Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Steroid Biochemistry & Molecular Biology 88 (2004) 53–59
The Journal of Steroid Biochemistry & Molecular Biology
www.elsevier.com/locate/jsbmb

# Effects of environmental estrogenic chemicals on AP1 mediated transcription with estrogen receptors α and β

Nariaki Fujimoto [a,*], Hiroaki Honda [a], Shigeyuki Kitamura [b]

[a] *Department of Developmental Biology, Research Institute for Radiation Biology and Medicine (RIRBM), Hiroshima University,1-2-3 Kasumi, Minami-ku, Hiroshima 734-8553, Japan*
[b] *Department of Xenobiotic Metabolism and Molecular Toxicology, Institute of Pharmaceutical Sciences, Hiroshima University School of Medicine, Kasumi 1-2-3, Minami-ku, Hiroshima 734-8551, Japan*

Received 7 July 2003; accepted 20 October 2003

**Abstract**

There has been much discussion concerning endocrine disrupting chemicals suspected of exerting adverse effects in both wildlife and humans. Since the majority of these compounds are estrogenic, a large number of in vitro tests for estrogenic characteristics have been developed for screening purpose. One reliable and widely used method is the reporter gene assay employing estrogen receptors (ERs) and a reporter gene with a *cis*-acting estrogen responsive element (ERE). Other elements such as AP1 also mediate estrogenic signals and the manner of response could be quite different from that of ERE. Since this has yet to be explored, the ER mediated AP1 activity in response to a series of environmental estrogens was investigated in comparison with ERE findings. All the compounds exhibited estrogenic properties with ERE-luc and their AP1 responses were quite similar. These was one exception, however, *p,p′*-DDT (1,1,1,-trichloro-2,2-bis(*p*-chlorophenyl)ethane) did not exert any AP1-luc activity, while it appeared to be estrogenic at $10^{-7}$ to $10^{-5}$ M with the ERE action. None of the compounds demonstrated ERβ:AP1 activity. These data suggest that significant differences can occur in responses through the two estrogen pathways depending on environmental chemicals.


*Keywords:* Environmental estrogens; Endocrine disruptors; Ap1 mediated estrogen responses; Estrogen receptors

## 1. Introduction

During the last decade, there has been much discussion as to the possibility that environmental chemicals which are capable of interfering with the endocrine system in organisms are responsible for a number of reproductive, developmental, and immunological anomalies and as well as cancers found in wildlife and man. Initially, this concern arose because of the presence of estrogen mimicking compounds in the environment, or environmental estrogens [1,2]. Although the issue has now become generalized to all types of agents with endocrine disrupting capability, the majority of the relevant chemicals are in fact estrogenic. A number of test methods for screening estrogenic compounds has already been developed, including receptor binding assays, hormone responsive cell proliferation assays and reporter gene assays, which has been widely used [3,4]. For screening, cells were usually transfected with estrogen receptor (ER) expression vectors, and a "reporter gene" linked the downstream of a *cis*-acting element, estrogen responsive element (ERE), to which the liganded receptor can bind, and recruit several transcriptional cofactors including p160/p300 complexes to initiate transcription [5,6].

Although the mechanisms operating with the ERE pathway are understand fairly well after intensive investigations, a number of estrogen inducible genes are regulated by other *cis*-elements, such as AP1 binding element for the collagenase and insulin-like growth factor 1 (IGF-1) cases [7–9]. When ER mediated transcription at AP1 sites is examined, responses to estrogenic compounds may be quite different from these with the ERE motif [10,11]. However, only the ERE dependent pathway has been utilized for reporter gene assays for screening. In the present study, therefore, estrogen dependent AP1 activity responding to a series of environmental estrogens was examined in comparison with ERE responses.

* Corresponding author. Tel.: +81-82-257-5820; fax: +81-82-256-7107.
*E-mail address:* nfjm@hiroshima-u.ac.jp (N. Fujimoto).

0960-0760/$ – see front matter © 2004 Elsevier Ltd. All rights reserved.
doi:10.1016/j.jsbmb.2003.10.006

## 2. Materials and methods

### 2.1. Chemicals

17β-Estradiol (E2), 17α-estradiol, estriol, 4-hydroxytamoxifen (OH-TAM), dienestrol, bisphenol A, *t*-methylbutylphenol, chlordecone, *p*,*p*′-biphenol, genistein, and zearalenol were purchased from Sigma Chemicals, St. Louis. The DDT isomers; *p*,*p*′-DDT (1,1,1,-trichloro-2,2-bis(*p*-chlorophenyl)ethane), *p*,*p*′-DDE (1,1,-dichloro-2,2-bis(*p*-chlorophenyl)ethylene), *p*,*p*′-DDD (1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethane), and *o*,*p*′-DDD (1,1-dichloro-2-(*p*-chlorophenyl)-2-(*o*-chlorophenyl)ethane) were from Wako-junyaku (Osaka, Japan). They were all dissolved in ethanol to give stock solutions.

### 2.2. Cell culture

The NIH 3T3 cell line was obtained from the Health Science Research Resources Bank (Osaka, Japan) and maintained in DME (Sigma Chemicals) containing penicillin and streptomycin with 10% calf serum (CS, Gibco/Invitrogen Corp., Carlsbad, CA, USA). For hormone treatment, the medium was changed to phenol red free DME (Sigma Chemicals) containing the same antibiotics along with dextran–charcoal treated CS for a week. The MCF-7 cell line was maintained in DME containing penicillin and streptomycin with 5% fetal bovine serum (FBS, Gibco/Invitrogen). The medium was then changed to phenol red free DME containing the antibiotics and dextran–charcoal treated FBS for a week.

### 2.3. Plasmids

pAP1-luc, which contains six tandem copies of the AP1 enhancer, was obtained from Clontech (Palo Alto, CA, USA). The construction of the $(ERE)_3$-SV40-luc plasmid and the expression vectors, pSG5-hERα and pSG5-hERβ was previously reported [12]. phRL-CMV (Promega, Madison, WI, USA) was used as the internal control.

### 2.4. ERE-luc and AP1-luc reporter assays

NIH 3T3 and MCF-7 cells were plated at $2 \times 10^4$ per well in 48-well plates (Nalgen Nunc International, Rochester, NY, USA). After 24 h, cells in each well were co-transfected with total 0.4 μg of plasmid DNA with TransFast transfection reagent (Promega), i.e. 0.3 μg of reporter plasmid, 0.1 μg of ER expression plasmid and 10 ng of phRL-CMV for NIH 3T3 and 0.4 μg of $(ERE)_3$-luc and 10 ng of phRL-CMV for MCF-7 cells. After 24 h incubation with chemicals, cells were harvested with 30 μl of cell lysis buffer (Promega). The firefly and renilla luciferase activities were determined with a Dual Luciferase Assay Kit (Promega) by measuring luminescence with a Wallac Micro-Beta scintillation counter (Perkin-Elmer Life Sciences, Boston, MA). Firefly luciferase activity was normalized to renilla luciferase activity from phRL-CMV.

### 2.5. Statistical analysis

Statistical comparisons were made using the Student's *t*-test.

## 3. Results

### 3.1. Estrogenic activity assayed with the ERE-luc reporter in MCF-7 cells

Estrogenic potency of test chemicals was examined with ERE responses in MCF-7 cells (Fig. 1). The endogenous type of ER in MCF-7 cells was the α type, which accounted for 99% of total expression of ERs, according to our competitive RT-PCR quantification (data not shown). ERE-luciferase activity was induced by E2 at the maximum of 27-fold, while OH-tamoxifen did not exert any activity. All of the test chemicals stimulated ERE-luc activity within a variety of concentration ranges.

### 3.2. ER mediated AP1-luc and ERE-luc transcription due to E2 and OH-tamoxifen

When NIH 3T3 cells were co-transfected with pSG5-hERα, E2 induced both ERE and AP1 mediated transcription (Figs. 2 and 3). OH-tamoxifen suppressed ERE-luc activity to less than the control level, but activated AP1 mediated transcription. When the cells were transfected with ERβ, E2 activated the ERE response but failed to initiate AP1 dependent transactivation (Fig. 3). OH-tamoxifen, on the other hand, induced AP1 mediated transcription with ERα or ERβ.

### 3.3. The effects of test chemicals on ER mediated ERE-luc and AP1-luc activities

For each chemical, two doses that gave around the maximal response in the MCF-7 assay were selected and applied. In NIH 3T3 cells, all of the chemicals induced ERE-luc activity with both ERα and ERβ. These were no significant differences in ERE response between two receptors, except genistein which gave significantly higher induction with ERβ and chlordecone which was specific for ERα. When the cells were transfected with the AP-luc reporter, the ERα mediated activity was similar to ERE-luc activity with one exception, DDT, which did not induce any AP1-luc activity. With ERβ, all of the chemicals failed to induce any AP-luc activity.

### 3.4. Effect of DDTs on ERα mediated ERE-luc and AP1 activity

Data for the effects of DDT isomers on ERα mediated ERE-luc and AP1 activity in NIH 3T3 are summarized

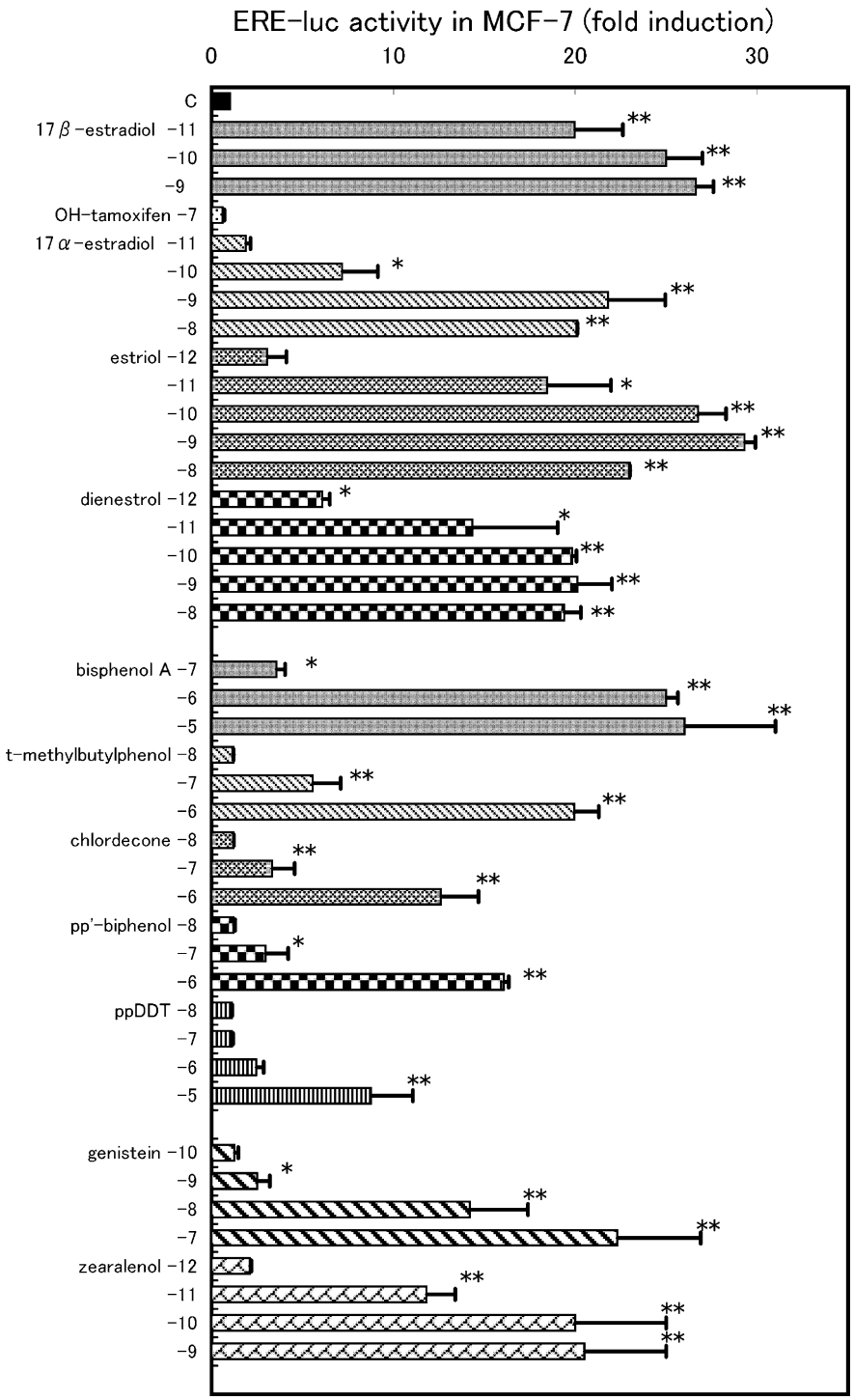


Fig. 1. Estrogenic property of a series of environmental chemicals measured by the ERE-luc reporter system in MCF-7 cells. The responses are expressed as fold changes relative to control activity (mean ± S.E.M.). Concentrations of chemicals were indicated in log[molar concentration]; (*) and (**) indicate significant difference from the control activity.

in Fig. 4. All four chemicals were estrogenic based on ERE-luc induction, *p,p′*-DDT and *o,p*-DDD having higher activity than the others. Although *p,p′*-DDT did not show any ERα:AP1-luc activity, the other three compounds expressed similar potency to ERα:ERE-luc case.

## 4. Discussion

The present study demonstrated that for the majority of test chemicals of endocrine disruptor type, induction of ERE-luc and AP1-luc activities though ERα is similar with

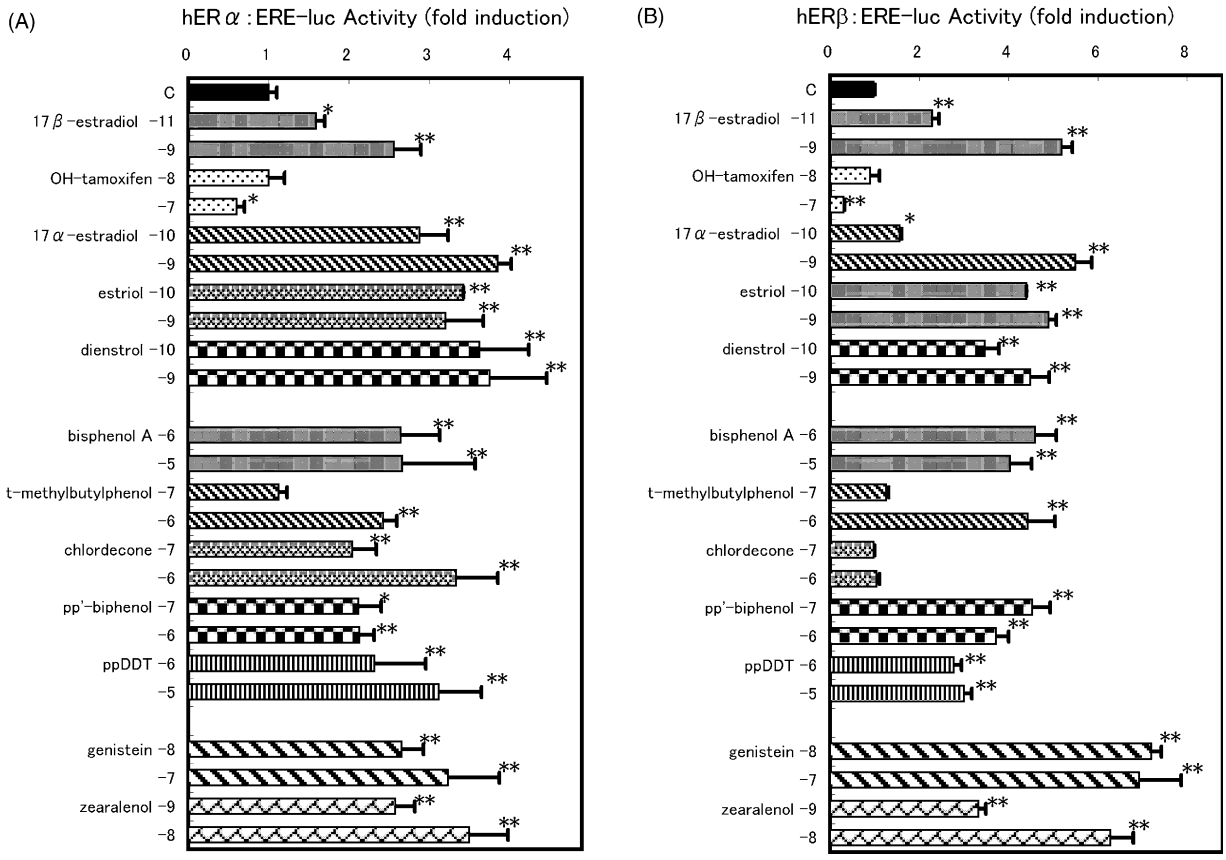


Fig. 2. ERE-luc reporter assays of a series of environmental chemicals in NIH 3T3 cells. NIH 3T3 cells were co-transfected with human ERα or ERβ. The responses are expressed as fold changes relative to each control activity (mean ± S.E.M.). Concentrations of chemicals were indicated in log[molar concentration]; (*) and (**) indicate significant difference from the control activity.

the exception of DDT. In contrast, only ERE-luc induction was observed though action of ERβ.

A number of in vitro detection methods have been developed for screening of endocrine disrupting chemicals, including receptor binding assay, hormone responsive cell proliferation assays, and reporter gene assays. Since binding to the receptor does not necessarily result in transactivation, assessment of this in itself has limitations [13]. For cell proliferation assays of estrogenic activity, human breast cancer cell lines such as MCF-7 and T47D have been used as well as rat pituitary cells as proposed by ourselves and others [14–17]. Because the mechanisms of hormone responsive cell growth are rather complex and are not fully understood, there is a problem with numbers of false positives and negatives. Thus, assays which can detect biologically relevant hormonal activity with a simple mechanism have become widely sought after [18,19]. Most of the assays utilize the classical estrogen pathway involving on ER and an ERE responsive element in either mammalian cells or yeast. Upon the ligand binding, the interaction of liganded receptor and the ERE results in recruitment of several transcriptional cofactors, including p160/p300 complexes, to initiate transcription of specific genes [6].

However, some genes such as collagenase, insulin-like growth factor 1 and ovalbumin genes, are known to be regulated by estrogen without containing any ERE motifs in their gene promoters and it has been found that their regulation is mediated through the AP1 motif [6–8,20]. Although several other promoter/enhancer motifs, such as Alu DNA repeats and the Sp1 element, may also be involved in estrogen dependent transactivation, AP1 mediated action seems to be the most common for the non-ERE estrogen responses. When ER mediated transcription at collagenase promoter was examined, the response for estrogen was observed only with ERα but not with ERβ [21], which suggested differential biological roles of ERα and ERβ regarding AP1. Interestingly, partial estrogen antagonists such as tamoxifen induce both ERα:AP1 and ERβ:AP1 initiated transcription. As we have reported previously, ER mediated AP1dependent transcription can be reconstructed with a simple AP1 reporter containing only consensus AP1 motifs, the approach utilized in the present study. The mechanisms of ER action at AP1 sites remain unclear, but it is interesting that substantial transcriptional differences exist between ERα and ERβ limited to the AP1 and not in the classical ERE pathway. We previously reported suppressive effects of ERβ co-existing with ERα regarding AP1 mediated

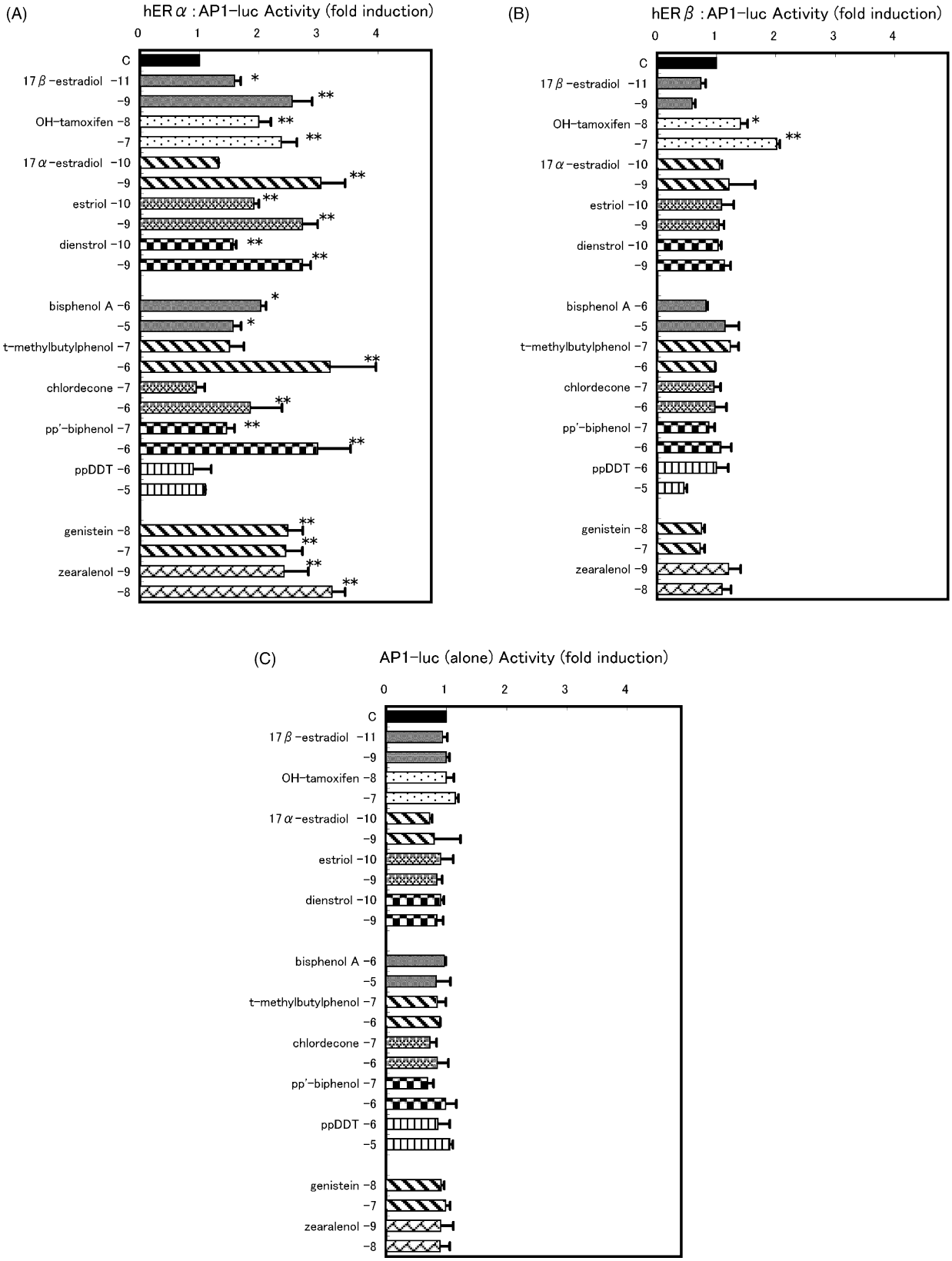


Fig. 3. AP1-luc activity in NIH 3T3 cells of a series of environmental chemicals. NIH 3T3 cells were co-transfected with human ERα or ERβ. The responses are expressed as fold changes relative to each control activity (mean ± S.E.M.). Concentrations of chemicals were indicated in log[molar concentration]; (*) and (**) indicate significant difference from the control activity.

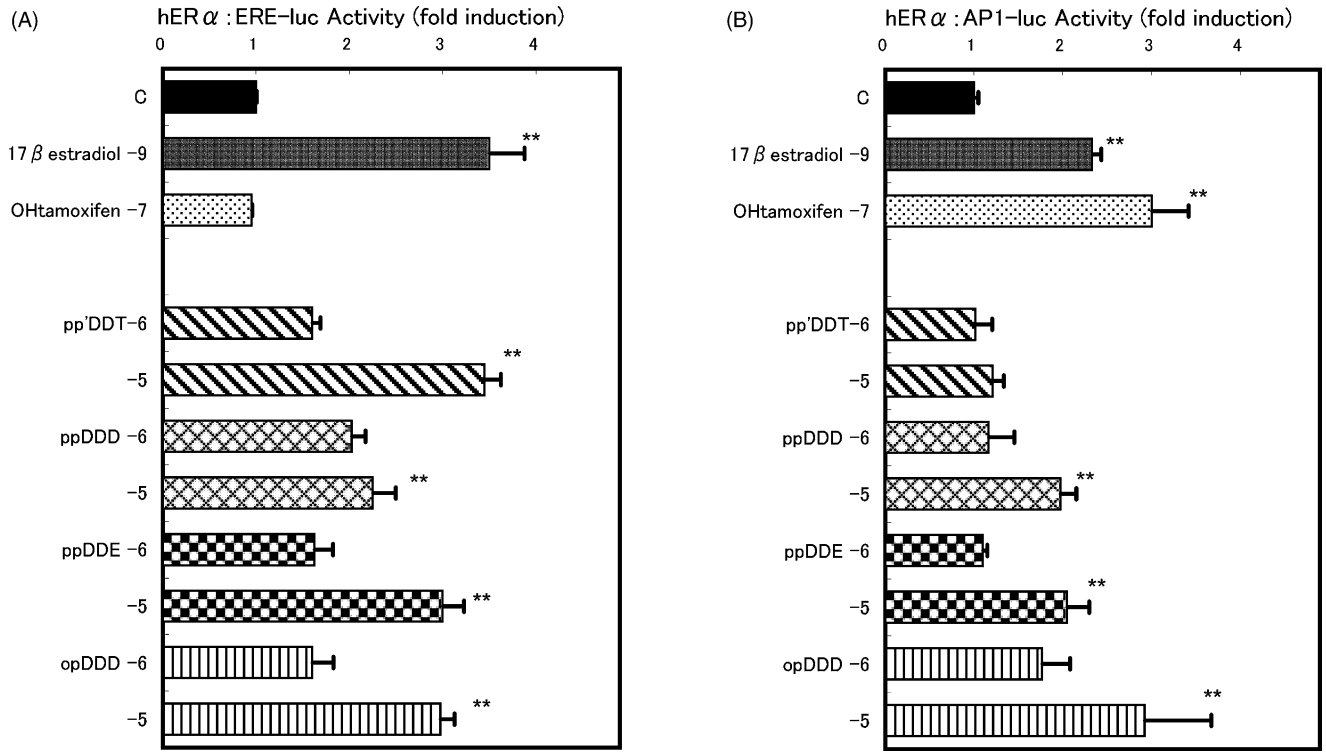


Fig. 4. ERE- and AP1-luc activities in NIH 3T3 cells induced by *p*,*p*′-DDT and isomers. The responses are expressed as fold changes relative to each control activity (mean ± S.E.M.). Concentrations of chemicals were indicated in log[molar concentration]; (*) and (**) indicate significant difference from the control activity.

transactivation [22]. The findings thus clearly indicated that responses to environmental estrogenic chemicals potentially differ between the AP1 and ERE pathways.

Here we chose a series of environmental chemicals previously reported to be estrogenic, including physiological estrogens, 17β-estradiol, 17α-estradiol, estroidol and dienstrol, environmental chemicals, bisphenol A, methylbutylphenol, cholordecon, *p*,*p*′-biphenol and DDTs, and two phytoestrogens, genistein (isoflavonoid) and zearelenol (mycotoxin). We first confirmed that all the compounds were indeed estrogenic with the ERα:ERE pathway in MCF-7 cells and determined doses for sub-maximal and maximal responses for application in the reporter assay with NIH 3T3 cells. Comparison of ERα:ERE and ERβ:ERE responses demonstrated close similarities except chlordecon which was a specific activator for ERα and genistein activating ERβ mediated response more effectively. These results were consistent with a report by Kuiper et. al. [23]. When ERα:ERE and ERα:AP1 responses were compared, a substantial difference was found for *p*,*p*′-DDT, which was clearly estrogenic at $10^{-7}$ to $10^{-5}$ M in the ERE system, but did not exert any AP1-luc activity. None of the compounds in the present study caused any ERβ:AP1 transcription. A reporter experiment with transfected of AP1-luc alone to examine non-ER mediated AP1 actions confirmed no activity.

Since no ERα:AP1 responses were found with *p*,*p*′-DDT, three isomers were also examined, all of them reported to bind to ERα. In the ERα:ERE-luc assay, the activity of *o*,*p*′-DDD was relatively high, similar to *p*,*p*′-DDT, while *p*,*p*′-DDD and *p*,*p*′-DDE showed lower potency [24]. However, unlike *p*,*p*′-DDT, all three isomers also exhibited ERα:AP1 activity. The reason for the lack of action of *p*,*p*′-DDT remains unclear.

Despite the limited number of compounds tested, the hypothesis that there might be differences in responses between ER:ERE and ER:AP1 to environmental endocrine disrupting chemicals was clearly proven. All of them failed to induce ERβ:AP1 transcription. Whether any compounds can evoke ERβ:AP1 action remains to be clarified.

## Acknowledgements

We thank Mr. Y. Mizuno for his expert technical assistance and Dr. M.A. Moore for reading the manuscript and suggesting English clarification. This work was supported in part by Grant-in-Aid (H13-Seikatsu) from the Ministry of Health, Labor and Welfare, Japan and a Grant-in-Aid (#14042241) from the Ministry of Education, Culture, Sports, Science, and Technology, Japan.

## References

[1] T. Colborn, S.F. Vom, A.M. Soto, Developmental effects of endocrine-disrupting chemicals in wildlife and humans, Environ. Health Perspect. 101 (1993) 378–384.

[2] V. Krishnan, S. Safe, Polychlorinated biphenyls (PCBs), dibenzo-*p*-dioxins (PCDDs), and dibenzofurans (PCDFs) as antiestrogens in MCF-7 human breast cancer cells: quantitative structure–activity relationships, Toxicol. Appl. Pharmacol. 120 (1993) 55–61.
[3] V.A. Baker, Endocrine disrupters-testing strategies to assess human hazard, Toxicol. In Vitro 15 (2001) 413–419.
[4] L. Rhomberg, Beyond screening: problems and prospects for risk characterization of endocrine disruptors, Regul. Toxicol. Pharmacol. 26 (1997) 74–79.
[5] S.M. Hyder, G.L. Shipley, G.M. Stancel, Estrogen action in target cells: selective requirements for activation of different hormone response elements, Mol. Cell Endocrinol. 112 (1995) 35–43.
[6] N.J. McKenna, J. Xu, Z. Nawaz, S.Y. Tsai, M.J. Tsai, B.W. O'Malley, Nuclear receptor coactivators: multiple enzymes, multiple complexes, multiple functions, J. Steroid Biochem. Mol. Biol. 69 (1999) 3–12.
[7] M.P. Gaub, M. Bellard, I. Scheuer, P. Chambon, C.P. Sassone, Activation of the ovalbumin gene by the estrogen receptor involves the fos–jun complex, Cell 63 (1990) 1267–1276.
[8] A. Philips, D. Chalbos, H. Rochefort, Estradiol increases and anti-estrogens antagonize the growth factor-induced activator protein-1 activity in MCF-7 breast cancer cells without affecting *c*-fos and *c*-jun synthesis published, J. Biol. Chem. 268 (1993) 14103–14108.
[9] Y. Umayahara, R. Kawamori, H. Watada, E. Imano, N. Iwama, T. Morishima, Y. Yamasaki, Y. Kajimoto, T. Kamada, Estrogen regulation of the insulin-like growth factor I gene transcription involves an AP1 enhancer, J. Biol. Chem. 269 (1994) 16433–16442.
[10] P. Webb, G.N. Lopez, R.M. Uht, P.J. Kushner, Tamoxifen activation of the estrogen receptor/AP1 pathway: potential origin for the cell-specific estrogen-like effects of antiestrogens, Mol. Endocrinol. 9 (1995) 443–456.
[11] P. Webb, P. Nguyen, C. Valentine, G.N. Lopez, G.R. Kwok, E. McInerney, B.S. Katzenellenbogen, E. Enmark, J.A. Gustafsson, S. Nilsson, P.J. Kushner, The estrogen receptor enhances AP1 activity by two distinct mechanisms with different requirements for receptor transactivation functions, Mol. Endocrinol. 13 (1999) 1672–1685.
[12] S. Maruyama, N. Fujimoto, K. Asano, A. Ito, Suppression by estrogen receptor beta of AP1 mediated transactivation through estrogen receptor alpha, J. Steroid Biochem. Mol. Biol. 78 (2001) 177–184.
[13] V.A. Baker, P.A. Hepburn, S.J. Kennedy, P.A. Jones, L.J. Lea, J.P. Sumpter, J. Ashby, Safety evaluation of phytosterol esters. Part 1. Assessment of oestrogenicity using a combination of in vivo and in vitro assays, Food Chem. Toxicol. 37 (1999) 13–22.
[14] C.W. Chen, C. Hurd, D.P. Vorojeikina, S.F. Arnold, A.C. Notides, Transcriptional activation of the human estrogen receptor by DDT isomers and metabolites in yeast and MCF-7 cells, Biochem. Pharmacol. 53 (1997) 1161–1172.
[15] N. Fujimoto, S. Maruyama, A. Ito, Establishment of an estrogen responsive rat pituitary cell sub-line MtT/E-2, Endocr. J. 46 (1999) 389–396.
[16] S. Maruyama, N. Fujimoto, H. Yin, A. Ito, Growth stimulation of a rat pituitary cell line MtT/E-2 by environmental estrogens in vitro and in vivo, Endocr. J. 46 (1999) 513–520.
[17] A.M. Soto, C. Sonnenschein, K.L. Chung, M.F. Fernandez, N. Olea, F.O. Serrano, The E-SCREEN assay as a tool to identify estrogens: an update on estrogenic environmental pollutants, Environ. Health Perspect. 103 (Suppl. 7) (1995) 113–122.
[18] J.S. Edmunds, E.R. Fairey, J.S. Ramsdell, A rapid and sensitive high throughput reporter gene assay for estrogenic effects of environmental contaminants, Neurotoxicology 18 (1997) 525–532.
[19] K.W. Gaido, L.S. Leonard, S. Lovell, J.C. Gould, D. Babai, C.J. Portier, D.P. McDonnell, Evaluation of chemicals with endocrine modulating activity in a yeast-based steroid hormone receptor gene transcription assay, Toxicol. Appl. Pharmacol. 143 (1997) 205–212.
[20] L. Biegel, S. Safe, Effects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) on cell growth and the secretion of the estrogen-induced 34, 52, and 160 kDa proteins in human breast cancer cells, J. Steroid Biochem. Mol. Biol. 37 (1990) 725–732.
[21] K. Paech, P. Webb, G.G. Kuiper, S. Nilsson, J. Gustafsson, P.J. Kushner, T.S. Scanlan, Differential ligand activation of estrogen receptors ERalpha and ERbeta at AP1 sites, Science 277 (1997) 1508–1510.
[22] S. Maruyama, N. Fujimoto, K. Asano, A. Ito, Suppression by estrogen receptor beta of AP1 mediated transactivation through estrogen receptor alpha, J. Steroid Biochem. Mol. Biol. 78 (2001) 177–184.
[23] G.G. Kuiper, B. Carlsson, K. Grandien, E. Enmark, J. Haggblad, S. Nilsson, J.A. Gustafsson, Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors alpha and beta, Endocrinology 138 (1997) 863–870.
[24] R.J. Kavlock, G.P. Daston, C. DeRosa, P. Fenner-Crisp, L.E. Gray, S. Kaattari, G. Lucier, M. Luster, M.J. Mac, C. Maczka, R. Miller, J. Moore, R. Rolland, G. Scott, D.M. Sheehan, T. Sinks, H.A. Tilson, Research needs for the risk assessment of health and environmental effects of endocrine disruptors: a report of the US EPA-sponsored workshop, Environ. Health Perspect. 104 (1996) 4715–4740.