AhR-activated genes is an important prerequisite for 3-MC-induced activation of ER$\alpha$ target genes. In control experiments, the ability of the ER$\alpha$ agonist DES to activate pS2 expression was unaffected by the siRNA against the AhR, showing that ER$\alpha$ is not dependent per se on the presence of AhR for its ability to induce transcription of pS2 (Fig. 4D, 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.

**HepG2 but Not HC11 Cells Generated Metabolites with ER-Activating Properties after 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 an agonist for ER$\alpha$ 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 after exposure to 3-MC was performed. HC11 or HepG2 cells were exposed to low (0.1 $\mu$M) or high (10 $\mu$M) concentrations of [$^3$H]3-MC for 24 h. After exposure, the growth media were collected and fractionated using an HPLC system developed for the study. In this system, the retention time of 3-MC was approximately 63 min. 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 retention time of 52 min, without any major difference observed between the analysis of media from 10 $\mu$M incubations (Fig. 5A) or from 0.1 $\mu$M incubations (data not shown), respectively. Similar radiochromatograms 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 $\mu$M media analysis is shown in Fig. 5B. For these incubations, unchanged 3-MC accounted for only 1.3 $\pm$ 0.3% of the radioactivity after 24 h. Radiochromatograms obtained after a 24-h exposure of HepG2 cells to 0.1 $\mu$M 3-MC were qualitatively similar, with unchanged 3-MC accounting for 0.6 $\pm$ 0.3% of the detected radioactivity.

**Metabolic Conversion of 3-MC Was Required for Its Ability to Induce ER-Dependent Transcription.** The ability of 3-MC to induce ER$\alpha$-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 $\mu$M 3-MC for 24 h were collected. Separation was achieved by collecting the eluted material each 5 min, resulting in 13 fractions in total. Each fraction was pooled (three incubations), concentrated, and dissolved in 1 ml of dimethyl sulfoxide. The ability of the individual fractions to induce ER activity was assessed in the H-ERE cells and compared with 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 (Fig. 6A). A similar fractionation was carried out for incubation media from HepG2 cells incubated with 0.1 $\mu$M 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 $\mu$M incubation media, it was concluded that the amount of active compounds present in fractions 2 and 3 of 0.1 $\mu$M incubation media was too small to induce reporter activity. However, the negative results obtained for 0.1 $\mu$M 3-MC incubations rules out the possibility that the observed ER activity induction was due to endogenous compounds present in HepG2 cell incubates. These experiments suggest that metabolic conversion of 3-MC generates compounds that can activate ER$\alpha$ and that HC11 cells in contrast to HepG2 cells seem to lack the capacity to perform such conversion. To further assess the 3-MC conversion products as ER$\alpha$ ligands, H-ERE cells were cotreated 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, whereas the original 3-MC did not (Fig. 6B). It is interesting that cotreatment with ICI resulted in a clear reduction of ER$\alpha$ transcriptional activity (Fig. 6B). These observations strengthen the hypothesis that the fractions from 10 $\mu$M 3-MC HepG2 cells incubation media contain compounds that may act as ligands for the ERs.

## 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 proestrogenic function of the activated AhR/ARNT complex (Ohtake et al., 2003). This study is inconsistent with numerous epidemiological studies and biological and experimental data demonstrating an antiestrogenic effect of AhR ligands (Zacharewski et al., 1994; Kharat and Saatcioglu, 1996; Safe et al., 2000; Chen et al., 2001; Safe, 2001). In addition, another recently published study suggests that 3-MC does not require the AhR to activate ER$\alpha$-dependent transcription (Abdelrahim et al., 2006).

In this study, we show that the ability of 3-MC to activate the ERs and in particular ER$\alpha$ 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 h, we fail to observe any activation of an ERE-regulated reporter gene construct. In contrast, using HepER$\alpha$ cells and RT-PCR analysis, induction of the pS2 gene

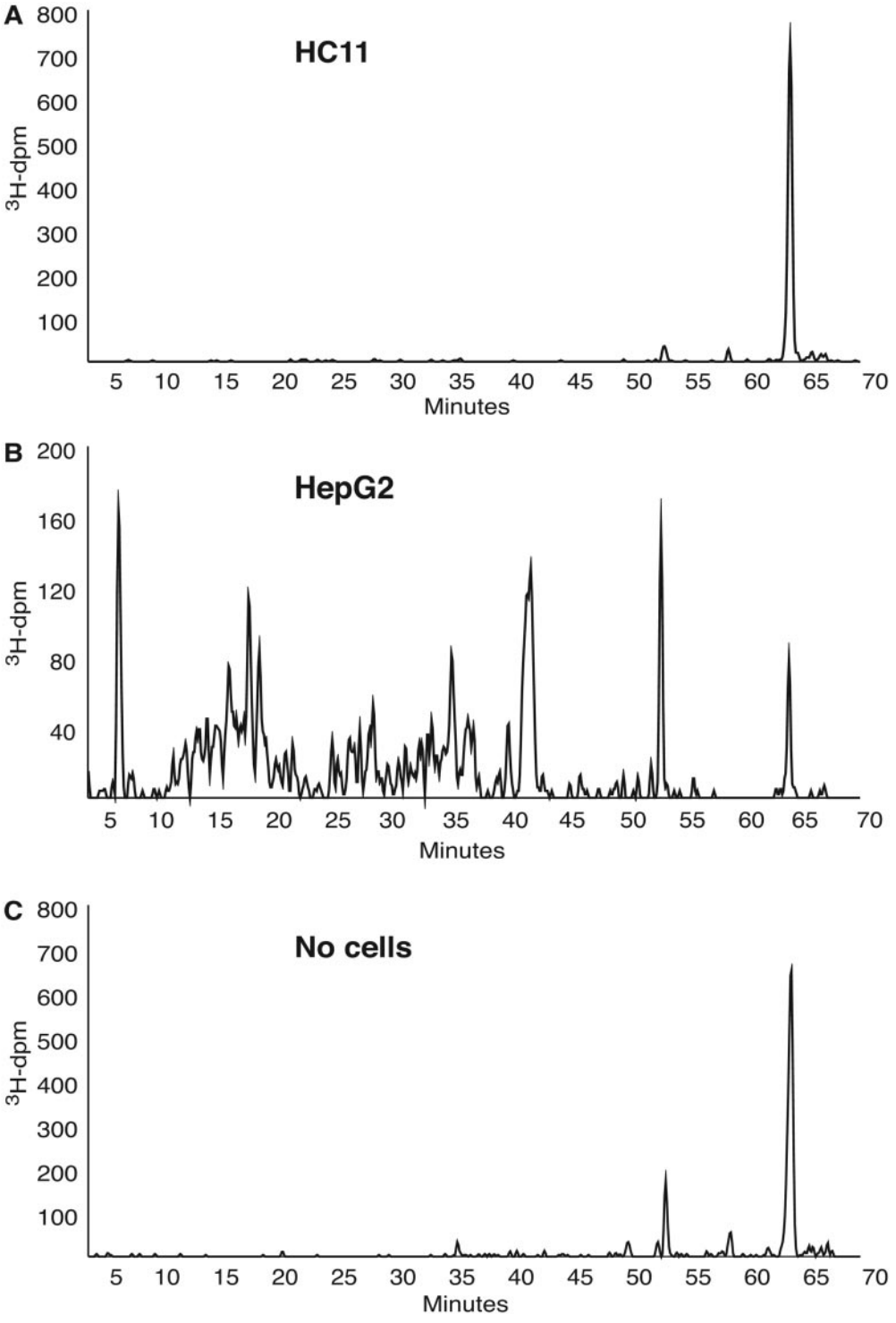


**Fig. 5.** Radiochromatographic profiles of 3-MC collected from medium from HepG2 and HC11 cells. HC11 (A) or HepG2 (B) cells were treated with 10 $\mu$M 3-MC for 24 h, and the medium was collected and fractionated as described under *Materials and Methods*. The chromatograms were run in triplicate. C, medium incubated without any cells serves as a control. Representative radiochromatograms are presented.

can be observed already after 6-h 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 ER$\alpha$. In Hep-ER$\alpha$ 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 ER$\alpha$ or ER$\beta$ transcriptional activity, and coexposure of cells to $E_2$ and TCDD resulted in a decrease in ER transcriptional response, demonstrating the antiestrogenic effects. Depending on the cellular system, different ligands of the AhR can either repress or activate the transcriptional activity of ER$\alpha$ and ER$\beta$. 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 ER$\alpha$ or ER$\beta$. 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 ER$\alpha$-mediated pS2 expression in response to 3-MC, but not DES, was blunted. It is interesting that 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 also has been shown to occur with benzo($\alpha$)pyrene (BaP), a compound related to 3-MC, and an AhR agonist. It is interesting that both 3-MC and BaP are so-called procarcinogens, which require metabolic activation to reactive intermediates to cause toxic effects. In the case of BaP, the parent compound displays little affinity for the ERs. However, after P450 activation, monohydroxylated derivatives with estrogenic activity are formed (Arcaro et al., 1999). These derivatives of BaP

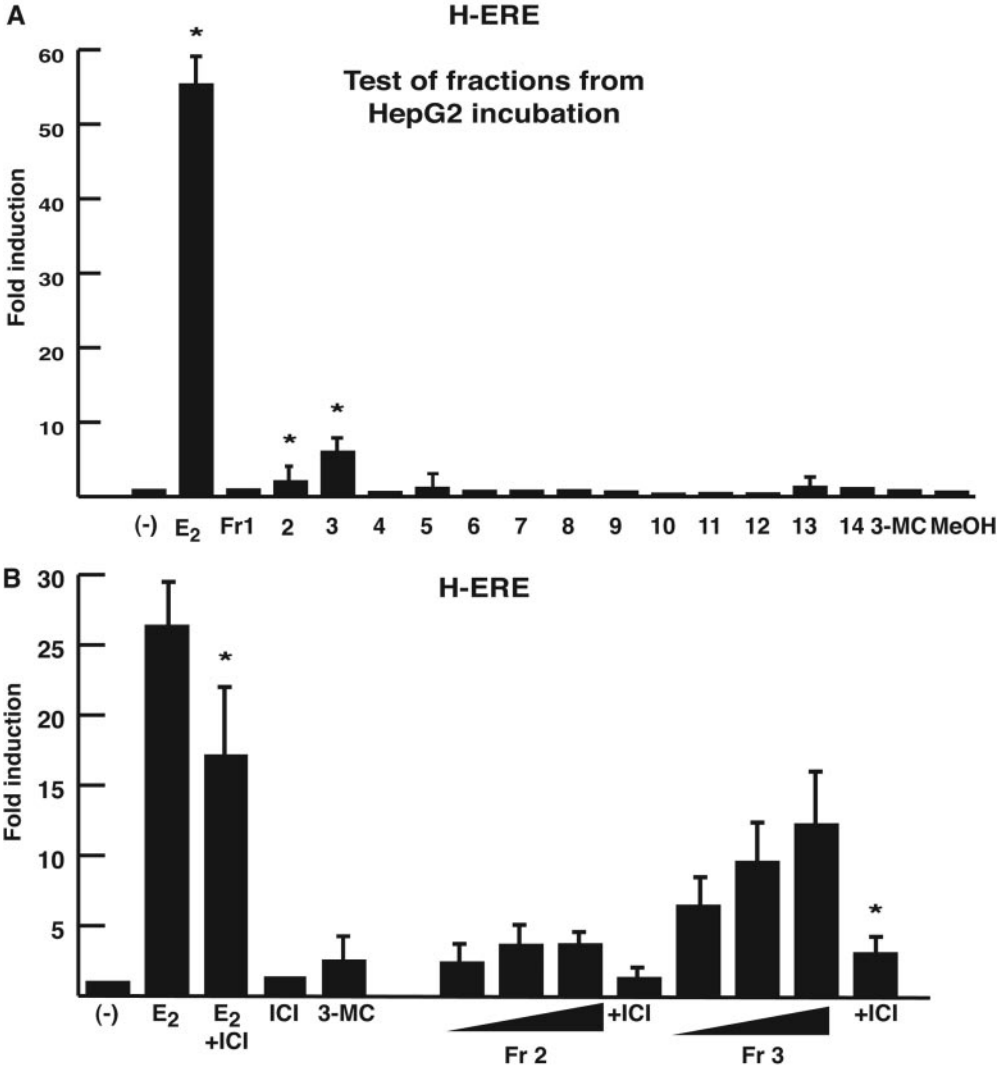


**Fig. 6.** Metabolic conversion of 3-MC is required to activate ER transcriptional activity. A, HepG2 cells were treated with 10 $\mu$M 3-MC for 24 h, and the medium was collected and fractionated as described under *Materials and Methods*. After fractionation, fractions were collected, pooled, evaporated, and resuspended in 200 $\mu$l of methanol. The different fractions (5 $\mu$l) were subsequently used to treat HC11 3xERE-Luc reporter cells. 10 nM $E_2$, 10$\mu$M 3-MC, and 5 $\mu$l of methanol were included as controls. After 24 h, the cells were harvested, and luciferase activity was determined. $*$ indicates significant changes compared with control. B, H-ERE reporter cells were treated with 5, 7.5, and 10 $\mu$l of fractions 2 and 3 in combination with 10 nM ICI for 24 h. 10 nM $E_2$, 10 nM ICI, 10 $\mu$M 3-MC, and 10 $\mu$l of methanol were included as controls. After 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 $p < 0.05$.

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 although 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 and 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 antiestrogens. 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.

It is intriguing that 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 radiochromatographic 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 in 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 with, 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, whereas 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.

One study suggested that the AhR is able to activate ERα in the absence of $E_2$ (Ohtake et al., 2003). Furthermore, a different study suggested that 3-MC is able to displace $E_2$ and thus act as ligand to ERα (Abdelrahman et al., 2006). 3-MC induced ERα transcriptional activation at far lower concentration compared with its ligand binding ability. Our study presented here provides a mechanistic explanation for 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 whereas 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. Although 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 dependent on the cellular context and, in particular, the cellular ability to transform the parent 3-MC compound to alternative compounds.

It is interesting that 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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