AC, Santa Cruz) for immunoprecipitation; a monoclonal antibody against haemagglutinin A (HA; 1867423, Roche), a polyclonal antibody against Myc (SC789, Santa Cruz), and antibodies against phosphylated (Ser 473) or total Akt (9270, New England Biolabs).

Statistical analysis

Results shown are the mean ± s.d. We analysed data by one-way analysis of variance (ANOVA). Individual statistical differences were determined by Scheffe's multiple range comparison test.

Accession numbers

The sequences of mouse and human ERas can be retrieved from DDBJ/GenBank/EMBL with accession numbers AB093573 and AB093575.

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Modulation of oestrogen receptor signalling by association with the activated dioxin receptor

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Environmental contaminants affect a wide variety of biological events in many species. Dioxins are typical environmental contaminants that exert adverse oestrogen-related effects[1]. Although their anti-oestrogenic actions[2,3] are well described, dioxins can also induce endometriosis[4–7] and oestrogen-dependent tumours[8,9], implying possible oestrogenic effects. However, the molecular mechanism underlying oestrogen-related actions of dioxins remains largely unknown. A heterodimer of the dioxin receptor (AhR) and Arnt, which are basic helix–loop–helix/PAS-family transcription factors, mediates most of the toxic effects of dioxins[10,11]. Here we show that the agonist-activated AhR/Arnt heterodimer directly associates with oestrogen receptors ER-α and ER-β. This association results in the recruitment of unliganded ER and the co-activator p300 to oestrogen-responsive gene promoters, leading to activation of transcription and oestrogenic effects. The function of liganded ER is attenuated. Oestrogenic actions of AhR agonists were detected in wild-type ovariectomized mouse uteri, but were absent in $AhR^{-/-}$ or ER-$\alpha^{-/-}$ ovariectomized mice. Our findings suggest a novel mechanism by which ER-mediated oestrogen signalling is modulated by a co-regulatory-like function of activated AhR/Arnt, giving rise to adverse oestrogen-related actions of dioxin-type environmental contaminants.

ERs, which are members of the nuclear receptor (NR) family[12,13], and AhR/Arnt are both ligand-dependent transcription factors. Ligand-activated AhR heterodimerizes with Arnt and activates the transcription of dioxin target genes such as *CYP1A1* (refs 10,11) through xenobiotic response elements (XREs). ERs bind to oestrogen response elements (EREs) and activate transcription in an oestrogen-dependent manner. This transcriptional activation

requires the recruitment of co-activator complexes[13–18], including histone acetyltransferase (HAT) complexes containing p300 and CREB binding protein (CBP). In view of previous reports that AhR ligands exhibit oestrogen-related adverse effects, it is possible that ER-mediated oestrogen signalling might cross-talk with AhR-mediated signalling through an unknown mechanism that regulates transcription. We therefore decided to examine whether AhR/Arnt heterodimer could transcriptionally affect ER transactivation functions, thereby modulating oestrogen signalling.

To monitor the transactivation function of endogenous receptors, luciferase reporter plasmids bearing consensus binding elements—ERE for ERs, and XRE for AhR/Arnt—were transfected into MCF-7 cells, a breast cancer cell line known to express both receptors endogenously[2]. Although the synthetic AhR ligand 3-methylcholanthrene (3MC) effectively activated transcription through XRE[10], 17β-estradiol (E2) did not, as expected (Fig. 1a). However, to our surprise, 3MC alone activated ERE-mediated transcription in the absence of E2 (Fig. 1a). In the presence of E2, ERE-mediated transcription was decreased by the addition of 3MC. Western blotting showed that the amount of ligand-induced transactivation did not simply reflect variations in receptor numbers (Fig. 1a). 3MC alone decreased AhR and ER-α protein levels, in agreement with previous reports[19].

We then examined the effect of AhR/Arnt on ER-mediated transcription by using exogenous receptors in Ishikawa cells, a uterine tumour cell line. Again, 3MC potently stimulated ERE-mediated transcription in the absence of E2 when both ER (either ER-α or ER-β) and AhR/Arnt were expressed, whereas it lowered the E2-induced transactivation function of ERs (Fig. 1b) without binding directly to ERs (Supplementary Fig. 1a) or affecting expression levels of ERs (data not shown). This activation effect of 3MC requires ERE (Fig. 1b, lanes 1–4), ER-α (lanes 7 and 8), AhR (lanes 9 and 10) and Arnt (lanes 11 and 12). To verify that an AhR ligand does indeed exert oestrogenic action through direct binding to AhR, other AhR ligands were further tested. More stable ligands such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), benzo[*a*]-pyrene and β-naphthoflavone acted as agonists, like 3MC (Fig. 1c), whereas the oestrogenic action of 3MC was blocked by either a known AhR antagonist, α-naphthoflavone or a pure oestrogen antagonist, ICI182,780 (Fig. 1d). The modulation of transcription activity by AhR/Arnt observed with ERs was not detected on other NRs including glucocorticoid receptor, progesterone receptor, vitamin D receptor (VDR), retinoic acid receptor and peroxisome proliferator activated receptor-γ (PPAR-γ) (data not shown).

Because ERs possess two transactivation functions, AF-1 and AF-2, in the amino-terminal A/B and carboxy-terminal E/F regions, respectively[16,20], we examined the functional association of AhR/Arnt with these two regions using ER deletion mutants (HE15 for AF-1 domain, and HE19 for AF-2 domain) (Supplementary Fig. 1b) in Ishikawa cells. The N-terminal A/B regions of ER-α and ER-β were required for stimulation of ERE-mediated transcription by AhR/Arnt, whereas we detected no modulation of AF-2 functions (Fig. 1e)[20]. Thus, 3MC-bound AhR/Arnt might modulate the functions of ERs through association with the N-terminal A/B regions. This possibility was supported by the observation that

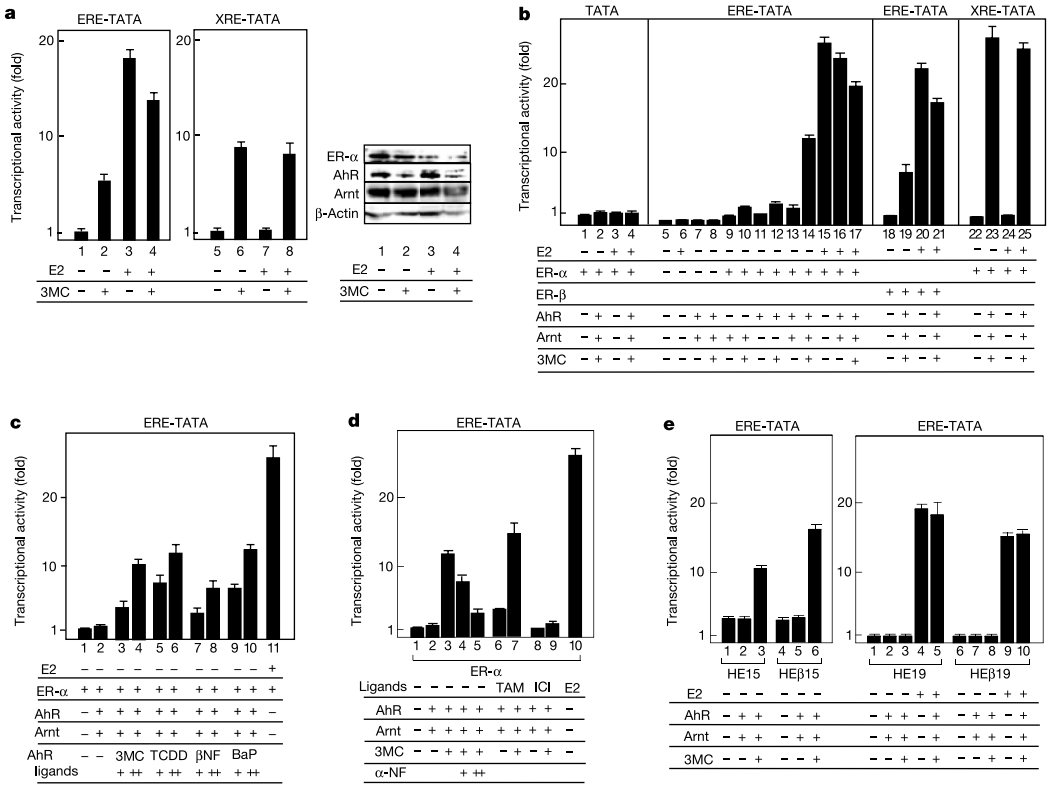

Figure 1 Activation of unliganded ER function by liganded dioxin receptor heterodimer. **a**, A dioxin receptor ligand activates transcription mediated through an ERE. MCF-7 cells were transfected with the reporter plasmids ERE-luciferase or XRE-luciferase in the presence or absence of E2 (10 nM) and 3MC (1 μM). Luciferase assays were performed with the cell extracts. All values are means ± s.d. for at least three independent experiments. **b**, Liganded AhR/Arnt induces the transactivation function of ERE-bound unliganded ER. Ishikawa cells transfected with the indicated plasmids were subjected to luciferase assays. **c**, Transactivation of unliganded ER by the other AhR agonists. **d**, Potentiation of ERE-mediated transcription by liganded AhR/Arnt is blocked by an antagonist for either ER-α or AhR. Cells treated with tamoxifen (TAM; 100 nM), ICI182,780 (ICI; 100 nM), 3MC (+, 100 nM; ++, 1 μM), TCDD (+, 10 nM; ++, 100 nM), β-naphthoflavone (β-NF; +, 100 nM; ++, 1 μM), benzo[*a*]pyrene (BaP; +, 10 nM; ++, 100 nM), α-naphthoflavone (α-NF; +, 100 nM; ++,1 μM). **e**, Potentiation of ERE-mediated transcription by AhR/Arnt is mediated by the ERs A/B regions.

3MC-bound AhR/Arnt potentiates the transactivation function of ER-α in the presence of the ER-α AF-1 agonist/AF-2 antagonist tamoxifen (Fig. 1d)[16].

We then tested whether a 3MC-dependent physical interaction occurred between AhR/Arnt and ERs. Irrespective of E2 binding, endogenous ER-α in MCF-7 cells, and tagged ER-α overexpressed in COS-1 cells, were found to co-immunoprecipitate with 3MC-bound AhR, but not with unliganded AhR, only when Arnt was co-expressed (Fig. 2a and b). In agreement with the functional interaction between AhR/Arnt and the A/B region of ER-α (Fig. 1e), a 3MC-dependent interaction between AhR/Arnt and HE15 was observed, but not between AhR/Arnt and HE19 (ref. 12). Although ER-β, like ER-α, also associated with AhR in a 3MC-dependent fashion, no other receptors tested showed such an association (Fig. 2b).

Moreover, a direct interaction between AhR, but not Arnt, and A/B regions from both ER-α and ER-β could be mapped by an *in vitro* glutathione *S*-transferase (GST) pull-down assay (Fig. 2c). It therefore seems that, upon ligand binding and nuclear translocation[10], AhR heterodimerizes with nuclear Arnt and then associates with unliganded ER-α or ER-β, which are constitutively in the nucleus[16], through direct interaction with their A/B regions. Further analyses by GST pull-down assay mapped the small regions of the A/B region of ER-α (residues 40–120), the A/B region of ER-β (residues 33–55)[21], and the helix–loop–helix/PAS domain of AhR[22], which are indispensable for direct interaction *in vitro* (Fig. 2d and Supplementary Fig. 2a). An ER-α mutant lacking the AhR-interacting region (ER-α ΔAhR) failed to be activated by AhR/Arnt but responsiveness to E2 was still retained, supporting the idea that the interaction is required for AhR ligand-induced activation of the ER function (Fig. 2e).

To explore the molecular mechanisms of the 3MC-dependent transactivation function of AhR and ERs, we used co-immunoprecipitation to examine whether p300 was recruited to the complex, because both AhR and ERs have been independently reported to require p300/CRB as a co-activator[10,16,18,23]. p300 was recruited to ER-α in the presence but not the absence of E2 (Fig. 2f, lanes 2 and 4). However, even in the absence of E2, p300 associated with 3MC-bound AhR/Arnt and unliganded ER-α to form a complex (Fig. 2f, lane 3). Recruitment of the p160 family co-activator SRC-1 (ref. 13; Fig. 2f, lane 3), TIF2 or AIB1 (data not shown) to AhR/Arnt-associated ERs were not detected. Thus, the co-activator complex required to activate transcription by the unliganded ERs associated with liganded AhR/Arnt might be distinct from both co-activator

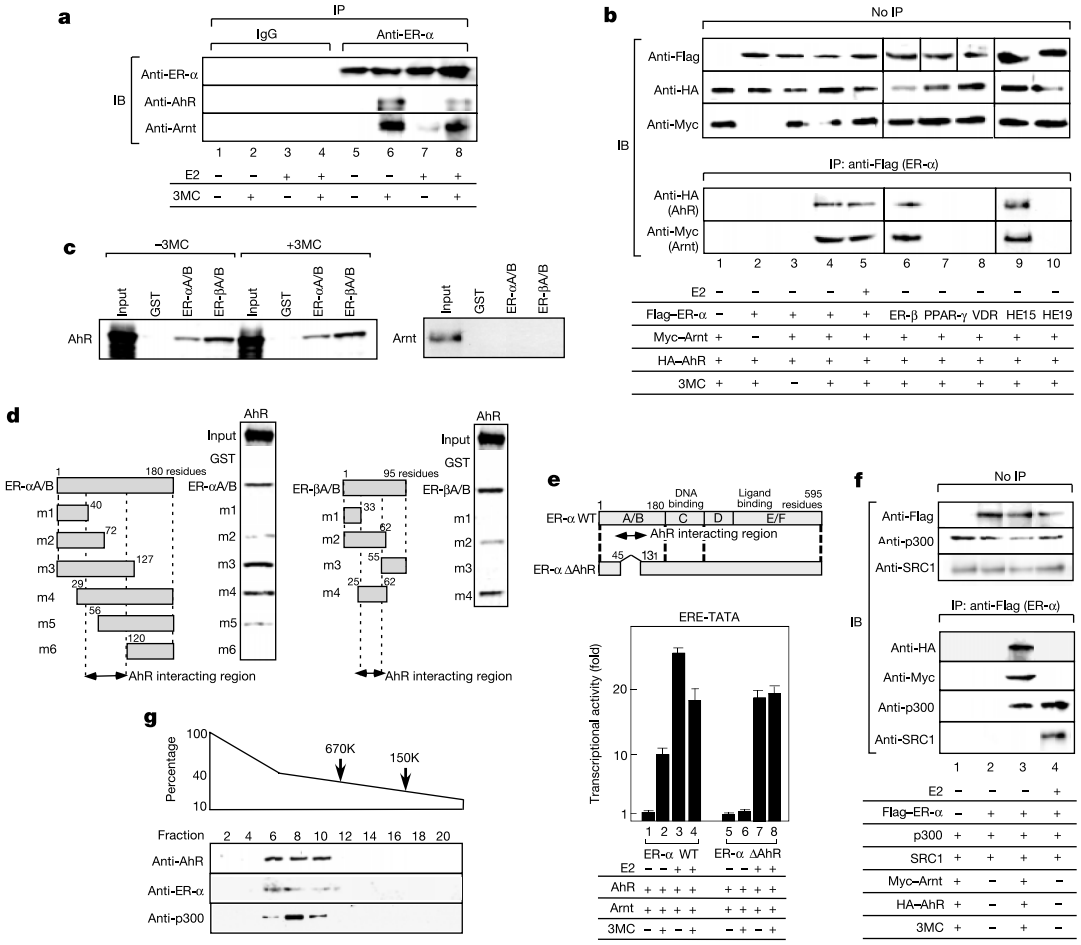

Figure 2 3MC-dependent interaction of ERs with AhR/Arnt. **a**, 3MC-dependent but E2-independent interaction of endogenous ER-α with AhR/Arnt in MCF-7 cells. Cells were subjected to immunoprecipitation (IP) with mouse anti-ER-α or normal mouse immunoglobulin as a control. The immunoprecipitates were western blotted (IB) with specific antibodies as indicated. **b**, E2-independent, 3MC-dependent interaction of exogenous ERs with AhR/Arnt in COS-1 cells. The transfected cells were subjected to immunoprecipitation and then western blotting. PPAR, peroxisomeproliferatoractivated receptor; VDR, vitamin D receptor. **c**, Direct but 3MC-independent interaction of AhR with ER-α and ER-β in an *in vitro* GST pull-down assay. **d**, Mapping the interaction domains of ER-α and ER-β with AhR. **e**, The AhR-interacting core region in the ER-α A/B domain is required for ER-α activation by AhR/Arnt. Luciferase assays with the indicated ER derivative. **f**, Recruitment of p300 co-activator to a complex containing unliganded ER-α and 3MC-bound AhR/Arnt. **g**, AhR/ER–α/p300 form a complex on glycerol gradient analysis. The Flag–AhR associated proteins in stable transformant HeLa cells were fractionated by molecular mass by a glycerol gradient assay.

complexes for the unassociated receptors. Indeed, ER-α and p300 were detected in the same fractions as Flag ($[EYKEEEK]_2$)-tagged AhR fractionated by a glycerol gradient, suggesting that they form a complex with a relative molecular mass (M_r) larger than 670,000 (670K) (Fig. 2g).

To investigate whether the observed association between AhR and ERs occurred on EREs in endogenous target gene promoters of MCF-7 cells, we performed a chromatin immunoprecipitation (ChIP) analysis with *pS2* and c-*fos* gene promoters[17]. Interestingly, 3MC induced binding of ER-α to ERE, as did E2, with AhR/Arnt recruitment. As expected, 3MC induced the recruitment of AhR/Arnt, but not ER-α, to the *CYP1A1* promoter XRE (Fig. 3a). Reflecting the recruitment of the receptors, acetylation of histone H4 was observed in the promoters (Fig. 3a), indicating the possible recruitment of a HAT co-activator complex to the receptors. The expression of these genes was accordingly induced by 3MC or E2 (Fig. 3d). Thus, the 3MC-dependent association between AhR/Arnt and ER-α seems to promote the binding of unliganded ER-α to EREs.

A ChIP assay involving sequential immunoprecipitation confirmed the 3MC-dependent association of AhR/Arnt with ER-α on

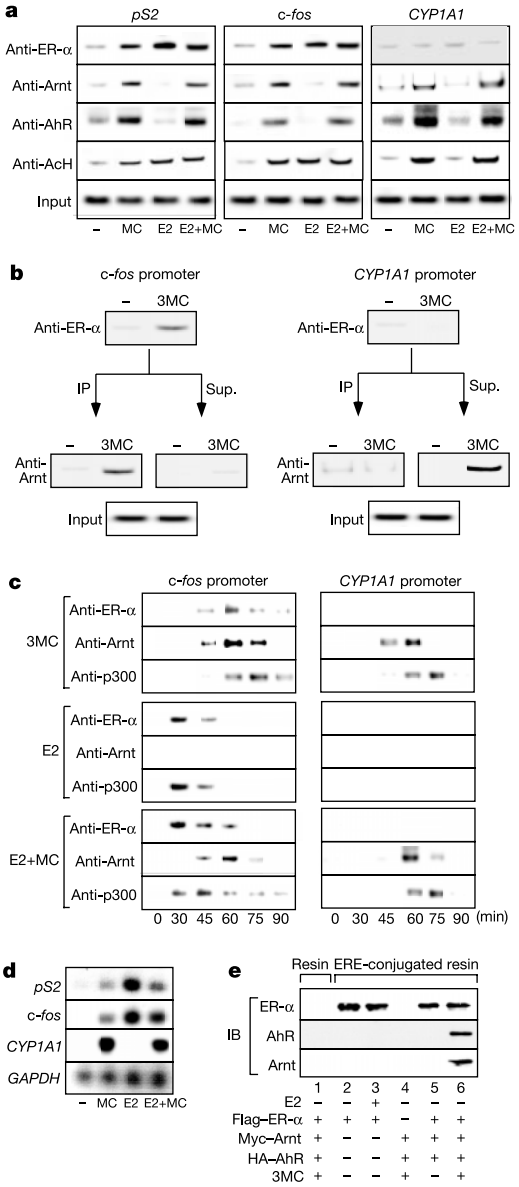

Figure 3 3MC-dependent recruitment of AhR/Arnt to ER-α bound on oestrogen-responsive gene promoters. **a**, 3MC-dependent interaction with AhR/Arnt induces ERE binding of unliganded ER-α to E2 responsive gene promoters in MCF-7 cells. For ChIP analyses, soluble chromatin prepared from MCF-7 cells treated with ligands for 45 min was immunoprecipitated with the indicated antibodies. The final DNA extracts were amplified using specific sets of primer pairs to detect the c-*fos*, *pS2* and *CYP1A1* gene promoters as indicated. **b**, 3MC-dependent association of AhR/Arnt with ER-α bound to E2-responsive gene promoters. The immunoprecipitates and their supernatants were sequentially applied for ChIP analysis as indicated. **c**, Dynamics of ER-α–Arnt–p300 assembly on ligand-responsive gene promoters. Occupancy of the c-*fos* and *CYP1A1* promoters by ER-α, Arnt and p300 at different times after ligand treatments. **d**, Induction of target genes examined by northern blot analysis. **e**, Complex formation of AhR–Arnt–ER-α on ERE through ER-α as revealed by ABCD assay.

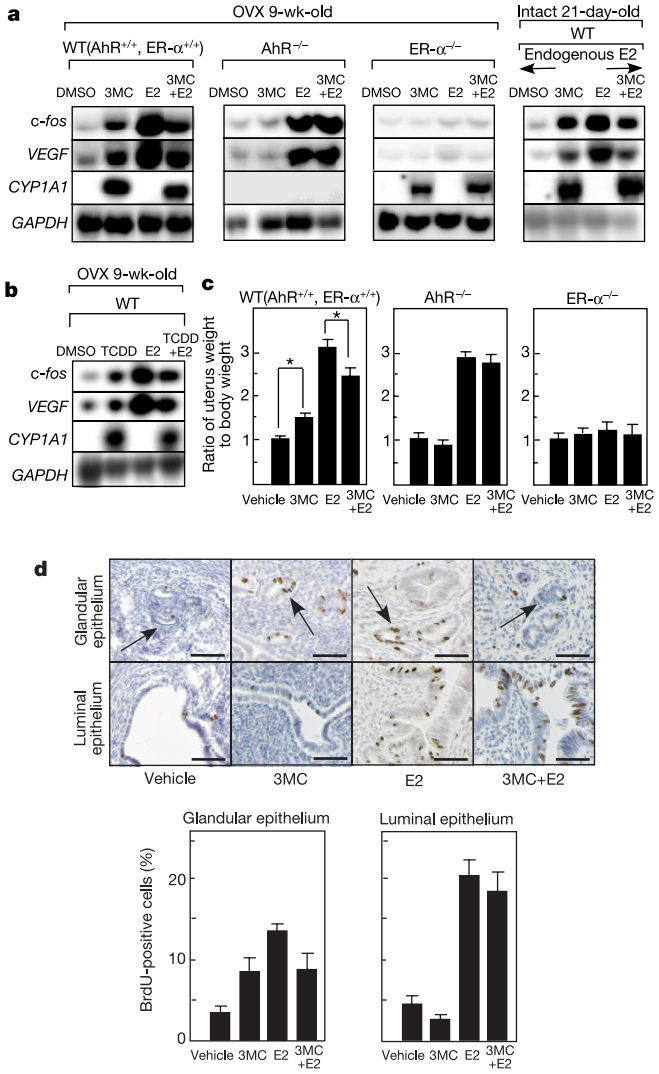

Figure 4 Oestrogenic actions of 3MC in mouse uterus are mediated by AhR and ER-α. **a**, **b**, Induction of E2-responsive genes by AhR agonists is mediated by both AhR and ER-α. Nine-week-old ovariectomized (OVX) mice and intact 21-day-old female mice of the indicated genotypes were injected with the ligands. Three hours later, total RNA was extracted from the uterus, then subjected to northern blot analysis with cDNAs for the target genes for E2 (c-*fos*, *VEGF*) and for 3MC (*CYP1A1*); *GAPDH* cDNA was used as an internal control. WT, wild type. **c**, The 3MC-induced increase in uterine wet weight (measured as the ratio of uterine wet weight in milligrams to body weight in grams) in ovariectomized mice was abolished by inactivation by either AhR or ER-α. The *t*-test shows a significant difference ($P < 0.01$) between 3MC-treated ($n = 9$) and olive-oil-treated ($n = 9$) wild-type mice. There is no significant difference ($P > 0.2$) between 3MC-treated ($n = 4$) and olive-oil-treated ($n = 4$) animals in either $AhR^{-/-}$ and $ER\text{-}\alpha^{-/-}$ mice. All values are means ± s.e.m. **d**, Induction of endometrial cell proliferation by 3MC and E2. BrdU-positive cells (brown) are indicated by arrows.

ERE (Fig. 3b). A time-course ChIP assay showed that ER-α, AhR and p300 HAT were simultaneously recruited to the c-*fos* promoter, presumably upon the binding of 3MC to AhR (Fig. 3c).

To verify the interaction of AhR/Arnt with ER-α bound to ERE in the promoters, the formation of a complex with ERE was tested by avidin–biotin-conjugated DNA(ERE) (ABCD) precipitation[24] (Fig. 3e). ER-α bound to consensus ERE (Fig. 3e, lanes 2, 3, 5 and 6), whereas AhR/Arnt alone did not (Fig. 3e, lane 4). However, in the presence of ER-α, AhR/Arnt was recruited to ERE in a 3MC-dependent manner (Fig. 3e, lanes 5 and 6). In the transient luciferase assay, the binding of ER-α to ERE and the activation function of both AhR and Arnt were required for the activation of ER-α through ERE by AhR/Arnt (Supplementary Fig. 3a, lanes 3, 7, and 8), whereas the AF-1 and AF-2 activities of ER-α and the DNA-binding capacity of the AhR/Arnt heterodimer were dispensable (Supplementary Fig. 3a, lanes 4–6).

Finally, we tested whether AhR-ligand-dependent AhR–ER interaction was responsible for the oestrogenic actions of AhR agonists in the absence of oestrogens on gene expression in intact animals. In addition to the induction of the *CYP1A1* gene, treatments with 3MC (Fig. 4a) and TCDD (Fig. 4b) for 3 hours stimulated the expression of the oestrogen-responsive genes c-*fos*[25] and *vascular endothelial growth factor* (*VEGF*)[26] in the uteri of ovariectomized wild-type mice (Fig. 4a, b). This oestrogenic action of 3MC in the uterus was also detected in intact 21-day-old female mice, whereas the AhR agonists exhibited anti-oestrogenic activities in the presence of high doses of oestrogen (Fig. 4a). There have been conflicting reports on the induction of c-*fos* by AhR ligands: one is that AhR ligands repress the E2-induced expression of c-*fos*[3]; the other is that AhR ligands themselves induce the expression of c-*fos*[27]. The 3MC-mediated activation of oestrogen target genes was completely abolished in both AhR$^{-/-}$ (ref. 28) and ER-α$^{-/-}$ (ref. 29) ovariectomized mice, although each receptor knockout mouse strain retained ligand responsiveness (Fig. 4a) and the expression (Supplementary Fig. 4a) of the other intact receptor. The injection of 3MC led to increases in uterine wet weight, as did that of E2 (Fig. 4c). This action of 3MC was again abolished in both AhR$^{-/-}$ and ER-α$^{-/-}$ mice (Fig. 4c).

To examine whether the increased uterine wet weight was due to the proliferation of endometrial cells, DNA synthesis in uterine epithelial cells was examined by labelling with bromodeoxyuridine (BrdU). Ovariectomized mice treated with 3MC exhibited enhanced cell proliferation in the glandular epithelium, as did E2-treated mice (Fig. 4d). Proliferation of the luminal epithelium was enhanced by E2 but not by 3MC.

The present findings indicate that the oestrogenic action of AhR agonists might be exerted through a direct interaction between AhR/Arnt and unliganded ER and by the formation of functional units bound to EREs that activate transcription, at least in uterine gene induction and cellular proliferation. The most marked manifestation of the possible oestrogenicity of dioxins could be seen as their linking to endometriosis[4–7], because oestrogen is the major factor in the stimulation of proliferation of these cells. Thus, AhR expressed in the uterine glandular epithelium[30] might respond to dioxins by associating with unliganded ERs, which then stimulates oestrogen-dependent cell proliferation. In contrast, AhR agonists exhibit anti-oestrogenic activities in the presence of high doses of E2 in animals[3] and cultured cell lines[2]. We also found that AhR/Arnt repressed E2-bound ER function, which is consistent with these previous reports. However, whereas most previous studies have not examined or mentioned the effects of AhR ligands in the absence of E2, we addressed this issue carefully in the present study. Thus, oestrogen concentrations, which vary with age, oestrous cycle, tissues and other factors, might define the oestrogenic/anti-oestrogenic actions of the AhR ligands in intact animals. Our present model, in which AhR potentiates unliganded ERs but represses liganded ER, might be an explanation of these previous findings, and it will be of interest to identify the other components of the liganded AhR–ER-α complex involved in the oestrogenic/anti-oestrogenic actions of dioxins. Our proposal is that one of the molecular mechanisms for the oestrogen-related adverse effects of dioxin-type environmental contaminants is the modulation of oestrogen receptor signalling by dioxin-dependent association with dioxin receptor. □

Methods

Plasmids

Full-length complementary DNAs of AhR and Arnt were inserted into pcDNA3 vectors (Invitrogen). Three consensus EREs[16] and XREs[22] were inserted into the promoter of luciferase pGL3-basic vector to generate ERE-TATA–luciferase and XRE-TATA–luciferase, respectively. ER-α ΔAhR was generated by the deletion of 45–131 residues from ER-α. The other mutants of ER-α and ER-β were as described previously[21].

Transfection and luciferase assay

Human endometrium cancer-derived Ishikawa cells, human breast cancer-derived MCF-7 cells, green monkey COS-1 cells and human 293T cells maintained in DMEM supplemented with 10% FBS were cultured in phenol-red-free DMEM containing 0.2% charcoal-stripped FBS before assays. Cells at 40–50% confluence were transfected with the indicated plasmids (0.25 μg ERE-Luc, 0.1 μg XRE-Luc, 0.025 μg ER-α, AhR and Arnt were transfected) using Lipofectamine reagent (Gibco BRL) in 12-well Petri dishes. Total amounts of cDNA were adjusted by supplementing with empty vector up to 1.0 μg. Cells were treated with E2 (100 nM) and 3MC (1 μM). Luciferase activity was determined with the Luciferase Assay System (Promega)[16]. As a reference plasmid to normalize transfection efficiency, 25 ng pRL-CMV plasmid (Promega) was co-transfected in all experiments. Results are given as means ± s.d. for at least three independent experiments.

Immunoprecipitation and GST pull-down assay

Whole cell extracts[17] were used for immunoprecipitation with either anti-ER-α or anti-Flag antibody (anti-ER-α Ab-4 from Neo Markers; anti-Flag from Santa Cruz Biotechnology) after western blotting with anti-ER-α (Chemicon), anti-Arnt (Santa Cruz Biotechnology), anti-AhR (Santa Cruz Biotechnology), anti-p300 (Upstate Biotechnology), anti-SRC-1 (Santa Cruz Biotechnology), anti-Flag, anti-haemagglutinin and anti-Myc (Invitrogen) antibodies. Normal mouse immunoglobulin was used as a control. For immunoprecipitation of overexpressed proteins, cells were transfected as indicated with Flag-tagged ERs (5 μg), haemagglutinin-tagged AhR (3 μg), Myc-Arnt (5 μg), SRC-1 (0.7 μg) and p300 (0.7 μg) in the presence or absence of 3MC and E2. For the GST pull-down assay, AhR and Arnt were translated *in vitro* and incubated with either GST, GST–ER-α(A/B) or GST–ER-β(A/B) immobilized on glutathione–Sepharose beads[17].

Purification and separation of AhR-interacting complexes

HeLa nuclear extracts were loaded on an M2 anti-Flag agarose gel (Kodak). After being washed with binding buffer, the bound proteins were eluted from the agarose by incubation overnight with 2.5–5.0 ml of the Flag peptide (Kodak) in binding buffer (0.2 mg ml^{-1}). For fractionation on a glycerol gradient, eluents were layered on the top of a 13-ml linear 100–10% glycerol gradient and centrifuged for 16 h at 40,000 r.p.m. in an SW40 rotor (Beckman). Each fraction was western blotted with anti-AhR, anti-ER-α and anti-p300 antibodies. The protein standards used were β-globulin (M_r 158K) and thyroglobulin (667K)[17].

Chromatin immunoprecipitation

Soluble chromatins of MCF-7 cells prepared with the acetyl-histone H4 immunoprecipitation assay kit (Upstate Biotechnology) were immunoprecipitated with antibodies against the indicated proteins. Specific primer pairs were designated to amplify the promoter regions of the c-*fos* (5′-GAAGAGTGGAGAAGGG-3′ and 5′-GAAGCTGTGCTTACGG-3′), *pS2* (5′-AAAGAATTAGCTTAGGCC-3′ and 5′-ACCTTAATCCAGGTCC-3′) or *CYP1A1* (5′-CTTCGCCATCCATTCC-3′ and 5′-GGGACTCCTCTTCGAC-3′) genes from the extracted DNA. Optimal PCR conditions to allow semiquantitative measurement were used on 2% agarose/Tris-acetate-EDTA gels[17]. As a usual condition, cells were treated with ligands for 45 min. The inductions of the target genes were examined by northern blot analysis in MCF-7 cells treated with the ligands for 3 h.

ABCD precipitation

Avidin resin (Promega) was incubated with biotin-conjugated consensus ERE oligonucleotides, followed by incubation with cell lysates in lysis buffer (20 mM HEPES, 100 mM KCl, 0.5 mM EDTA, 0.1% Triton X-100 and 1 mM dithiothreitol) for 30 min. The subsequent ERE–protein complexes trapped on the resin were then eluted and western blotted[24].

Oestrogen responses in uterus

Nine-week-old female C57BL/6 mice with the indicated genotypes were ovariectomized. After 2 weeks the mice were treated with 3MC (4 mg kg^{-1}), TCDD (40 μg kg^{-1}), and/or E2 (20 μg kg^{-1}) in olive oil for 3 h. Total RNA was extracted from the uteri by Isogen (Wako Co.) and then subjected to northern blot analysis with cDNAs for the target genes for E2 (c-*fos*, *VEGF*) and for 3MC (*CYP1A1*), with *GAPDH* cDNA (encoding glyceraldehydes-3-

phosphate dehydrogenase) as an internal control[16]. For experiments with intact mice, 21-day-old female mice were used.

For uterine weight analysis, mice were treated with ligands for 3 days, and the ratio of uterine wet weight to body weight was calculated, followed by *t*-test analysis. Results are given as means ± s.e.m.

For the BrdU labelling experiment, ovariectomized mice were treated with ligands for 3 days, then injected with BrdU (30 mg kg^{-1}). Paraffin sections from the uteri 8 h after BrdU injection were immunostained with anti-BrdU monoclonal antibody by using the BrdU Labeling and Detection Kit 1 (Roche), and the percentage of BrdU-positive epithelial cells in the sections was calculated.

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Insulin-regulated hepatic gluconeogenesis through FOXO1–PGC-1α interaction

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Hepatic gluconeogenesis is absolutely required for survival during prolonged fasting or starvation, but is inappropriately activated in diabetes mellitus. Glucocorticoids and glucagon have strong gluconeogenic actions on the liver. In contrast, insulin suppresses hepatic gluconeogenesis[1–3]. Two components known to have important physiological roles in this process are the forkhead transcription factor FOXO1 (also known as FKHR) and peroxisome proliferative activated receptor-γ co-activator 1 (PGC-1α; also known as PPARGC1), a transcriptional co-activator; whether and how these factors collaborate has not been clear. Using wild-type and mutant alleles of FOXO1, here we show that PGC-1α binds and co-activates FOXO1 in a manner inhibited by Akt-mediated phosphorylation. Furthermore, FOXO1 function is required for the robust activation of gluconeogenic gene expression in hepatic cells and in mouse liver by PGC-1α. Insulin suppresses gluconeogenesis stimulated by PGC-1α but co-expression of a mutant allele of FOXO1 insensitive to insulin completely reverses this suppression in hepatocytes or transgenic mice. We conclude that FOXO1 and PGC-1α interact in the execution of a programme of powerful, insulin-regulated gluconeogenesis.

Two transcriptional components that are targets of insulin signalling, and that can activate the process of gluconeogenesis in liver, are FOXO1 and PGC-1α. FOXO1 has been shown to bind directly to the promoters of gluconeogenic genes and activate the process of glucose production[4–6]. FOXO1 is directly phosphorylated by Akt, a key protein kinase downstream of the insulin receptor[7,8]. This phosphorylation results in exclusion of FOXO1 from the nucleus. A second transcriptional component controlled by insulin and having a role in gluconeogenesis is the co-activator PGC-1α. PGC-1α is induced in liver on fasting, and is elevated in several models of diabetes or deficiency in insulin signalling. Notably, expression of PGC-1α at physiological levels turns on the entire programme of gluconeogenesis[9,10].

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