

# Aryl hydrocarbon receptor-independent activation of estrogen receptor-dependent transcription by 3-methylcholanthrene<sup>☆</sup>

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## Abstract

Aryl hydrocarbon receptor (AhR) is a ligand-activated transcription factor that stimulates transcription directed by xenobiotic response elements upstream of target genes. Recently, AhR ligands were reported to induce formation of an AhR–estrogen receptor (ER) complex, which can bind to estrogen response elements (EREs) and stimulate transcription of ER target genes. Presently, we investigate the effect of the AhR ligands 3-methylcholanthrene (3MC), 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and 3,3',4,4',5-pentachlorobiphenyl (BZ126) on ERE-regulated luciferase reporter activity and endogenous ER target gene expression. In MCF-7 human breast cancer cells, 3MC induced transcription of ER reporter genes containing native promoter sequences of the ER-responsive genes *complement 3* and *pS2* and heterologous promoters regulated by isolated EREs. Dose–response studies revealed that the concentration of 3MC required to half-maximally activate transcription (EC<sub>50</sub>) was >100-fold higher for an ER reporter (27–57 μM) than for an AhR reporter (86–250 nM) in both MCF-7 cells and in human endometrial cancer Ishikawa cells. 3MC also stimulated expression of the endogenous ER target genes *amphiregulin*, *cathepsin D* and *progesterone receptor*, albeit to a much lower extent than was achieved following stimulation with 17β-estradiol. In Ishikawa cells, 3MC, but not BZ126 or TCDD, stimulated ERα-dependent reporter activity but did not induce expression of endogenous ER target genes. Finally, studies carried out in the AhR-positive rat hepatoma cell line 5L and the AhR-deficient variant BP8 demonstrated that ER reporter activity could be induced by 3MC in a manner that was independent of AhR and thus distinct from the AhR-ER ‘hijacking’ mechanism described recently. 3MC may thus elicit estrogenic activity by multiple mechanisms.

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**Keywords:** Ah receptor; 3-methylcholanthrene; Estrogen receptor; AhR-ER cross-talk

## Introduction

Aryl hydrocarbon receptor (AhR) is a ligand-activated transcription factor that mediates the induction of gene expression by diverse environmental chemicals, including polychlorinated dibenzo-*p*-dioxins, dibenzofurans and polychlorinated biphenyls (PCBs) (Abnet et al., 1999). Following ligand binding, the AhR translocates to the nucleus, where it heterodimerizes with the aryl hydrocarbon nuclear translocator (Arnt), binds to xenobiotic response elements (XREs) and activates aryl hydrocarbon-responsive genes, including *CYP1A1* (Whitlock et al., 1996) and *CYP1B1* (Larsen et al., 1998). Cross-talk has been observed between the AhR and

multiple intracellular signaling pathways, such as those involving NFκB, cell cycle regulators and steroid hormone receptors, including estrogen receptor α (ERα) (Safe et al., 2000; Carlson and Perdew, 2002). AhR ligands, such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), can exert anti-estrogenic effects by a variety of mechanisms, including suppression of ERα mRNA (Tian et al., 1998), inhibition of ERα binding to estrogen response elements (EREs) found adjacent to, or overlapping with XREs (Klinge et al., 1999), competition for a common pool of transcriptional coactivators (Reen et al., 2002) and stimulation of ERα degradation (Wormke et al., 2003). Anti-estrogens such as the ER antagonist tamoxifen can be used therapeutically to protect from the recurrence of breast cancer (Jordan, 1994). Similarly, the ER inhibitory activity of ligand-activated AhR has provided a basis for the development of selective AhR modulators for treatment of ER-positive breast cancer (Safe and McDougal, 2002; Safe et al., 2000).

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In addition to the well-described inhibitory effects of ligand-activated AhR on ER-regulated transcription, recent studies have suggested that the AhR can mediate pro-estrogenic actions when stimulated by AhR ligands such as 3MC and TCDD (Ohtake et al., 2003). 3MC was shown to induce the formation of a complex between ER $\alpha$  the AhR–Arnt heterodimer, which can then bind to an ERE and stimulate transcription of ER target genes. 3MC-induced estrogenic responses were observed in cultured human breast cancer MCF-7 cells, which express AhR and ER $\alpha$ , and in an ovariectomized mouse model, where 3MC-induced expression of 17 $\beta$ -estradiol (E<sub>2</sub>)-responsive genes, endometrial cell proliferation and uterine growth were observed (Ohtake et al., 2003). AhR may thus ‘hijack’ ER $\alpha$ , and ER $\beta$ , in a manner that enables TCDD, polycyclic aromatic hydrocarbons and other classical AhR agonists to stimulate estrogenic responses in the absence of estrogen (Brosens and Parker, 2003).

In this study, we further investigate the estrogenic activity of AhR agonists, and show that ER $\alpha$ -dependent transcription can be induced by the AhR ligand 3MC, but only at concentrations far higher than those required to activate AhR-regulated transcription. Moreover, we report that two other AhR agonists, TCDD and 3,3',4,4',5-pentachlorobiphenyl (BZ126), do not activate ER reporter activity. Furthermore, we show that 3MC activation of ER reporter activity does not necessarily translate into a corresponding increase in expression of endogenous ER target genes. Finally, using the rat hepatoma cell line 5L and its AhR-deficient variant BP8 (Weiss et al., 1996; Reiners et al., 1999), we demonstrate estrogenic actions of the AhR agonist 3MC that are independent of AhR. These findings are discussed in the context of the AhR-ER ‘hijacking’ model proposed by Ohtake et al. (2003).

## Materials and methods

**Plasmids.** The ER-activated firefly luciferase reporter plasmid ERE-TATA-luc, containing three EREs, and the AhR-activated reporter plasmid XRE-TATA-luc, containing two XREs from the *CYP1A1* promoter, were obtained from Dr. S. Kato (University of Tokyo, Japan) (Ohtake et al., 2003). The reporter plasmid C3-luc, containing 1.8 kb of the human C3 gene promoter, the human ER $\alpha$  expression plasmid pRST7-ER $\alpha$  and the human ER $\beta$  expression plasmid pRST7-ER $\beta$  were provided by Dr. D. McDonnell (Duke University Medical Center, Durham, NC) (Tzukerman et al., 1994). Human AhR and ARNT expression plasmids were provided by Dr. C. Bradfield (University of Wisconsin Medical School, Madison, WI). The ER-responsive luciferase reporters ERE-tk-luc (containing two EREs) and pGL3-pS2-luc (containing 1.1 kb of the human pS2 promoter) were provided by Dr. M. Brown (Dana-Faber Cancer Institute, Boston, MA). The *Renilla* luciferase expression plasmid pRL-CMV was purchased from Promega (Madison, WI).

**Other materials.** Rat hepatoma 5L cells and BP8 cells were kindly provided by Dr. C. Elferink (University of Texas Medical Branch, Galveston, TX) (Weiss et al., 1996). Human breast carcinoma MCF-7 cells were purchased from ATCC (Manassas, VA). ER $\alpha$ -positive and ER $\alpha$ -negative human uterine cancer Ishikawa cells were obtained from Dr. J. McLachlan (Tulane University, New Orleans, LA) (Frigo et al., 2002a). 3MC, TCDD and E<sub>2</sub> were purchased from Sigma (St Louis, MO). The ER antagonist ICI-182,780 was purchased from Tocris Cookson Inc. (Ellisville, MO). BZ126 was purchased as a hexane stock (Ultra Scientific, North Kingstown, RI). The hexane was gently evaporated and the residue was dissolved in DMSO by vortexing for at least 1 h to give a 5 mM stock solution (stored in aliquots at –20 °C). Stock solutions of 3MC dissolved

in toluene (stock concentration 50 mM) and E<sub>2</sub> and ICI-182,780 dissolved in DMSO (stock concentration 100 mM) were prepared fresh daily.

**Cell culture and transfection.** 5L, BP8 and MCF-7 cells were grown in Dulbecco's modified Eagles medium (DMEM) containing 10% fetal bovine serum (FBS) (Gibco-BRL, Inc.). Ishikawa cells were maintained in a 1:1 mixture of Ham F12 medium and Iscove F12 medium, supplemented with 10% FBS, Basal Medium Eagle (BME) amino acids (10 ml of 50 $\times$  stock per liter medium; Sigma), sodium pyruvate (5 ml of 100 mM stock per liter; Gibco), Minimal Essential Medium (MEM) non-essential amino acids (5 ml of 10 mM stock per liter; Gibco) and insulin (6  $\mu$ l of 10 ng/ml stock per liter; Sigma) (Frigo et al., 2002a). In all experiments where ER reporter activity was assayed, the medium was changed to Ishikawa Treatment medium (IT medium) beginning 48 h prior to splitting the cells into 48-well plates for transfection. IT medium contains phenol red-free DMEM and 5% charcoal-stripped FBS (Biomed, Foster City, CA) supplemented with 5 ml of 20 mM L-glutamine (Gibco), 5 ml of 10 mM MEM non-essential amino acids (Gibco), 10 ml of 50 $\times$  BME amino acids (Sigma), 5 ml of 100 mM sodium pyruvate (Gibco), 24.65 ml of 7.5% (w/v) sodium bicarbonate (Gibco) and 6  $\mu$ l of 10 ng/ml insulin (Sigma) per liter of culture medium (Frigo et al., 2002b). Transfection efficiencies were lower in IT medium than in DMEM + 10% FBS, especially in 5L and BP8 cells. In experiments where AhR activation was assayed using the reporter plasmid XRE-TATA-luc, cells were cultured in DMEM + 10% FBS throughout the experiment, unless noted otherwise.

For transfection studies, cells were plated in 48-well tissue culture plates at  $3 \times 10^4$  cells/well in 250  $\mu$ l DMEM + 10% FBS, or at  $4.25 \times 10^4$  cells/well in 250  $\mu$ l of IT medium. Twenty-four hours later, the cells were transfected with a total of 250 ng DNA/well using 0.3  $\mu$ l of FuGENE 6 transfection reagent (Roche Molecular Biochemicals). Salmon sperm DNA was used as a carrier to adjust the total to 250 ng DNA/well. To improve the transfection efficiency of 5L, BP8 and ER $\alpha$ -negative Ishikawa cells, Hoechst dye 33258 (Molecular Probes, Eugene, OR) was included in the transfection mix at 100  $\mu$ M final concentration (1.6  $\mu$ l of a 10 mg/ml stock in water), unless indicated otherwise. DNA to be transfected (250 ng in 1–3  $\mu$ l of water per well) was added to DMEM minus serum to give a total volume of 8.4  $\mu$ l/well, followed by the addition of 1.6  $\mu$ l of 10 mg/ml Hoechst 33258 and incubation for 30 min at room temperature. Ten microliters comprised of FuGENE 6 (0.3  $\mu$ l/well) preincubated with DMEM minus serum (9.7  $\mu$ l/well) for 5 min was then added, and the DNA/DMEM/Hoechst 33258/FuGENE mix (20  $\mu$ l/well) was incubated for a further 15 min. The mix was then added to each individual well to be transfected. Cells were treated with hormones and chemicals (e.g., 3MC, E<sub>2</sub>) added to the cells in a medium change applied 24 h post-transfection. Cells were lysed 24 h later in 250  $\mu$ l passive lysis buffer (Promega), and firefly and *Renilla* luciferase activities were measured using a dual luciferase assay kit (Promega).

Transfections were performed using the following amounts of plasmid DNA/well of a 48-well tissue culture plate: 150 ng of ER reporter plasmid (ERE-TATA-luc, C3-luc, ERE-tk-luc or pGL3-pS2-luc), 50 ng of hER $\alpha$ , hER $\beta$ , hAhR or hARNT expression plasmid and 5 ng of the *Renilla* luciferase control reporter plasmid pRL-CMV. Twenty nanograms of the AhR reporter plasmid XRE-TATA-luc was used when cells were plated in DMEM + 10% FBS, whereas 150 ng of the AhR reporter was used when the cells were plated in IT medium. In some experiments, cells were transfected in 6-well plates as described above for 48-well plates, with the plasmid and reagent amounts scaled up 12.25-fold to account for the larger surface area of the 6-well plates. Firefly luciferase activity values were normalized by the activity of the *Renilla* luciferase internal control, and data are presented as the fold-increase in normalized reporter activity for chemical- or hormone-treated cells compared to untreated cells. EC<sub>50</sub> values were calculated by subtracting the background activity (untreated cells) and converting the normalized luciferase activity to a percentage of maximal activation. Percent activation data were plotted as sigmoidal dose–response curves and EC<sub>50</sub> values were determined using GraphPad-Prism software (version 4.0). Statistical significance was assessed using a one-way ANOVA test followed by Dunnett's Multiple Comparison test calculated using GraphPad-Prism software (version 4.0).

**Quantitative PCR (qPCR).** RNA expression levels were assayed by qPCR using an ABI 7900 Prism Sequence Detection System (Applied Biosystems).

qPCR primers were designed using Primer Express software (Applied Biosystems) as shown in Table 1. Primers for amphiregulin (schwannoma-derived growth factor; AREG) were based on Vendrell et al. (2004). Rat AhR was quantified using primers corresponding to mouse AhR (GenBank accession number NM\_013464), but containing a single 5' mismatch with rat AhR (GenBank accession number NM\_013149) in the anti-sense primer. MCF-7 cells were seeded in 6-well plates at  $4.2 \times 10^5$  cells/well in IT medium and treated for 48 h with E<sub>2</sub> (10 nM), with toluene as a vehicle control (0.2%), or with the indicated concentrations of 3MC beginning 24 h after transfection. Total RNA was extracted from the cells using TRIZOL reagent (Gibco) and then treated with DNase I to remove contaminating DNA. One microgram RNA was reverse-transcribed into cDNA in a 20 µl reaction that included random hexamer primers and MuLV reverse transcriptase (Applied Biosystems). The cDNA was diluted 100-fold into water containing 50 µg/ml yeast RNA (Ambion) and SYBR Green-based qPCR assays were then carried out in triplicate. Each qPCR reaction contained SYBR Green PCR Master Mix (Applied Biosystems) and 300 nM of each primer in a total volume of 5 µl/well of a 384-well plate. The PCR program consisted of one cycle at 50 °C for 2 min and then 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s, 60 °C for 1 min and 95 °C for 15 s. No PCR amplification was observed in control reactions that omitted reverse transcriptase or the cDNA template. The relative level of each RNA was calculated for each cDNA sample after subtracting the threshold cycle (C<sub>T</sub>) for 18S rRNA (determined in triplicate for each cDNA) from the C<sub>T</sub> values (determined in triplicate) (ΔC<sub>T</sub>). Dissociation curves were generated after each qPCR run to ensure that a single, specific product was amplified. The average ΔC<sub>T</sub> for untreated cells was then subtracted from the corresponding ΔC<sub>T</sub> for treated cells (3MC or E<sub>2</sub>) (ΔΔC<sub>T</sub>) and the values were back-transformed ( $2^{-\Delta\Delta C_T}$ ) to calculate the relative abundance of each RNA in treated cells compared with untreated controls.

## Results

### Trans-activation of ER reporter activity by 3MC

ER-dependent transcription was studied in MCF-7 cells treated with the AhR agonist 3MC. MCF-7 cells express AhR (Abdelrahim et al., 2003), ERα (Freddie et al., 2004) (Table 2) and ERβ (Power and Thompson, 2003). Initial experiments using cells cultured in DMEM + 10% FBS gave a modest, ≤2-fold activation of the ER reporter ERE-TATA-luc in response to 17β-estradiol (E<sub>2</sub>) treatment. This culture medium has been associated with high constitutive ER activity attributed to estrogenic compounds (e.g., phenol red and undefined constituents of FBS) present in the medium (Rogers and Denison, 2000). MCF-7 cells were therefore grown in DMEM + 10%

Table 2

ER and AhR mRNA levels and transcriptional activity in MCF-7, Ishikawa and rat hepatoma cell lines

| Cell line       | Cell type              | ERα RNA | AhR RNA   | ER activity | AhR activity |
|-----------------|------------------------|---------|-----------|-------------|--------------|
| Ishikawa ERα(−) | Human endometrial cell | 1 ± 0.3 | 1 ± 0.1   | (−)         | (+)          |
| Ishikawa ERα(+) | Human endometrial cell | 31 ± 6  | 1.1 ± 0.5 | (+)         | (+)          |
| MCF-7           | Human breast cancer    | 35 ± 22 | 4.1 ± 1.2 | (+)         | (+)          |
| BP8             | Rat hepatoma           | ND      | 1 ± 0.4   | (−)         | (−)          |
| 5L              | Rat hepatoma           | ND      | 126 ± 75  | (−)         | (+)          |

Relative RNA levels were determined by qPCR. Data shown are mean expression values based on at least four independent RNA samples from each cell line, with each assay based on triplicate qPCR reactions. Data are relative values, normalized to the 18S RNA content of each sample, mean ± SD. Thus, the ERα and AhR RNA content of Ishikawa ERα(−) cells was set to 1, as was the AhR RNA of BP8 cells. Analyses were carried out using either human or rat-specific qPCR primers (Table 1) as appropriate for each cell line. The endogenous ER and AhR transcriptional activity of each cell line (last two columns) was assessed by transfection of the cells with the ER luciferase reporter plasmid ERE-TATA-luc or with the AhR luciferase reporter plasmid XRE-TATA-luc, followed by treatment with E<sub>2</sub> or 3MC, respectively. Cells that showed ligand-activation of luciferase activity (i.e., endogenous, functional ER and AhR) are designated (+) whereas those that do not are designated (−). ND, not determined.

FBS, which is optimal for cell growth, followed by a change to a culture medium with reduced levels of estrogenic compounds (IT medium) 48 h prior to plating the cells for transfection. Cells were then transfected with the ER reporter ERE-TATA-luc and, 24 h later, stimulated with E<sub>2</sub> (10 nM). E<sub>2</sub> activated the luciferase reporter 1.3-fold in cells plated in DMEM + 10% FBS, whereas a 5-fold increase in reporter activity was obtained in cells maintained in IT medium, with the difference being due to the lower basal reporter activity in IT medium (Fig. 1A).

3MC (100 µM) treatment of MCF-7 cells cultured in IT medium induced up to a 5-fold activation of the ER reporter ERE-TATA-luc, similar to the 6-fold activation seen in cells treated with E<sub>2</sub> (10 nM) in parallel (Fig. 1B). To confirm that this response was not limited to the artificial reporter ERE-TATA-luc, which contains isolated ER binding sites, we

Table 1  
qPCR primers and GenBank accession numbers

| Gene                            | Oligo numbers | GenBank   | Nucleotides  | Forward primer          | Reverse primer           |
|---------------------------------|---------------|-----------|--------------|-------------------------|--------------------------|
| <i>Rat</i>                      |               |           |              |                         |                          |
| Aryl hydrocarbon receptor (AhR) | 981/1416      | NM_013149 | 260 to 341   | GGGCCAAGAGCTTCTTTGATG   | GCAAATCTCTGCCAGTCTCTGA   |
| <i>Human</i>                    |               |           |              |                         |                          |
| Aryl hydrocarbon receptor (AhR) | 1499/1500     | NM_001621 | 744 to 799   | ATCCTTCCAAGCGGCATAGA    | GCTAGCCAAACGGTCCAACCTC   |
| Amphiregulin (AREG)             | 1366/1367     | NM_001657 | 439 to 541   | CGGGAGCCGACTATGACTACTC  | GGGCTTAACTACTGTTCAACTCTG |
| Cathepsin D                     | 1305/1306     | BC016320  | 898 to 948   | CTGTGCAAGGAGGGCTGTG     | CATGAGGGAAGTGCTGTGTGTC   |
| P450 1A1 (CYP1A1)               | 1307/1308     | NM_000499 | 276 to 328   | ATGCTGACCCCTGGGAAAGAAC  | TGCTGGCTCATCTTGACAG      |
| Estrogen receptor α (ERα)       | 1429/1430     | NM_000125 | 1612 to 1663 | GTAGAGGGCATGGTGGAGATCTT | CCGAGATGATGTAGCCAGCAG    |
| Progesterone receptor           | 1291/1292     | NM_000926 | 1742 to 1795 | TGGAGGCAGCAGTTCTAGTCC   | ACACTGTCCAGCAGTCCGCT     |

qPCR primers were designed as described in Materials and methods. Nucleotide numbering indicates the location of the corresponding qPCR amplicon based on the indicated GenBank accession number. The underlined 'A' in the rat AhR Reverse Primer indicates a single base-pair mismatch (see Materials and methods).

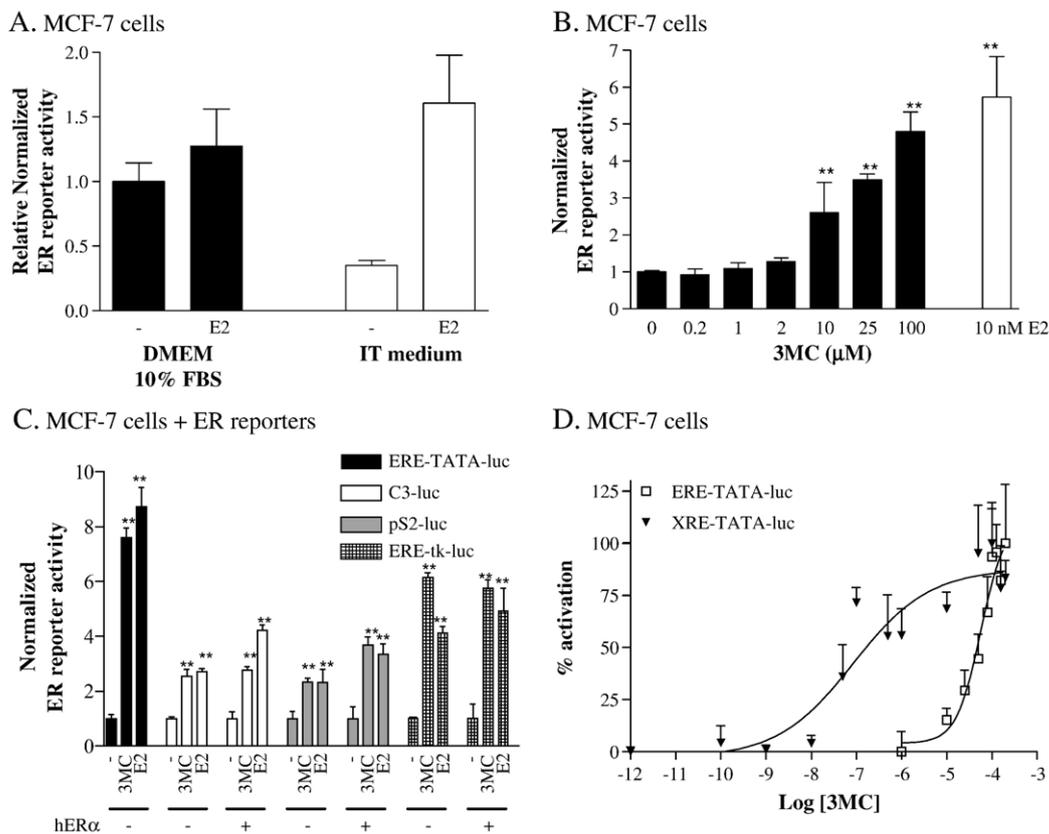


Fig. 1. 3MC activation of estrogen-responsive luciferase reporters in MCF-7 cells. MCF-7 cells were plated in 48-well plates in DMEM + 10% FBS (A, black bars) or in IT medium (A, white bars). Cells were transfected for 24 h with ERE-TATA-luc (A and B; C, black bars; and D, white squares), C3-luc (C, white bars), pGL3-pS2-luc (C, gray bars), ERE-tk-luc (C, hatched bars) or XRE-TATA-luc (D, inverted black triangles) (150 ng reporter plasmid/well), in the absence or presence of human ER $\alpha$  plasmid (50 ng) (as indicated in panel C) together with pRL-CMV as an internal control. Twenty-four hours after transfection, the cells were stimulated for 24 h with E<sub>2</sub> (10 nM), 3MC (100  $\mu$ M; A and C) or at the concentrations indicated (B and D). Cell lysates from triplicate wells were then prepared and assayed for luciferase activity. Activities are expressed as fold-activation of the firefly luciferase reporter compared with untreated cells (A–C), or as a percentage of maximal observed luciferase activity (D), after normalizing the activities of each sample for transfection efficiency, as indicated by the reporter activity of a *Renilla* luciferase internal standard (mean  $\pm$  SD values;  $n = 3$ ). Normalized activity presented in panel A is expressed relative to untreated cells plated in DMEM + 10% FBS. The concentration of 3MC required to elicit 50% of maximal ER transcriptional activity (EC<sub>50</sub>) was 57  $\mu$ M (squares), whereas the EC<sub>50</sub> for AhR transcriptional activity was 86 nM (inverted triangles) (D). Reporter activity was significantly different from that of untreated controls as indicated in panels B and C (\* $P < 0.05$ ) (\*\* $P < 0.01$ ).

assayed the ability of 3MC to stimulate ER transcriptional activity using three other ERE-regulated reporters. The reporter plasmid C3-luc contains the promoter of the human *complement 3* gene and pGL3-pS2-luc contains the promoter of the ER-responsive human *pS2* gene. A third reporter, ERE-tk-luc, contains two isolated EREs linked to a heterologous promoter. All three reporters were activated by 3MC, and by E<sub>2</sub>, although the overall increase in transcription was somewhat lower than that seen with ERE-TATA-luc (Fig. 1C). Little or no further increase in E<sub>2</sub>-stimulated reporter activity was obtained in cells transfected with an ER $\alpha$  expression plasmid, indicating that the endogenous ER $\alpha$  is sufficient to saturate the reporter plasmid.

Next, we investigated the dose–response for the estrogenic activity of 3MC. Comparison of the 3MC concentration-dependence of AhR and ER activation revealed that half-maximal activation of AhR reporter activity (XRE-TATA-Luc) was achieved at 86 nM, whereas half-maximal activation of the ER reporter occurred at 57  $\mu$ M 3MC, a 660-fold higher concentration (Fig. 1D).

#### ER $\alpha$ -dependence of 3MC-stimulated ER reporter activity

The Ishikawa cell line is an ER $\alpha$ -positive, estrogen-responsive human uterine tumor cell line (Nishida, 2002). An ER $\alpha$ -deficient Ishikawa cell sub-line (Table 2) is also available (Frigo et al., 2002a). These cell lines were used to investigate the ER $\alpha$ -dependence of 3MC-induced ERE-regulated transcription. E<sub>2</sub> stimulated an 83-fold increase in ER reporter activity in ER $\alpha$ -positive Ishikawa cells. By contrast, ER $\alpha$ -deficient Ishikawa cells were unresponsive to E<sub>2</sub> unless the cells were transfected with ER $\alpha$  (Fig. 2A). Thus, the endogenous level of ER $\beta$  expressed in these cells (Koda et al., 2004) is too low to support ER reporter activity. The AhR is expressed at similar levels in both Ishikawa cell lines (Table 2), which responded to 3MC by activation of an AhR-responsive reporter (Fig. 2B). Basal AhR firefly luciferase reporter activity was 3-fold higher in ER $\alpha$ (–) cells than in ER $\alpha$ (+) Ishikawa cells (data not shown), resulting in a lower fold-increase in normalized AhR reporter activity in the ER $\alpha$ (–) Ishikawa cells (Fig. 2B). ER $\alpha$ (+) Ishikawa cells were also highly responsive

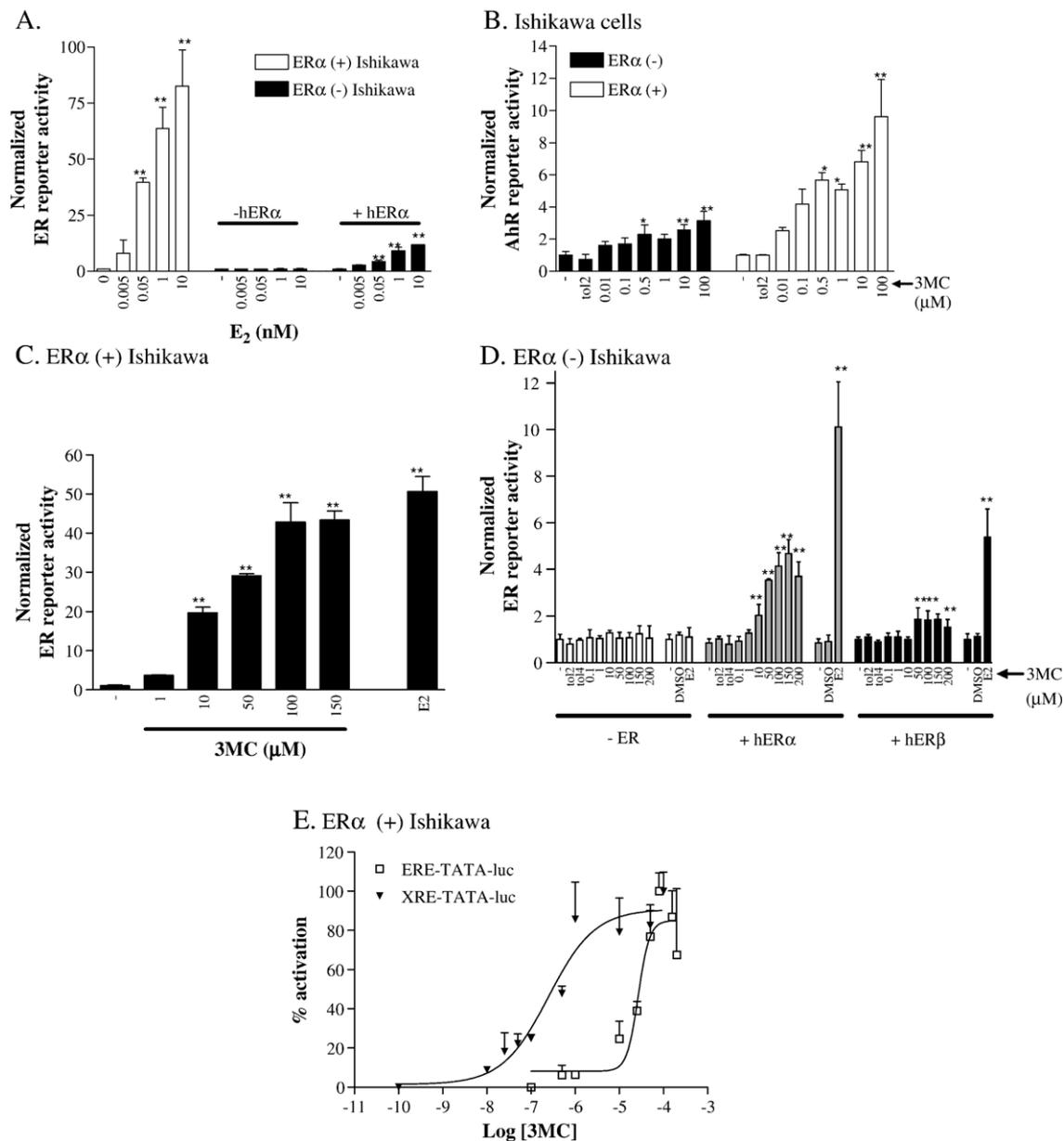


Fig. 2. ER-dependence of ERE and AhR reporter activity in Ishikawa cells. Ishikawa cells that express ERα (A and B, white bars; C and E) and ERα-deficient Ishikawa cells (A and B, black bars; and D) grown in 48-well plates were transfected for 24 h with the ERE-TATA-luc (150 ng/well) (A, C, D and E, open squares) or XRE-TATA-luc (20 ng/well) (B and E, inverted triangles) together with pRL-CMV as an internal control. ERα-deficient cells were cotransfected with an expression plasmid encoding human ERα or human ERβ (50 ng/well), as indicated. Twenty-four hours after transfection, the cells were stimulated for 24 h with 10 nM E<sub>2</sub>, or with 3MC and E<sub>2</sub> at the indicated concentrations. In D, DMSO, 2 μl toluene/ml culture medium (“tol2”) and 4 μl toluene/ml culture medium (“tol4”) were used as vehicle controls for E<sub>2</sub>, 3MC (≤100 μM) and 3MC (>100 μM), respectively, as indicated. Transfection of ERα(-) Ishikawa cells was carried out using FuGENE 6 together with either 83 μM (panel A) or 100 μM Hoechst dye 33258 (panel D) to increase transfection efficiency. Cell lysates from triplicate wells were assayed for luciferase activity. Activities are expressed as fold-activation of the firefly luciferase relative to untreated cells normalized by the reporter activity of a *Renilla* luciferase internal standard, ±SD values (n = 3). The concentration of 3MC required to elicit 50% of maximal ER transcriptional activity (EC<sub>50</sub>) in ERα(-) cells was 21 μM (data based on panel D) and was 27 μM in ERα(+) cells (E, white squares). The EC<sub>50</sub> for 3MC activation of ERα(+) Ishikawa cell AhR transcriptional activity was 250 nM (E, inverted black triangles). Reporter activity was significantly different from that of untreated controls as indicated in panels A–D (\*P < 0.05) (\*\*P < 0.01).

to 3MC, which induced a 20-fold increase in ER reporter activity at a concentration of 10 μM (Fig. 2C). Significant increases in ER activity were obtained at 3MC concentrations as low as 1 μM. In ERα(-) Ishikawa cells, ER reporter activity was not induced by 3MC or E<sub>2</sub>. However, following transfection of an ERα expression plasmid, 3MC induced ER reporter activity by ~5-fold, and with an EC<sub>50</sub> of 21 μM (cf., 10-fold increase in cells treated with E<sub>2</sub>) (Fig. 2D). A more

modest, 1.8-fold increase in ER reporter activity was stimulated by 3MC in ERα(-) Ishikawa cells transfected with ERβ (Fig. 2D). Similar to MCF-7 cells, much higher concentrations of 3MC were required to activate ER reporter activity (EC<sub>50</sub> = 27 μM) than to activate AhR reporter activity (EC<sub>50</sub> = 250 nM) in the ERα(+) Ishikawa cells (Fig. 2E). Finally, two other AhR ligands, BZ126 (Fig. 3A) and TCDD (Fig. 3B), did not induce ER reporter activity in ERα(+) Ishikawa cells under conditions

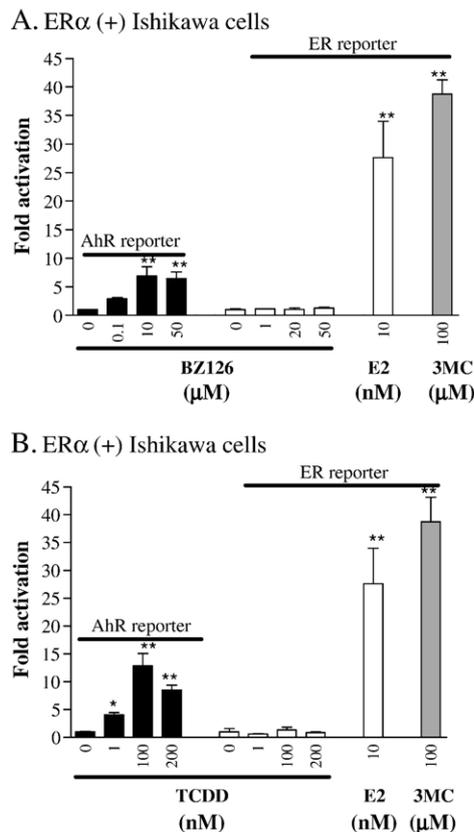


Fig. 3. TCDD and BZ126 do not activate the ER reporter activity in ER $\alpha$ -positive Ishikawa cells. Ishikawa cells that express ER $\alpha$  were transfected for 24 h with the ER reporter plasmid ERE-TATA-luc (white bars and gray bars) or the AhR reporter plasmid XRE-TATA-luc (150 ng/well of a 48-well plate) (black bars) together with pRL-CMV as an internal control. Twenty-four hours after transfection, cells were stimulated for 24 h with 3MC, BZ126, TCDD or E<sub>2</sub> at the concentrations indicated. Cell lysates from triplicate wells were then prepared and assayed for luciferase activity. Activities are expressed as fold-activation of the firefly luciferase relative to untreated cells normalized by the reporter activity of a *Renilla* luciferase internal standard,  $\pm$ SD values for  $n = 3$  replicates. The data presented are representative of four independent experiments. Reporter activity was significantly different from that of untreated controls as indicated (\* $P < 0.05$ ) (\*\* $P < 0.01$ ).

and at concentrations where induction of AhR reporter activity was maximal.

#### Effect of 3MC on ER target gene expression

Next, we investigated the effects of 3MC and E<sub>2</sub> on the expression of endogenous ER target genes in MCF-7 cells treated for times up to 48 h. qPCR was performed to monitor changes in expression of the established ER target genes *AREG*, *progesterone receptor* and *cathepsin D*. E<sub>2</sub> induced the expression of each of these genes, from 4.4-fold to ~350-fold at 48 h (Table 3 and Fig. 4). By contrast, more modest increases in gene expression were seen following 3MC-treatment. For example, 3MC (48 h) induced progesterone receptor RNA by ~2–3-fold, whereas E<sub>2</sub> stimulated a ~16-fold increase in expression of the same gene (Table 3), despite the similar fold-activation of ER reporter activity by E<sub>2</sub> and 3MC seen in parallel analyses (11–12-fold induction; data not

shown). Further studies revealed that 3MC activated the *AREG* gene ~7-fold after 12 h (Fig. 4A) and ~10-fold after 24 h (Fig. 4B), whereas three other estrogen-responsive genes (progesterone receptor, cathepsin D and pS2) failed to be activated by 3MC at these time points (data not shown). The induction of *AREG* expression was ER-dependent, as demonstrated by the inhibitory effects of the anti-estrogen ICI-182,780 on RNA induction by 3MC and E<sub>2</sub> (Fig. 4A). Interestingly, 3MC induction of *AREG* RNA decreased with time, to 2 to 4-fold at 48 h, whereas E<sub>2</sub> induction of *AREG* RNA continued to increase through the time period examined, reaching ~350-fold by 48 h (Fig. 4C). In studies carried out in ER $\alpha$ (+) Ishikawa cells, progesterone receptor RNA was induced 8–17-fold by E<sub>2</sub>; however, no induction was seen in cells treated with 3MC, TCDD or BZ126 (Fig. 4D). BZ126 also did not induce *AREG* RNA in MCF-7 cells under conditions (24 h treatment) where 3MC and E<sub>2</sub> were both active (data not shown), a finding that is consistent with the inactivity of this AhR ligand in ER reporter assays (Fig. 3A). As a positive control, we showed that 3MC induced the AhR-responsive CYP1A1 RNA, by ~60–80-fold, in MCF-7 cells (Table 3). Similarly, 3MC, BZ126 and TCDD induced CYP1A1 RNA ~20-fold in ER $\alpha$ (+) Ishikawa cells (Fig. 4E), whereas E<sub>2</sub> was without effect (Fig. 4E).

#### 3MC activates ER reporter activity in cells deficient in AhR

A key question is whether the ER $\alpha$ -dependent estrogenic responses that 3MC induces are mediated by AhR, as proposed in the AhR-ER ‘hijacking’ model (Ohtake et al., 2003), or whether other mechanisms are involved. To determine the role of the AhR in ER-mediated transcription, we used the rat hepatoma cell line 5L and its AhR-deficient variant, the BP8 cell line (Reiners et al., 1999). First, we

Table 3  
3MC activation of ER target gene expression in MCF-7 cells

| Treatment              | Progesterone receptor | Cathepsin D      | AREG              | CYP1A1  |
|------------------------|-----------------------|------------------|-------------------|---------|
| 3MC (1 μM)             | 2.9 ± 0.3 (12%)       | 1.2 ± 0.3 (6%)   | 0.9 ± 0.7 (0%)    | 26 ± 2  |
| 3MC (10 μM)            | 1.5 ± 0.7 (3%)        | 1.9 ± 0.1 (26%)  | 2.9 ± 0.8 (13%)   | 82 ± 11 |
| 3MC (100 μM)           | 2.2 ± 0.3 (8%)        | 1.9 ± 0.3 (26%)  | 4.9 ± 0.1 (27%)   | 62 ± 6  |
| E <sub>2</sub> (10 nM) | 16.5 ± 0.7 (100%)     | 4.4 ± 0.2 (100%) | 15.4 ± 2.2 (100%) | ≤1      |

MCF-7 cells cultured in IT medium were treated for 48 h with 3MC and E<sub>2</sub> at the concentrations indicated. Total RNA was isolated, reverse transcribed into cDNA and qPCR was performed to quantify RNA levels for *AREG*, cathepsin D, progesterone receptor and CYP1A1. Data are expressed as fold-increase in RNA level compared to toluene (vehicle)-treated cells for 3MC, or compared to untreated cells for E<sub>2</sub>. Data shown represent mean  $\pm$  half-the-range values for two independent RNA samples, each assayed in triplicate qPCR reactions. Values in parentheses are based on an average of the two samples, expressed as a percentage of the increase in expression observed for the E<sub>2</sub>-treated positive control (last line). For example, E<sub>2</sub> induced cathepsin D RNA by an average of 4.4-fold, corresponding to a 340% increase over the uninduced (basal) value, whereas 3MC (10 μM) increased this RNA 1.9-fold, or a 90% increase. The 3MC response is thus 26% (90/340) of the E<sub>2</sub>-induced increase.

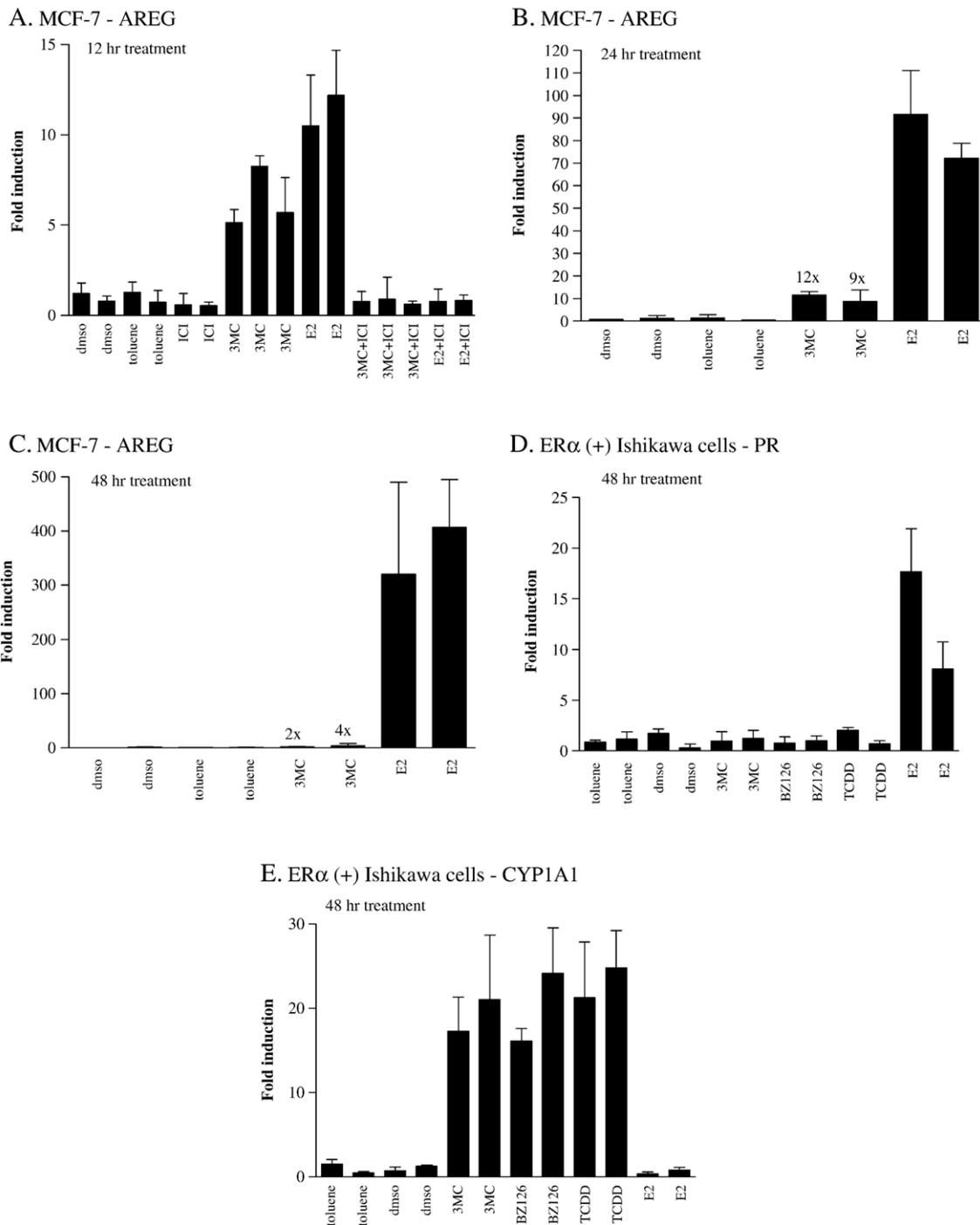


Fig. 4. Induction of ER target genes in AhR ligand-treated MCF-7 and ERα(+) Ishikawa cells. MCF-7 cells (A–C) and ERα(+) Ishikawa cells (D, E) were plated in 6-well plates and cultured in IT medium. Cells were treated for 48 h with 3MC (100 μM), BZ126 (10 μM), TCDD (100 nM), E<sub>2</sub> (10 nM) and/or the ER antagonist ICI-182,780 (1 μM), as indicated. DMSO was used as a vehicle control for BZ126, E<sub>2</sub> and ICI-182,780, and toluene was used as the vehicle control for 3MC and TCDD. Total RNA was isolated, reverse transcribed into cDNA and qPCR was performed to assay AREG (A–C), progesterone receptor (PR, D) and CYP1A1 RNA levels (E). Each bar represents a separate RNA preparation, each assayed in triplicate qPCR reactions (±SD). Data correspond to fold-increases in expression compared to vehicle (control)-treated cells (also see values above bars in panels B and C). Note y-axis scale differences for panels A–C. AREG RNA was not inducible by E<sub>2</sub> in Ishikawa cells (data not shown).

verified the AhR status of each cell line by transfection of the AhR reporter plasmid XRE-TATA-luc, to assay the responsiveness to 3MC. Both hepatoma lines proved very difficult to transfect. Transfection efficiency could be substantially improved, however, by including the DNA-binding dye Hoechst

33258 in the transfection mix (Fong et al., 2004). Under these conditions, 3MC (100 μM) increased XRE-TATA-luc reporter activity 25-fold in 5L cells, whereas no induction was seen in BP8 cells (Fig. 5A). Transfection of 5L cells with an expression plasmid for AhR increased AhR reporter activity

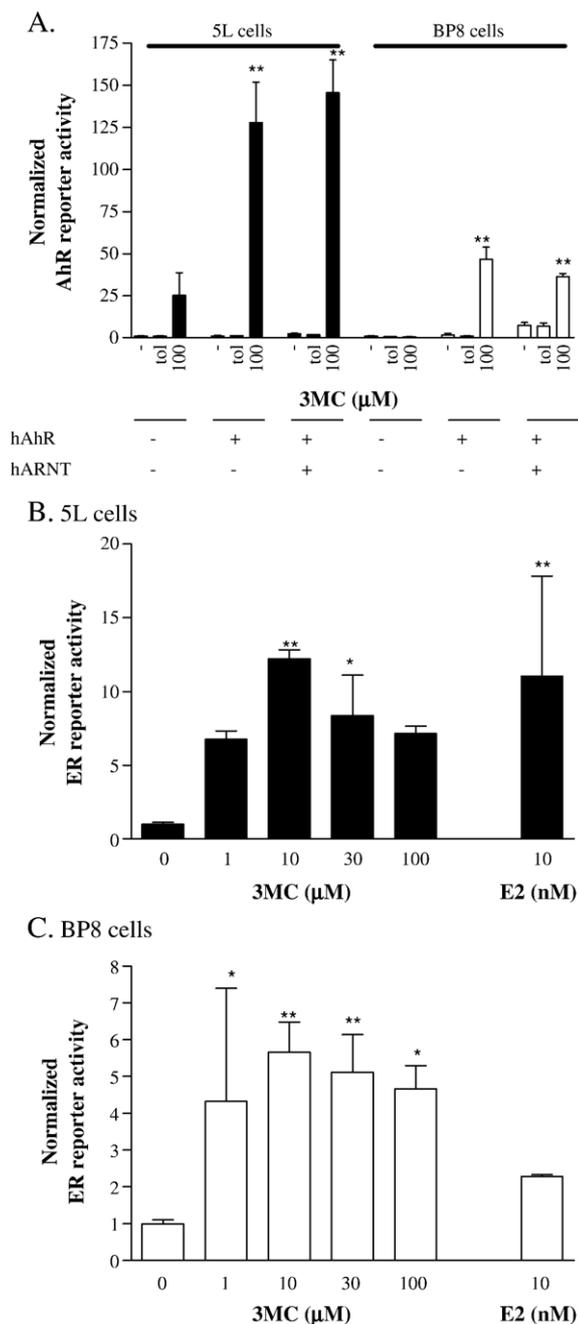


Fig. 5. AhR-dependence of AhR and ER reporter activity in 5L and BP8 hepatoma cells. 5L cells (black bars) and BP8 cells (white bars) seeded in 48-well plates were transfected for 24 h with XRE-TATA-luc (20 ng/well) alone or in combination with expression plasmids coding for human AhR (50 ng/well) and human ARNT (50 ng/well) (A) or ERE-TATA-luc in combination with an expression plasmid for human ER $\alpha$  (B and C), together with pRL-CMV as an internal control, as indicated. FuGENE 6 transfection reagent was used in combination with Hoechst 33258 to improve transfection efficiency as described under Materials and methods. Twenty-four hours after transfection, the cells were stimulated for 24 h with 3MC (100  $\mu$ M in panel A; other samples as indicated) or E<sub>2</sub>. Cell lysates from triplicate wells were assayed for luciferase activity. Activities are expressed as fold-activation of the firefly luciferase compared to untreated cells, after normalization to the reporter activity of a *Renilla* luciferase internal standard ( $\pm$ SD values for  $n = 3$  samples). Toluene ('tol') was used as the vehicle for 3MC (A). Reporter activity was significantly different from that of untreated controls as indicated (\* $P < 0.05$ ) (\*\* $P < 0.01$ ).

a further 5- to 6-fold, and in the case of BP8 cells, restored 3MC-inducible reporter activity. Cotransfection with an expression plasmid for ARNT, the heterodimerization partner of AhR, increased basal reporter activity in BP8 cells, but did not result in a further increase in AhR reporter activity (Fig. 5A). The AhR status of these cells was further confirmed by qPCR, which showed that AhR RNA was >100-fold higher in 5L cells than in BP8 cells (Table 2).

Next, we evaluated the effect of 3MC on ER reporter activity in both hepatoma cell lines. Given the apparent low expression of ER $\alpha$  (data not shown), 5L cells and BP8 cells were transfected with ER $\alpha$  in combination with ERE-TATA-luc reporter plasmid. Treatment of 5L cells with 3MC stimulated up to a 12-fold increase in ER reporter activity (Fig. 5B). Under these same conditions, a 5 to 6-fold increase in ER reporter activity was obtained in the AhR-negative BP8 cells (Fig. 5C). Thus, 3MC is able to activate ER-dependent gene expression, even in a cell line that is deficient in AhR and lacks functional AhR activity.

## Discussion

Many foreign chemicals exhibit estrogenic activity when assayed in cultured cells or animal models. In many cases, these xenoestrogens bind directly to ER $\alpha$ , or ER $\beta$ , to elicit estrogenic responses (Moggs and Orphanides, 2001; Mueller, 2004). Foreign chemicals may also exhibit anti-estrogenic activity, as shown previously for several ligands of AhR (Safe et al., 2000), which is an established mediator of the toxic and carcinogenic responses to TCDD and many polycyclic aromatic hydrocarbons. Recent studies have demonstrated, however, that AhR ligands can stimulate ER $\alpha$  and ER $\beta$  transcriptional activity by inducing formation of a complex between ER and ligand-bound AhR (Ohtake et al., 2003). This interaction was proposed to account for the increases in ER reporter activity and estrogen target gene expression seen in transfected cells and in ovariectomized mice, respectively. Notably, induction of these estrogenic responses did not occur in the absence of AhR or ER $\alpha$  (Ohtake et al., 2003).

In the present study, we confirmed that the AhR ligand 3MC can activate ER reporter activity in the human breast cancer cell line MCF-7, which expresses AhR (Abdelrahim et al., 2003), ER $\alpha$  (Freddie et al., 2004) and ER $\beta$  (Power and Thompson, 2003). This estrogenic response was observed with ER reporters containing isolated EREs, and with reporters containing EREs in the context of their natural, estrogen-responsive promoters. However, although 3MC treatment activated ER reporter activity, the concentration of 3MC required to induce a response ( $EC_{50} = 57 \mu$ M) was far greater than that required to bind and activate human AhR ( $K_d = 5$  nM) (Harper et al., 1991) or to stimulate AhR reporter activity ( $EC_{50} = 86$  nM) (Fig. 1). A similar result was obtained in ER $\alpha$ (+) Ishikawa cells, where the concentration of 3MC required to activate ER-regulated transcription ( $EC_{50} = 27 \mu$ M) was  $\sim$ 100-fold higher than that required to stimulate AhR reporter activity ( $EC_{50} = 250$  nM) (Fig. 2). The poor correlation between the concentration-dependence of 3MC activation of the AhR vs. activation of ER

transcriptional activity could indicate that efficient formation of a functional, ERE-interacting AhR-ER complex requires unusually high concentrations of 3MC. Alternatively, the ERE-dependent transcriptional activity seen in the present study might not involve direct binding of 3MC to AhR. Rather, it may proceed by an AhR-independent mechanism, e.g., direct binding of 3MC, or one of its metabolites, to ER.

Our finding that 3MC can activate ER-dependent transcription from the *Complement 3* and *pS2* promoters raised the possibility that 3MC may up-regulate these and other endogenous ER target genes. To investigate this possibility, qPCR was used to quantify the expression of ER target genes in 3MC-treated MCF-7 cells and Ishikawa cells. 3MC increased the expression of several estrogen-responsive genes in MCF-7 cells, but in general, the RNA increases obtained were substantially lower than those induced by E<sub>2</sub>. This finding is in contrast to the very similar fold-increases in ER reporter activity that were induced by 3MC and E<sub>2</sub> in the same cell model. A time-course study in MCF-7 cells showed that expression of the *AREG* gene was induced ~5–10-fold by 12 h after stimulation with 3MC and E<sub>2</sub>, with the ER-dependence of both responses verified using the ER antagonist ICI-182,780. Interestingly, whereas the E<sub>2</sub>-induced *AREG* response progressively increased with time, to ~350-fold at 48 h, the 3MC-stimulated increase was maximal at 24 h and decreased thereafter. Moreover, in ER $\alpha$ (+) Ishikawa cells, no increase in expression of the E<sub>2</sub>-responsive *progesterone receptor* gene was seen following stimulation with 3MC, BZ126 or TCDD, despite the strong activation of an ER reporter by 3MC ( $\geq 40$ -fold) seen in the same cell line. Discrepancies between ER reporter activation and endogenous ER-regulated gene transcription have been noted previously. For example, in a study characterizing the estrogenicity of a series of polycyclic aromatic hydrocarbons, five compounds showing activity in an ER reporter assay failed to activate endogenous E<sub>2</sub> responsive genes, as assessed by qPCR (Gozgit et al., 2004). Differences between reporter gene assays and endogenous target gene expression may be explained by several factors. For example, the introduction of multiple copies of the transfected ER reporter plasmid may increase the sensitivity of gene expression to levels of ER that are too low to activate a much lower copy number endogenous ER target gene. Alternatively, the conformational changes in ER that are induced in 3MC-treated cells may be sufficient for efficient binding to transfected reporter plasmid DNA, but not to ER target genes in their native chromatin structure. Finally, when activated by 3MC or other ligands of the AhR, ER may be unable to recruit essential coactivator proteins or remove repressor proteins from native ER target gene promoters.

AhR and ER $\alpha$  can both bind to the promoters of the ER target genes *pS2* and *c-fos* in MCF-7 cells, as revealed by chromatin immunoprecipitation analysis. Moreover, sequential immunoprecipitation has shown that AhR binding to the *c-fos* promoter is dependent on ER $\alpha$  and, therefore, is not likely to proceed *via* a direct AhR-XRE binding mechanism (Ohtake et al., 2003). In that same study, 3MC induction of the ER target genes *pS2* and *c-fos* was demonstrated, but analogous to the qPCR data

presented in the present study, the increase in expression was very low compared to the increase in response to E<sub>2</sub> (Ohtake et al., 2003).

The Ishikawa human endometrial cancer cell line (Nishida, 2002) expresses AhR, Arnt, ER $\alpha$  and ER $\beta$  and has been used to characterize the anti-estrogenic effects of certain AhR ligands (Wormke et al., 2000). A variant of this cell line that is deficient in ER $\alpha$  expression has been described (Frigo et al., 2002a) and was used in the present study to investigate the ER $\alpha$  dependence of the estrogenic activity of 3MC. ER $\alpha$ (-) Ishikawa cells were unresponsive to E<sub>2</sub>, despite their expression of ER $\beta$ , which may reflect the low intrinsic transcriptional activity of ER $\beta$  compared to ER $\alpha$  (Ramsey et al., 2004). 3MC strongly induced ERE-mediated transcription in ER $\alpha$ (+) Ishikawa cells, reaching 20- to 40-fold at 10–100  $\mu$ M. By contrast, ER $\alpha$ (-) Ishikawa cells were unresponsive to 3MC unless ER $\alpha$  was introduced into the cells by transfection, thus establishing the ER $\alpha$ -dependence of the 3MC response. Transfection of ER $\beta$  also resulted in a 3MC-stimulated increase in transcription, albeit at a lower level than was achieved in cells transfected with ER $\alpha$ .

The ability of AhR ligands to activate ER-regulated transcription by AhR ‘hijacking’ of ER (Ohtake et al., 2003) has raised the possibility that at least some of the myriad of chemicals classified as xenoestrogens may actually be acting as AhR ligands whose estrogenic responses are mediated by an AhR-ER complex. In our studies, 3MC, but not two other AhR ligands, BZ126 and TCDD, stimulated ER transcriptional responses. To investigate the role that the AhR plays in the responses to 3MC, we used the rat hepatoma cell line 5L and its AhR-deficient variant BP8 (Weiss et al., 1996; Reiniers et al., 1999). BP8 cells are insensitive to the potent AhR ligand TCDD, and were originally isolated by selection in benzo(a)-pyrene-containing growth medium (Wiebel et al., 1991). The absence of functional AhR in BP8 cells was demonstrated by the lack of binding of (<sup>3</sup>H)TCDD by cytosolic extracts (Gottlicher et al., 1990), by the absence of AhR mRNA on Northern blots and by the lack of TCDD-inducible XRE-dependent reporter activity (Weiss et al., 1996). These cell lines proved very difficult to transfect, requiring that we use the DNA-binding dye Hoechst 33258 (Fong et al., 2004) to improve the transfection efficiency. The AhR status of these cells was confirmed using functional, AhR reporter assays and by qPCR analysis of AhR RNA. Using this cell model, we observed 3MC-induced, ER-regulated transcription even in the cells deficient in AhR. This finding supports the hypothesis that 3MC, or perhaps a metabolite of 3MC, serves as a weak agonist that binds to and activates ER directly. Recent studies have demonstrated that another AhR ligand, 6-methyl-1,3,8-trichlorodibenzofuran (MCDF), can also activate ER reporter activity. MCDF induced ERE-luciferase activity and increased E<sub>2</sub>-responsive transforming growth factor- $\alpha$  RNA expression in an ER-dependent manner (Pearce et al., 2004). Partial knockdown of AhR expression in MCF-7 cells using siRNA (Abdelrahim et al., 2003) did not affect inducibility of ER activity by E<sub>2</sub> or MCDF, suggesting a mechanism in which MCDF serves as a partial agonist of ER (Pearce et al., 2004).

Other AhR agonists that exhibit estrogenic activity include certain polycyclic aromatic hydrocarbons (Gozgit et al., 2004) and indolo(3,2-*b*)-carbazole (Liu et al., 1994). However, two other AhR agonists investigated in the present study, TCDD and BZ126, failed to activate ER reporter activity or endogenous ER-stimulated gene expression. Thus, some, but not all, AhR agonists are able to activate ER and induce estrogenic responses. Further studies are required to define the structural features required for induction of ER-dependent responses by certain AhR ligands.

In conclusion, 3MC, but not two other AhR ligands, activated ER $\alpha$ -dependent, and ERE-directed reporter gene expression. 3MC also effected a modest increase in ER target gene expression in cultured cells, albeit at much higher concentrations than were needed to activate the AhR. Moreover, using a hepatoma cell model, 3MC was shown to activate ER-regulated transcription even in the absence of AhR. Together, these findings indicate that 3MC has the potential to induce estrogenic responses by an AhR-independent mechanism. This conclusion contrasts with that of a previous study, where an AhR-hijacking mechanism was proposed to account for 3MC-stimulated estrogenic responses (Ohtake et al., 2003). Further studies are needed to ascertain whether 3MC and other AhR ligands may, in part, act as agonists of ER $\alpha$ , in addition to any indirect, AhR-dependent mechanisms of action they may exert, and to elucidate the biological and toxicological significance of their estrogenic activities.

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