

The different effects of endocrine-disrupting chemicals on estrogen receptor-mediated transcription through interaction with coactivator TRAP220 in uterine tissue

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Abstract

An endocrine-disrupting chemical (EDC) can alter endocrine functions through a variety of mechanisms, including nuclear receptor-mediated changes in protein synthesis, interference with membrane receptor binding, steroidogenesis or synthesis of other hormones. Although major chemicals have been shown to disrupt estrogenic actions mainly through their binding to estrogen receptor (ER) or androgen receptor, it is not clear how EDCs affect endocrine functions *in vivo*. We present evidence that the EDCs bisphenol A and phthalate activate ER-mediated transcription through interaction with TRAP220. Moreover, bisphenol A had positive effects on the interaction between ER- β and TRAP220 and on the expression of ER- β and TRAP220 compared with phthalate and estradiol in uterine tissue. These data suggested that some EDCs might alter endocrine function through the change of the receptor and coactivator levels in uterine tissue and through the different effect on the interaction between ERs and coactivator TRAP220.

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Introduction

An endocrine-disrupting chemical (EDC) has been defined as an exogenous agent that interferes with the synthesis, secretion, transport, binding, action or elimination of natural hormones in the body; these are responsible for the maintenance of homeostasis, reproduction, development and/or behavior (Kavlock *et al.* 1996). These chemicals can alter endocrine functions through a variety of mechanisms, including steroid hormone receptor-mediated changes in protein synthesis, interference with membrane receptor binding, steroidogenesis or synthesis of other hormones (Cooper & Kavlock 1997). Although major chemicals such as phthalates, alkylphenols, bisphenol A and dichlorodiphenyltrichloroethane (DDT) have been shown to disrupt estrogenic actions mainly through their binding to estrogen receptors (ER) or androgen receptors (Cooper & Kavlock 1997), it is not clear

how EDCs directly affect endocrine functions through receptor-mediated transcription *in vivo*.

The nuclear receptor superfamily consists of over 150 different proteins that have evolved to mediate a complex array of extracellular signals into transcriptional responses. There are many ligands for these nuclear receptors, including steroid hormones, such as estradiol, progesterone and glucocorticoid, and non-steroid hormones, such as vitamin D, retinoids, thyroid hormone and prostanoids. These receptors form homodimers or heterodimers with retinoid X receptor and directly associate with specific DNA sequences known as hormone-responsive elements located in the promoters of specific genes (Evans 1988, Tsai & O'Malley 1994, Mangelsdorf & Evans 1995). The DNA–receptor complex interacts with basal transcriptional machinery and nuclear receptor coactivator proteins, resulting in the ligand-dependent induction of transcription (Mangelsdorf

& Evans 1995, Horwitz *et al.* 1996, Masuyama *et al.* 1997a, McKenna & O'Malley 2002).

It is becoming increasingly clear that protein–protein contacts between the receptor and the basal transcriptional machinery are important for ligand-mediated transactivation or repression by nuclear receptors. Nuclear receptors directly contact several general transcription factors (GTFs) in the preinitiation complex (PIC). The interaction of receptors with these GTFs is thought to either recruit these limiting factors to PIC assembly or to stabilize the PIC itself (Tsai & O'Malley 1994). In addition, a large number of coactivator proteins have been isolated through interactions with nuclear receptors. One group of related proteins are termed the p160 coactivators, including steroid receptor coactivator-1/nuclear coactivator-1, transcriptional intermediary factor-2/glucocorticoid receptor-interacting protein 1/nuclear coactivator-2 and p300/CREB binding protein (CBP) interacting protein/activator of thyroid and retinoic acid receptor/amplified in breast cancer-1 (Freedman 1997, McKenna *et al.* 1999). They have an intrinsic histone acetyl transferase (HAT) activity and the ability to interact with other acetyl transferase coactivators, such as CBP/p300 and p300/CBP-associated factor (Freedman 1997, McKenna *et al.* 1999). In contrast, mediator-like coactivator complexes, referred to as thyroid hormone receptor-associated protein-220 (TRAP220)/vitamin D receptor interacting protein-205 complexes, which have no HAT activity, are believed to function at different steps by directly associating with the basal transcription machinery (Rachez *et al.* 2000).

We have examined how EDCs affect endocrine function especially in reproductive tissues. First, we checked the effect of EDCs on the ER-mediated transcription and the effect of coactivator TRAP 220 on this transcription in cell lines derived from endometrial cancer in a transient transfection assay. We also used a two-hybrid protein interaction assay to determine whether the EDCs tested in this study affected the interaction between ERs and TRAP220. Finally, we checked the effect of these EDCs on the expression of ERs and TRAP220 in murine uterine tissue *in vivo* and in endometrial cancer cells. The results suggested that some EDCs may affect endocrine function through the change of ERs and coactivator TRAP220 expressions in uterine tissue and through the different effect on the interaction between ERs and TRAP220.

Materials and methods

Materials

Isopropylidenediphenol (bisphenol A), phthalic acid bis (2-ethylhexyl ester; phthalate), and 17 β -estradiol (1,3,5[10]-estratriene-3,17 β -diol; estradiol) were purchased from Sigma Co. Ltd (St Louis, MO, USA). DDT was obtained from Tokyo Kasei Kogyo Co. Ltd (Tokyo, Japan). All EDCs and ligands were added simultaneously to the culture media at the concentrations indicated. Primary rabbit polyclonal antibody for ER- α (MC-20), which reacts with mouse, rat and human ER- α , but is non-cross-reactive with ER- β , that for ER- β (H-150), which reacts with mouse, rat and human ER- β , but is non-cross-reactive with ER- α , and that for TRAP220 (M-255), which reacts with mouse, rat and human TRAP220, were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Two plasmids, pSG5-ER- α and (ERE)²-G-chloramphenicol acetyltransferase (CAT) were kindly provided by Dr S Kato (Tokyo University, Tokyo, Japan), and cDNA for human ER- β was a gift from Dr S Mosselman (N.V. Organon, Oss, Netherlands) and subcloned into pSG5 expression vector (Stratagene, La Jolla, CA, USA). TRAP220 cDNA was generated by RT-PCR and subcloned into pcDNA3 (Invitrogen, San Diego, CA, USA) and pAD-gal4 (Stratagene) expression vectors. Ishikawa cells were kindly provided by Dr M Nishida (Tukuba University, Tukuba, Japan).

Cell culture and transient transfection studies

COS-7 cells and Ishikawa cells were cultured in Dulbecco's modified Eagle's medium without phenol red supplemented with 10% charcoal-stripped fetal bovine serum. COS-7 cells and Ishikawa cells were cotransfected with 1 μ g of a reporter gene construct ((ERE)²-G-CAT) and 0.5 μ g of a receptor expression vector (pSG5-ER- α or ER- β) or empty vector (pSG5). Ishikawa cells were also transfected with 1 μ g of a reporter gene construct ((ERE)²-G-CAT) or empty vector (G-CAT) without the ER expression vector. For coactivator expression, pcDNA3-TRAP220 or pcDNA3 alone were also transfected into the cells. In all transfections, liposome-mediated transfections were accomplished by using lipofectamine

(Life Technologies Inc., Gaithersburg, MD, USA) according to the manufacturer's protocol. Transfected cells were treated either with vehicle alone or with the indicated concentrations of steroid hormones or EDCs for 36 h. Cell extracts were prepared and assayed for CAT activity. The amount of CAT was determined using a CAT enzyme-linked immunosorbent assay (ELISA) kit (Roche Diagnostics Co., Tokyo, Japan) according to the manufacturer's protocol.

Preparation of two-hybrid expression vectors and β -galactosidase assays

All two-hybrid plasmids constructs used the pAS1 (Durfee *et al.* 1993) and pAD-gal4 yeast expression vectors. The pAS1-ER- α and -ER- β constructs have been described previously (Masuyama *et al.* 2000). The pAD-TRAP220 was cotransformed with pAS1-ER- α or ER- β into the yeast strain Hf7c. Transformants were plated on media lacking leucine and tryptophan (SC-leu-trp) and were grown for 4 days at 30 °C to select for yeast that had acquired both plasmids. Triplicate independent colonies from each plate were grown overnight in 2 ml SC-leu-trp with or without the indicated concentrations of progesterone, phthalic acid, nonylphenol or bisphenol A. Cells were harvested and assayed for β -galactosidase activity as described previously (Masuyama *et al.* 1997b).

Procedure for *in vivo* experiment

Three-week-old ICR mice were given estradiol, phthalate, bisphenol A (0.3 mg/kg) or vehicle via intraperitoneal injection every 24 h for 5 days. The animals were killed under ether anesthesia and the uterine tissues were collected 24 h after the final administration. Tissue samples were immediately frozen and stored at -80 °C until assay.

Nuclear extracts and Western analysis

Nuclear extracts were obtained from Ishikawa cells or uterine tissues using NE-PER nuclear and cytoplasmic extraction reagents (Pierce Chemical Co., Rockford, IL, USA) according to the manufacturer's protocol and stored at -80 °C until analysis. Equivalent amounts of nuclear protein (25 μ g/sample) from each extract were determined by bicinchoninic acid protein assay

(Pierce Chemical Co.), solubilized in SDS buffer (0.05 M Tris-HCl, 2% SDS, 6% mercaptoethanol, 10% glycerol, pH 6.8) and analyzed by Western blot analysis as previously described (Masuyama & MacDonald 1998) using rabbit polyclonal antibodies for ER- α (1:1000 dilution), ER- β (1:1000 dilution) and TRAP220 (1:200 dilution). The amount in each band was quantitated densitometrically using Image Scanner GT-9500 (Epson, Suwa, Japan) and Bio Image BQ 2.0 software (Bio Image, Ann Arbor, MI, USA).

RT-PCR

Frozen murine uterine tissues were homogenized and total RNA was extracted using the RNeasy Mini Kits (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's instructions. Total RNA was also extracted from Ishikawa cells using Trizol reagent (Life Technology, Inc., Grand Island, NY, USA). Each sample was treated with DNase I to remove genomic DNA contamination. According to the protocol of the RNA PCR kit (TAKARA Co. Ltd, Kyoto, Japan), 0.1 μ g total RNA was reverse transcribed at 42 °C for 20 min in 20 μ l of reaction solution containing 1 \times PCR buffer, 5 mM MgCl₂, 1 mM dNTPs, 2.5 μ M random 9 mers primer, 10 U RNase inhibitor and 5 U AMV reverse transcriptase. The primers for human ER- α , ER- β , TRAP220 and β -actin were as follows: ER- α sense: 5'-agcacctgaagtctctgga-3', antisense 5'-gatgtgggagaggatgagga-3'; ER- β sense: 5'-aagaagattcccggctttgt-3', antisense 5'-tctacgattccctcatc-3'; TRAP220 sense: 5'-tgcgtcaagtcattgag aag-3', antisense 5'-ccactggcactgagatgaga-3'; β -actin sense: 5'-agaaaatctggcaccacacc-3', antisense 5'-cctg cagagtccaaaggag-3'. The primers for mouse ER- α , ER- β , TRAP220 and β -actin were as follows: ER- α sense: 5'-aatgaaatgggtccttcagg-3', antisense 5'-ataga tcatgggctggtcag-3'; ER- β sense: 5'-gaagctggctga caaggaac-3', antisense 5'-aacgaggtctggagcaaga-3'; TRAP220 sense: 5'-gcaacaaggccaagaagaag-3', anti- sense 5'-agctggggtgatagagtg-3'; β -actin sense: 5'-ctaaggccaaccgtgaaaag-3', antisense 5'-tctcagct gttggtggaag-3'. Amplification was carried out on a GeneAmp PCR system 9700 (Applied Biosystems, Foster City, CA, USA) with initial denaturation at 94 °C for 2 min, followed by 25 cycles of 94 °C for 30 s, 60 °C for 30 s, 72 °C for 30 s and a final extension at 72 °C for 2 min. The number of PCR cycles resulting in PCR products in the linear

logarithmic phase of the amplification curve was determined. PCR samples were electrophoresed on 3% Nu-Sieve agarose gel (FMC BioProducts, Rockland, ME, USA) and visualized by ethidium bromide. The amount of each electrophoretically separated cDNA was quantitated densitometrically using an Image Scanner GT-9500 and Bio Image

BQ 2.0 software. The housekeeping gene, β -actin, was used to determine the relative levels of ERs and TRAP220 gene transcription and to control for variations in RNA recoveries from each specimen. Normalization of the data was accomplished by quantifying the amount of amplified cDNA products by calculating the ratio of the amount of ERs and TRAP220 cDNA relative to the amount of β -actin cDNA. This ratio was used to compare the relative amounts of ERs and TRAP220 mRNA in each sample.

Statistical analysis

Statistical analysis was evaluated by one-factor ANOVA followed by Dunnett's test. Data are the means \pm s.d. $P < 0.05$ denotes the presence of a statistically significant difference.

Results

EDCs, bisphenol A and phthalate stimulate ER- α - and ER- β -mediated transcription in COS-7 cells and the endometrial cancer cell line, Ishikawa cells

Transient reporter expression assay was analyzed in COS-7 cells and Ishikawa cells to examine

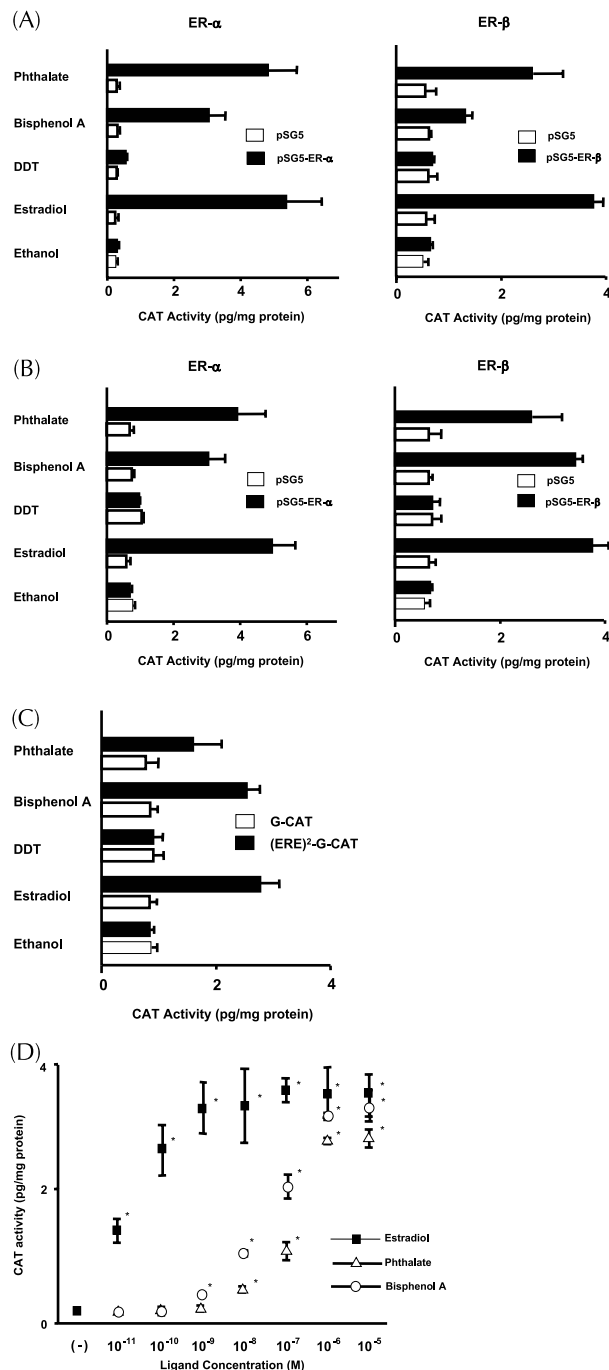


Figure 1 The effect of EDCs on ER- α - and ER- β -mediated transactivation. (A) COS-7 cells and (B) Ishikawa cells were transfected with 1 μ g (ERE)²-G-CAT reporter gene construct and 0.5 μ g pSG5-ER or pSG5 expression vector together. The cells were treated with ethanol vehicle or 10⁻⁶ M estradiol, DDT, bisphenol A or phthalate for 24 h. The amount of CAT was determined with a CAT ELISA kit (5'Prime-3'Prime, Inc., Boulder, CO, USA) according to the manufacturer's protocol. The results represent the means \pm s.d. of triplicate determinations. (C) Ishikawa cells were cotransfected with 1 μ g (ERE)²-G-CAT or G-CAT reporter gene constructs. The cells were treated with ethanol vehicle or 10⁻⁶ M estradiol, DDT, bisphenol A or phthalate for 24 h. The amount of CAT was determined with a CAT ELISA kit. The results represent the means \pm s.d. of triplicate determinations. (D) Ishikawa cells were cotransfected with 1 μ g (ERE)²-G-CAT or G-CAT reporter gene constructs and incubated in the absence and presence of increasing concentrations of estradiol or EDCs for 24 h. The amount of CAT was determined with a CAT ELISA kit. The results represent the means \pm s.d. of triplicate determinations (* $P < 0.01$ compared with ethanol treatment).

whether several of the EDCs enhanced ER- α - and ER- β -mediated transcription. Bisphenol A and phthalate as well as estradiol enhanced both ER- α - and ER- β -mediated transcription in COS-7 cells (Fig. 1A) and Ishikawa cells (Fig. 1B). In contrast, DDT had no effect on either transcription. Moreover, bisphenol A and phthalate had positive effect on endogenous ER-mediated transcription in Ishikawa cells (Fig. 1C). These effects were dependent on the ligand concentration and significantly increased at 1 nM bisphenol A and 10 nM phthalate in Ishikawa cells compared with ethanol treatment ($P < 0.01$). The median effective concentration (EC_{50}) values of estradiol, bisphenol A and phthalate for ER-mediated transcription in Ishikawa cells were about 50 pM, 50 nM and 500 nM respectively (Fig. 1D).

Effect of bisphenol A and phthalate on the interaction between ER- α , ER- β and TRAP220

We used the two-hybrid protein interaction assay to examine whether ER- α and ER- β interacted with the coactivator protein TRAP220, which is very important for ER-mediated transcription (Burakov *et al.* 2000, Kang *et al.* 2002), in the presence of EDCs. TRAP220 interacted with ER- α and ER- β in the presence of estradiol, bisphenol A and phthalate, which stimulated ER-mediated transcription, and the β -galactosidase activity with ER- β was much higher than that with ER- α . However, DDT, which had no effect on ER-mediated transcription, did not affect this interaction (Fig. 2A). The effect on the interactions of TRAP220 with ER- α was dependent on the concentration of the ligands and significantly increased at 100 nM bisphenol A and 10 nM phthalate, which is compatible with the transcriptional activity ($P < 0.01$ compared with ethanol treatment, Fig. 2B). In addition, the effect on the interactions of TRAP220 with ER- β was dependent on the concentration of the ligands and significantly increased at 1 nM bisphenol A and 100 nM phthalate ($P < 0.01$ compared with ethanol treatment, Fig. 2C). The EC_{50} of the interaction between ER- β and TRAP220 in the presence of bisphenol A (about 10 nM) was lower than that in the presence of phthalate (about 50 nM) (Fig. 2C). In contrast, phthalate strongly enhanced the interaction between ER- α

and TRAP 220 compared with bisphenol A (Fig. 2B).

The effect of TRAP220 on ER- α - and ER- β -mediated transcription

We used the transient reporter expression assay in COS-7 cells and Ishikawa cells to examine whether TRAP220 affected ER- α - and ER- β -mediated transcription and found that TRAP220 enhanced both ER- α - and ER- β -mediated transcription in the presence of bisphenol A and phthalate as well as estradiol in COS-7 cells (Fig. 3A) and Ishikawa cells (Fig. 3B). The effect of TRAP220 on ER- β -mediated transcription in the presence of bisphenol A was more efficient than that in the presence of phthalate. Moreover, TRAP220 had a positive effect on endogenous ER-mediated transcription in the presence of bisphenol A, phthalate and estradiol in Ishikawa cells (Fig. 3C). The effect of TRAP220 on ER-mediated transcription in the presence of bisphenol A was significantly increased at 10 nM, which is the physiological concentration, compared with that without TRAP220 overexpression ($P < 0.01$, Fig. 3D).

The effect of EDCs on the expression of ER- α , ER- β and TRAP220 *in vivo*

We checked the expression of ER- α , ER- β and TRAP220 in several cell lines and uterine tissue to examine whether EDCs affect the expression of ER- α , ER- β and TRAP220. ER- α , ER- β and TRAP220 were abundant in the human endometrial cancer cell line, Ishikawa cells, and uterine tissue (Fig. 4A). We then checked the expression of ER- α , ER- β and TRAP220 in murine uterine tissue after exposure to estradiol, phthalic acid, bisphenol A or ethanol *in vivo*. The protein level of ER- α was increased only in the presence of estradiol, but there was a significant increase in ER- β protein level in the presence of bisphenol A as well as estradiol. Moreover, a significant increase of TRAP220 was observed only in the presence of bisphenol A ($P < 0.01$, Fig. 4B). The mRNA levels of these receptors and coactivator were also examined by semi-quantitative RT-PCR. The effects of estradiol and EDCs on mRNA level of ERs and TRAP220 were compatible with these effects on protein levels ($P < 0.01$, Fig. 4C).

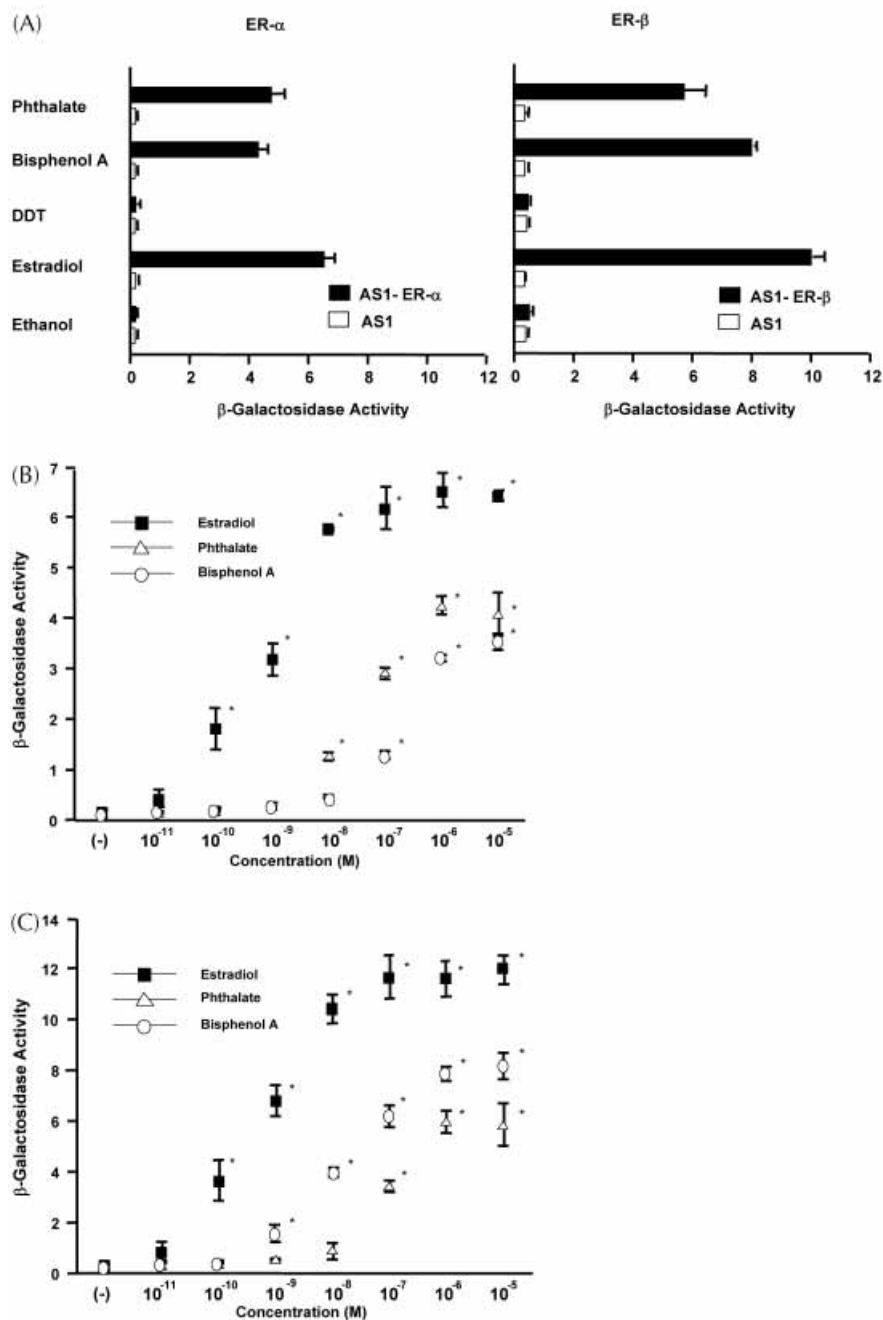
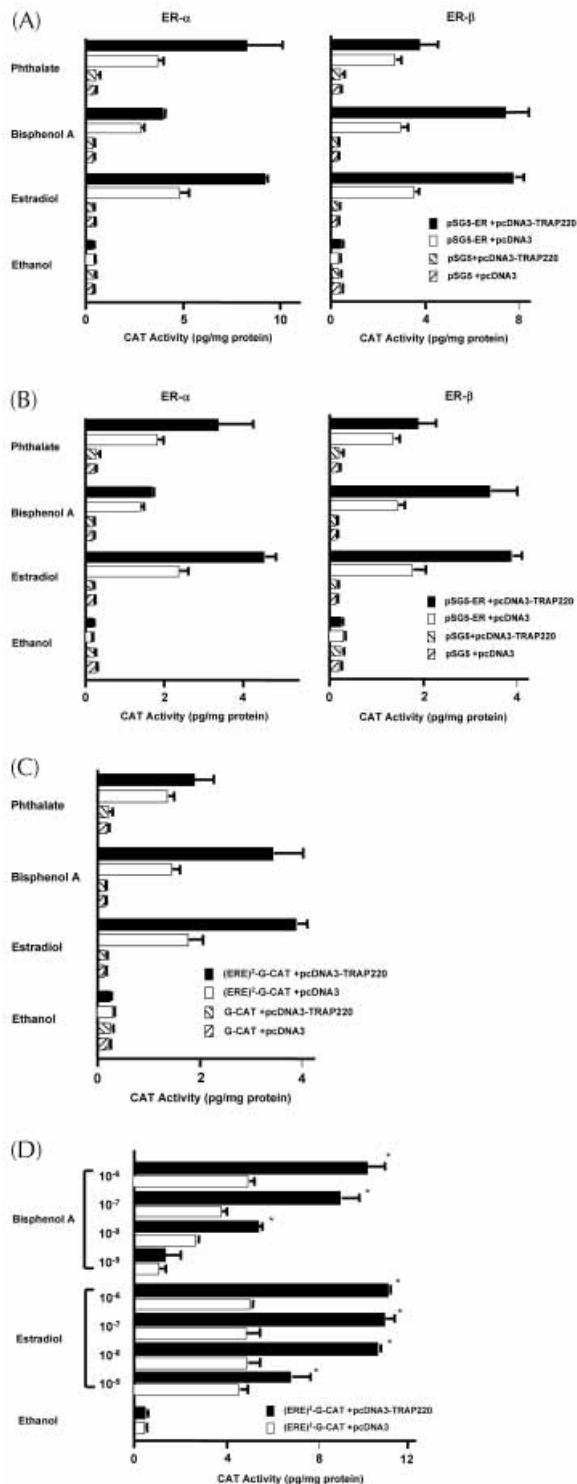


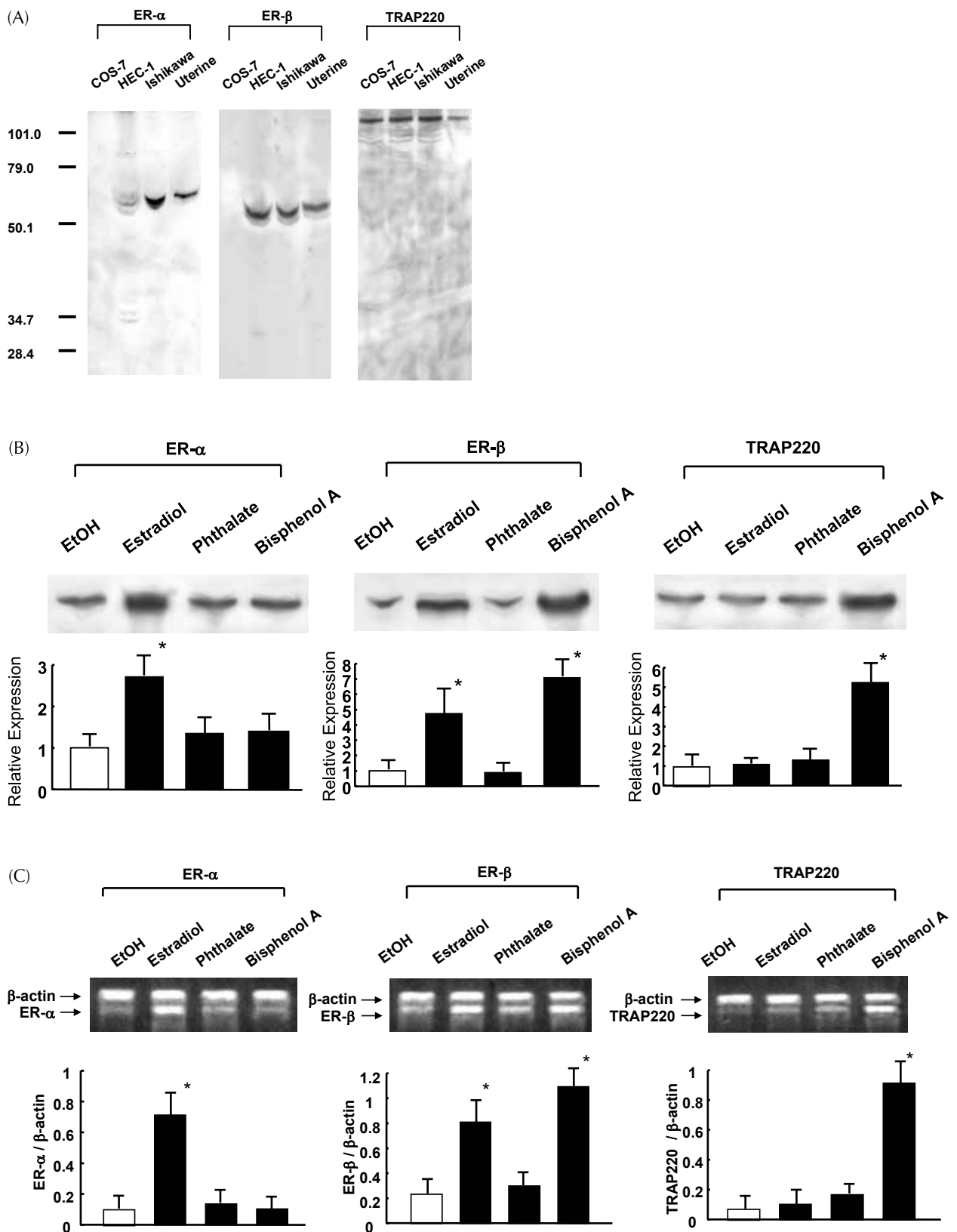
Figure 2 The interaction between ERs and TRAP220 in the presence of EDCs. (A) Yeast expressing the pAS1-ER- α or ER- β and pAD-TRAP220 two-hybrid plasmids were grown for 24 h at 30 °C in the presence of ethanol vehicle or 10^{-6} M estradiol, DDT, bisphenol A or phthalate. The ER-TRAP220 interaction was assessed in a β -galactosidase assay. Results are presented as the means \pm s.d. of triplicate independent cultures. (B) Yeast expressing the pAS1-ER- α and pAD-TRAP220 two-hybrid plasmids were grown for 24 h at 30 °C in the absence and presence of increasing concentrations of estradiol, bisphenol A or phthalate. The ER- α -TRAP220 interaction was assessed in a β -galactosidase assay. Results are presented as the means \pm s.d. of triplicate independent cultures (* P <0.01 compared with ethanol treatment). (C) Yeast expressing the pAS1-ER- β and pAD-TRAP220 two-hybrid plasmids were grown for 24 h at 30 °C in the absence and presence of increasing concentrations of estradiol, bisphenol A or phthalate. The ER- β -TRAP220 interaction was assessed in a β -galactosidase assay. Results are presented as the means \pm s.d. of triplicate independent cultures (* P <0.01 compared with ethanol treatment).



The effect of EDCs on the protein levels of ER- α , ER- β and TRAP220 in the endometrial cancer cell, Ishikawa cells

As described in Fig. 4A, ER- α , ER- β and TRAP220 were abundant in Ishikawa cells. Thus, the protein levels of ER- α , ER- β and TRAP220 were quantitatively examined in Ishikawa cells that had been exposed to endogenous steroid hormones or EDCs. The ER- α protein was slightly increased in the presence of estradiol, but there was no significant increase among each of the groups. However, ER- β protein level was significantly increased in the presence of bisphenol A as well as estradiol compared with the vehicle, but phthalate had no effect on the ER- β level. In addition, TRAP220 was not affected by estradiol, bisphenol A or phthalate. There were no effects of these steroids and EDCs on the expression of ER- α , ER- β and TRAP220 in COS-7 cells ($P < 0.01$, Fig. 5A). The mRNA levels of these receptors and coactivator in Ishikawa cells that had been exposed to estradiol, bisphenol A or phthalate were also examined by semi-quantitative RT-PCR. The effect of estradiol and EDCs on mRNA level of ERs and TRAP220 were compatible with these effects on protein levels in Ishikawa cells ($P < 0.01$, Fig. 5B).

Figure 3 The effect of TRAP220 on ER- α and ER- β -mediated transactivation. (A) COS-7 and (B) Ishikawa cells were transfected with 1 μ g (ERE)²-G-CAT reporter gene construct and 0.5 μ g pSG5-ER or pSG5 expression vector together with 1 μ g pcDNA3-TRAP220 or pcDNA3 expression vector. The cells were treated with ethanol vehicle or 10⁻⁶ M estradiol, DDT, bisphenol A or phthalate for 24 h. The amount of CAT was determined with a CAT ELISA kit. The results represent the means \pm s.d. of triplicate determinations. (C) Ishikawa cells were cotransfected with 1 μ g (ERE)²-G-CAT or G-CAT reporter gene constructs with 1 μ g pcDNA3-TRAP220 or pcDNA3 expression vector. The cells were treated with ethanol vehicle or 10⁻⁶ M estradiol, DDT, bisphenol A or phthalate for 24 h. The amount of CAT was determined with a CAT ELISA kit. The results represent the means \pm s.d. of triplicate determinations. (D) Ishikawa cells were cotransfected with 1 μ g (ERE)²-G-CAT reporter gene constructs with 1 μ g pcDNA3-TRAP220 or pcDNA3 expression vector. The cells were treated with ethanol vehicle or the indicated concentrations of estradiol or bisphenol A for 48 h. The amount of CAT was determined with a CAT ELISA kit. The results represent the means \pm s.d. of triplicate determinations (* $P < 0.01$ compared with results without TRAP220).



Discussion

It has been shown that the EDCs nonylphenol, bisphenol A and daidzein can bind to ER- α and ER- β and that relatively high concentrations of these chemicals are needed for the activation of ER-mediated transcription (Kuiper *et al.* 1998). We have shown that bisphenol A and phthalate activated endogenous ER- α - and ER- β -mediated transcription in a transient transfection assay using an endometrial cancer cell line. In addition, these EDCs, which activated ER- α - and ER- β -mediated transcription, enhanced the interaction between ERs and coactivator TRAP220 at a concentration compatible with the transcriptional activity. Because TRAP220 has been demonstrated to function as an important coactivator for nuclear receptor, especially in ER-mediated transcription (Burakov *et al.* 2000, Rachez *et al.* 2000, Kang *et al.* 2002), we carried out additional experiments to check whether TRAP220 had any effect on ER-mediated transcription in the presence of EDCs. As expected, TRAP220 augmented ER- α - and ER- β -mediated transcription in the presence of bisphenol A and phthalate as well as estradiol in both COS-7 cells and Ishikawa cells. These data suggested that the EDCs bisphenol A and phthalate might be ligands for both ER- α and ER- β and that they may mediate the transactivation through the interaction with TRAP220 in uterine tissue. However, bisphenol A and phthalate had different positive effects on both the interaction between ERs and TRAP220 and ER-mediated transcription in the presence of overexpressed TRAP220. There may be varied conformational changes of bisphenol A-occupied receptor from those of phthalate-occupied or estradiol-occupied receptors because

crystal structure analysis has shown that the structural features of the ER ligand-binding domain bound to tamoxifen and raloxifene, partial agonists for ER, were different from that bound to estradiol (Brzozowski *et al.* 1997, Shiau *et al.* 1998). In addition, TRAP220 has been demonstrated to interact with ER- β preferably compared with ER- α (Warnmark *et al.* 2001). Our data showed that bisphenol A enhanced both the interaction between ER- β and TRAP220 and ER- β -mediated transcription more efficiently compared with those with ER- α , suggesting that the coactivator selectivity of the ER subtypes might be an additional specificity that influences the transcriptional response in the presence of EDCs.

A variety of putative pathways by which EDCs have effects on the endocrine system have been reported (Kavlock *et al.* 1996, Cooper & Kavlock 1997). We have already shown one potential pathway by which EDCs may affect endocrine function through pregnane X receptor (PXR)-mediated changes of steroidogenesis (Masuyama *et al.* 2000). Moreover, we have also demonstrated that phthalate, an EDC, blocked the degradation of PXR by proteasome, which results in the relative up-regulation of PXR protein levels that have positive impacts on PXR-mediated gene regulation (Masuyama *et al.* 2002). Thus, it may be a potential mechanism of EDCs that affects endocrine function. We have presented some evidence that EDCs, especially bisphenol A, affect the expression of ER- β and TRAP220 in uterine tissue *in vivo*, which results in the relative up-regulation of receptors and coactivator protein levels that affect ER-mediated gene expressions. We have also shown that bisphenol A directly enhanced ER- β expression

Figure 4 The effect of EDCs on the expression of ERs and TRAP220 in the mouse. (A) Representative Western blotting of ER- α , ER- β and TRAP220 proteins in nuclear extracts of several cell lines and uterine tissue (25 μ g/sample). (B) Three-week-old ICR mice were treated with estradiol, phthalate, bisphenol A (0.3 mg/kg) or vehicle (EtOH) via intraperitoneal injection every 24 h for 5 days. Nuclear extracts were prepared as described in the Materials and methods. Equivalent amounts of each extract (25 μ g/sample) were resolved by 10% SDS-PAGE, and ER- α , ER- β and TRAP220 protein levels were determined by Western blotting using anti-ER- α , -ER- β and -TRAP220 antibody. The band intensities were densitometrically measured and quantified using Image Scanner GT-9500 and Bio Image software. The results represent the means \pm s.d. of triplicate determinations (* P < 0.01 compared with ethanol-treated control). (C) Three-week-old ICR mice were treated with estradiol, phthalate, bisphenol A (0.3 mg/kg) or vehicle (EtOH) via intraperitoneal injection every 24 h for 5 days. The total RNA was obtained from uterine tissues and analyzed for the expression of ERs, TRAP220 and β -actin mRNA using semi-competitive RT-PCR. The PCR products were separated on 3% Nu-Sieve agarose gels and visualized by ethidium bromide. The band intensities were densitometrically measured and quantified using Image Scanner GT-9500 and Bio Image software. The results represent the means \pm s.d. of triplicate determinations (* P < 0.01).

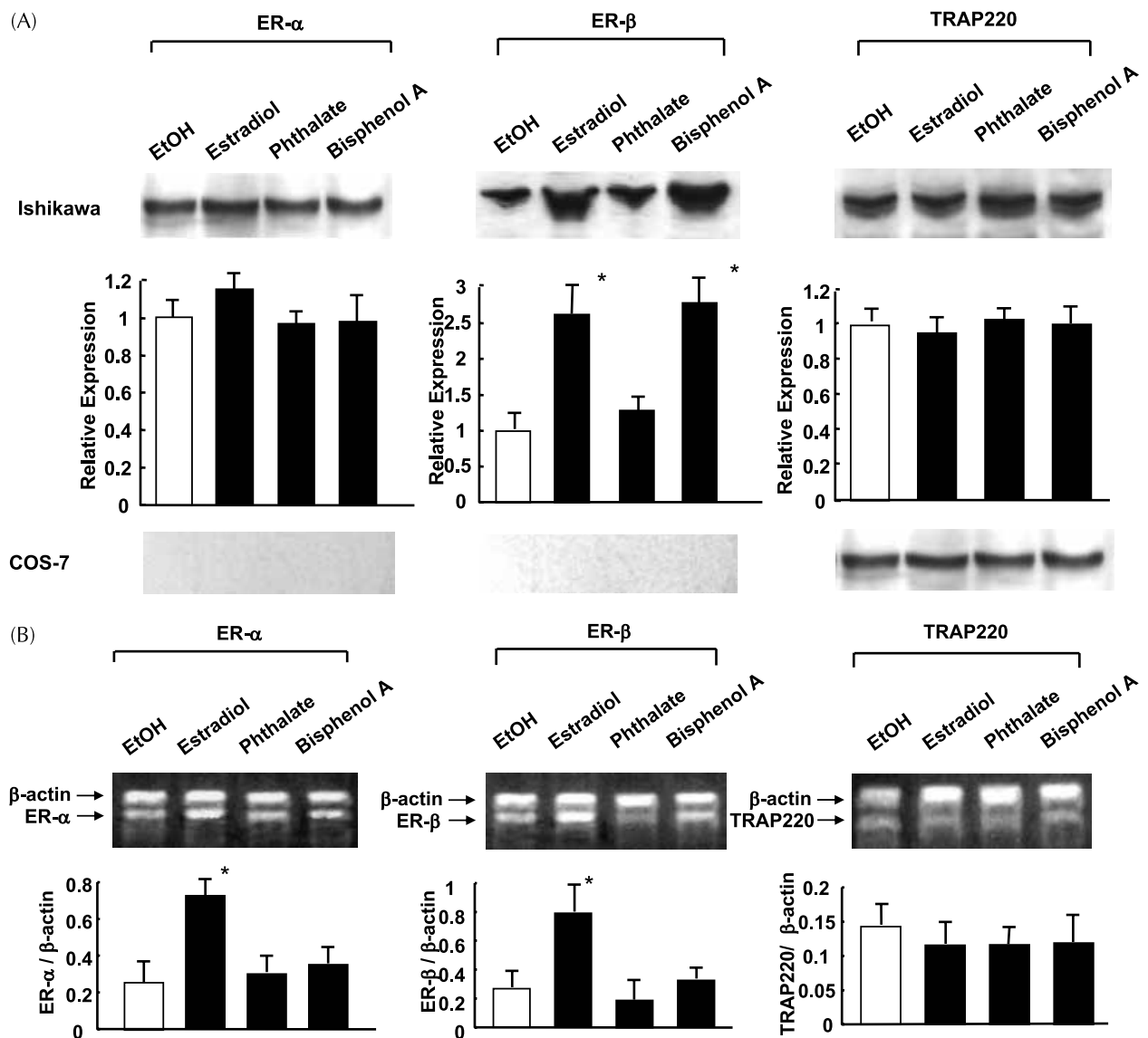


Figure 5 The effect of EDCs on the expression of ERs and TRAP220 in endometrial cancer cell. (A) Subconfluent Ishikawa cells were treated with ethanol (EtOH) or 10^{-6} M estradiol, phthalate or bisphenol A for 24 h and nuclear extracts were prepared as described in the Materials and methods. Equivalent amounts of each extract (25 μ g/sample) was resolved by 10% SDS-PAGE, and ER- α , ER- β and TRAP220 protein levels were determined by Western blotting using anti-ER- α , -ER- β and -TRAP220 antibodies. The band intensities were densitometrically measured and quantified using Image Scanner GT-9500 and Bio Image software. The results represent the means \pm s.d. of triplicate determinations (* P <0.01 compared with ethanol-treated control). (B) Subconfluent Ishikawa cells were treated with ethanol or 10^{-6} M estradiol, phthalate or bisphenol A for 24 h and the total RNA was obtained from Ishikawa cells and analyzed for the expression of ERs, TRAP220 and β -actin mRNA using semi-competitive RT-PCR. The PCR products were separated on 3% Nu-Sieve agarose gels and visualized by ethidium bromide. The band intensities were densitometrically measured and quantified using Image Scanner GT-9500 and Bio Image software. The results represent the means \pm s.d. of triplicate determinations (* P <0.01).

but did not affect TRAP220 expression in an endometrial cancer cell line, suggesting that bisphenol A has different mechanisms to affect

the expressions of ER- β and TRAP220. Further analysis will be required to investigate this mechanism.

In summary, we present here evidence that the EDCs bisphenol A and phthalate activate ER-mediated transcription through the interaction with TRAP220. Moreover, bisphenol A had positive effects on the interaction between ER- β and TRAP220 and on the expression of ER- β and TRAP220 compared with phthalate and estradiol in uterine tissue. These data suggested that some EDCs might alter endocrine function through the change of the receptor and coactivator levels in uterine tissue and through the different effect on the interaction between ERs and TRAP220.

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