

## The Activity of Bisphenol A Depends on Both the Estrogen Receptor Subtype and the Cell Type

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**Abstract.** Bisphenol A (BPA), a monomer of plastic used in consumer products, is abundant in the environment and enters the body by ingestion or adsorption. In order to characterize the estrogenic effect of BPA, we performed luciferase assay on three independent cell lines derived from different tissues transfected with either human ER $\alpha$  cDNA or ER $\beta$  cDNA. The estrogenic activities of BPA were detectable in all cell lines via both ER $\alpha$  and ER $\beta$ . In 293T cells and Hec-1 cells, the estrogenic activities were significantly decreased when cells expressing ER $\alpha$  were incubated with 10<sup>-6</sup> M BPA in the presence of 10<sup>-8</sup> M 17 $\beta$ -estradiol (E2) while the activities via ER $\beta$  were essentially unchanged in the same conditions. Interestingly, no reduction of estrogenic activity was detected in HOS-TE85 cells via either ER $\alpha$  or ER $\beta$ . Our results indicate that BPA only acts as an agonist of estrogen via ER $\beta$  whereas it has dual actions as an agonist and antagonist in some types of cells via ER $\alpha$ . Thus, the activity of BPA may depend on the ER subtype and the tissue involved.

**Key words:** Bisphenol A, Estradiol, Estrogen receptor  $\alpha$ , Estrogen receptor  $\beta$ , Endocrine disruptor  
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**ENVIRONMENTAL** estrogens (xenoestrogens) are nonsteroidal, man-made chemicals that enter the body by ingestion or adsorption, bind to estrogen receptors and mimic estrogen actions [1, 2]. Bisphenol A (BPA) is one of the best known environmental estrogens. BPA has two unsaturated phenol rings and has no structural homology with estrogen. However, the structure of BPA is similar to diethylstilbestrol (DES), a potent synthetic estrogen, prenatal exposure to which in utero is known to cause genital abnormalities and carcinomas [3]. BPA is a monomer of polycarbonate plastics and BPA-based epoxy

together with polystyrene resins are used in many products, such as the inner coating of food cans, dental composites and drug delivery systems. BPA can be liberated from polycarbonate plastics subjected to high temperature [4] or from incomplete polymerized epoxy resins. Indeed, a significant amount of BPA was detected in the liquid from canned vegetables (20  $\mu$ g/can) that had been exposed to high temperature during autoclaving [5] and in the saliva (20–30  $\mu$ g/ml) of dental patients fitted with restorative materials [6].

In previous studies, several lines of evidence indicated that BPA has estrogenic activities [4, 7, 8]. The estrogenic activity of BPA was shown by an experiment in which BPA induced the expression of estrogen responsive genes and promoted cell proliferation in MCF-7, a breast cancer cell line [4]. In another experiment using rats, BPA also induced

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prolactin gene expression as well as its release both *in vivo* and *in vitro* [7]. Furthermore, BPA treatment induced the growth differentiation and *c-fos* gene expression in rat uterus and vagina, with similar effects to estrogen [8].

Estrogen receptor (ER), a ligand dependent transcription factor, specifically binds to estrogen, and regulates gene transcription via the estrogen responsive element (ERE). ER had been assumed to exist as a single species, until a novel estrogen receptor (ER $\beta$ ) was recently isolated in rats [9], humans [10, 11], and mice [12]. ER $\beta$  has a high degree of sequence homology with the classical estrogen receptor (ER $\alpha$ ). ER $\beta$  mRNA was detected predominantly in rat ovary, prostate, lung, brain, bladder, uterus and bone [9, 13–15], as well as in human breast cancer tissues [13]. ERs are even expressed in the preimplantation embryos [16]. Although ER $\beta$  has a slightly lower binding affinity for 17 $\beta$ -estradiol (E2) than ER $\alpha$  [9, 12, 13], its transactivating manner via ERE is similar to ER $\alpha$  [17, 18]. On the other hand, some of the transcription activating functions of ER $\beta$  are different from that of ER $\alpha$ , which depend on the ligand and its responsive element [12, 19, 20].

Previously, we reported that BPA in HeLa cell only exhibits an agonistic action of estrogen via ER $\beta$  whereas it has dual actions as both an agonist and antagonist via ER $\alpha$  [21]. In this study, we used the luciferase assay to evaluate the estrogenic and anti-estrogenic activity of BPA with ER $\alpha$  and ER $\beta$  using three cell lines derived from different tissues.

## Materials and Methods

### *Plasmid Construction*

The ER $\alpha$  cDNA originated from HEG0 [22] was cloned into pCXN2 expression vector [23], which can also express the neomycin resistance gene, to construct pCXN2-hER $\alpha$  [11]. The reverse transcription polymerase chain reaction (RT-PCR) product of ER $\beta$  cDNA was cloned into pCXN2 to construct pCXN2-hER $\beta$  [11]. The oligonucleotide containing the wild-type ERE of the *Xenopus vitellogenin* gene A2 enhancer was synthesized and inserted at the upstream position of the reporter plasmid for the luciferase assay [24]. The expression vector of sea pansy luciferase was also used as an internal control

(Picagene; dual luciferase assay kit, Toyo Inc, Tokyo, Japan).

### *Cell Culture and Luciferase Assay*

17 $\beta$ -estradiol (E2) was obtained from Sigma Chemical Company and dissolved with ethanol in a 1.5 ml microreaction tube. BPA was obtained from Aldrich Chemical Company and dissolved with ethanol in a 1.5 ml microreaction tube. They were diluted with cell culture medium to yield the desired final concentrations. The concentration of ethanol in the culture medium was kept at 0.1%.

In this study, the following human cell lines were used: 293T cells which is a human embryonic kidney cell line, Hec-1 cells from human endometrial cancer and HOS-TE85 cells from human osteosarcoma. They were grown in Dulbecco's modified Eagle's medium containing 10% fetal calf serum. 293T cells were transfected with the human ER $\alpha$  or ER $\beta$  expression vector, luciferase reporter plasmid and the plasmid with luciferase of sea pansy for the internal control as described previously [19]. One hour before transfection, cell culture medium was replaced with phenol red-free Dulbecco's modified Eagle's medium (GIBCO BRL, Rockville, MD, USA) containing 10% dextran-coated charcoal treated fetal bovine serum (HyClone, Logan, UT, USA). After 12 h of incubation, the cells were cultured further with or without 10<sup>-8</sup> M E2 or with 10<sup>-6</sup> to 10<sup>-10</sup> M of BPA for 24 h. Cell extracts were prepared and assayed for luciferase activity following the manufacture's instruction (Picagene; dual luciferase assay kit, Toyo Inc, Tokyo, Japan). Luciferase assay was performed with a MicroLumat Plus (EG&G BERTHOLD, Bad Wildbad, Germany). All data were standardized with the sea pansy luciferase activity.

### *Statistical Analysis*

Data are presented as means  $\pm$  standard deviation (SD) of three independent experiments. The differences between each treated value were evaluated by analysis of variance and Student's *t*-test.

## Results

We determined the estrogenic activity of BPA employing a cell based transcription assay system in which luciferase assays were carried out. A significant increase in estrogenic activity was detectable at  $10^{-6}$  M BPA concentration in the three kinds of cell line transfected with ER $\alpha$  (Fig. 1A, B, C), or ER $\beta$  (Fig. 1D, E, F).

The combination of E2 and BPA exhibited differential actions depending on which subtype of ER was expressed. In 293T cells expressing ER $\alpha$ , the luciferase activity with  $10^{-8}$  M of E2 and  $10^{-6}$  M of BPA was significantly reduced to 77.5% as compared to the luciferase activity with E2  $10^{-8}$  M alone (Fig. 2A). On the other hand, in 293T cells expressing ER $\beta$ , the luciferase activity with  $10^{-8}$  M of E2 and  $10^{-6}$  M of BPA was essentially the same as when incubated with E2  $10^{-8}$  M alone (Fig. 2D). Almost the same results were observed using Hec I cells. In Hec I cells expressing ER $\alpha$ , luciferase activity with  $10^{-8}$  M E2 and  $10^{-6}$  M BPA was significantly decreased to 72.9% as compared to the luciferase activity with E2  $10^{-8}$  M alone (Fig. 2B), while cells expressing ER $\beta$ , luciferase activities were essentially the same under both conditions (Fig. 2E). Interestingly, no anti-estrogenic activity was detected in HOS-TE85 cells expressing either ER $\alpha$  or ER $\beta$  (Fig. 2C, F).

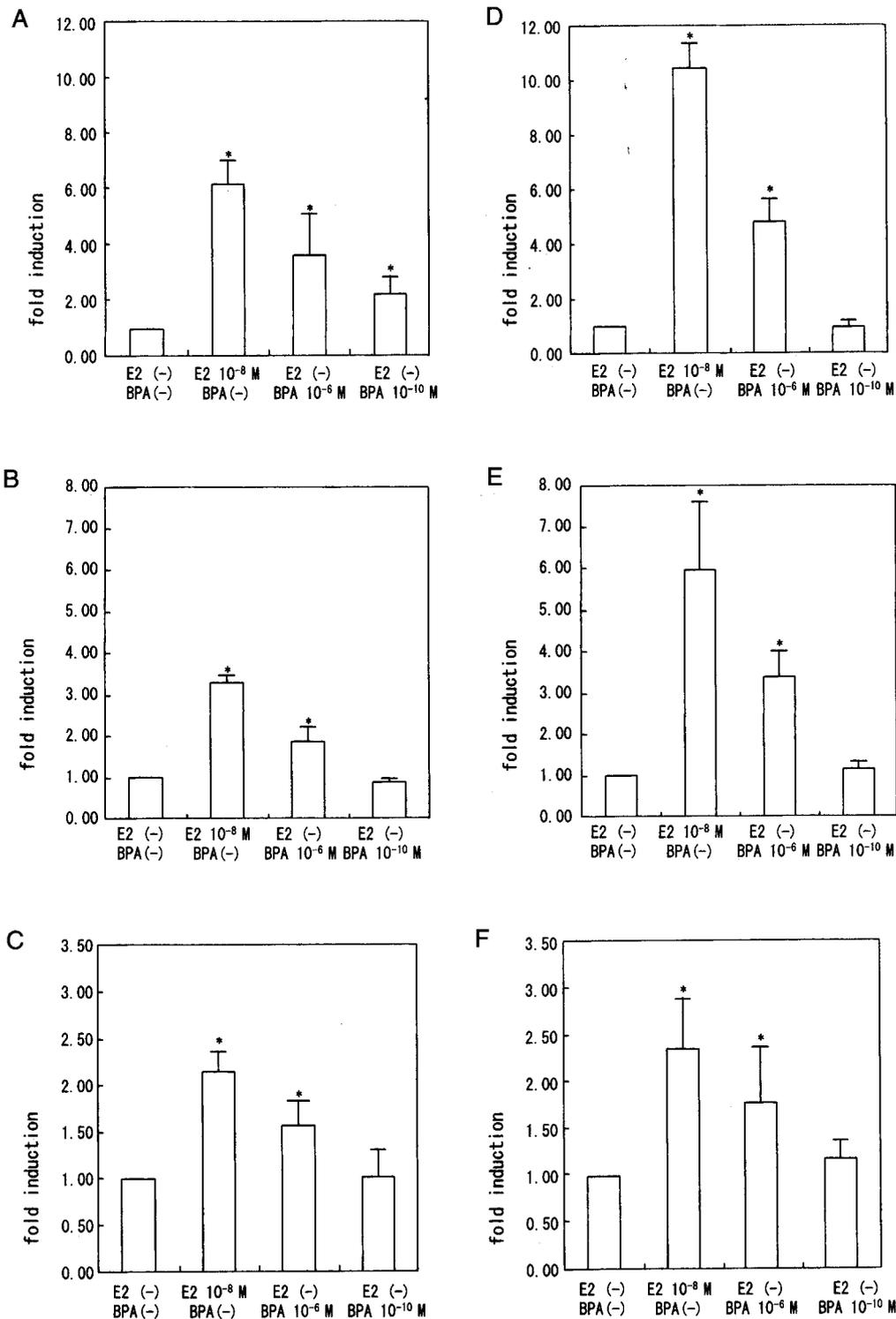
## Discussion

Previously, several lines of evidence indicated that BPA has estrogenic activities [4, 7, 8]. Kuiper *et al.* recently examined the estrogenic potency of various estrogenic chemicals via both ER $\alpha$  and ER $\beta$ , and found that the relative transactivation activities of  $10^{-6}$  M of BPA with ER $\alpha$  and ER $\beta$  were 50 and 41, respectively, when the relative transactivation activity of  $10^{-6}$  M E2 was set at 100 [2]. It was reported that  $10^{-9}$  M of BPA caused PRL release in a dose dependent manner in primary anterior pituitary cells [7]. Previously, we reported that  $10^{-8}$  M BPA exhibited approximately half of the estrogenic activity produced by E2 at equimolar concentrations, via either ER $\alpha$  and ER $\beta$  in HeLa cells, derived from uterine cervical carcinoma [21]. In the present study, the estrogenic activities of BPA were detectable in all cell lines via both ER $\alpha$  and ER $\beta$ . At the

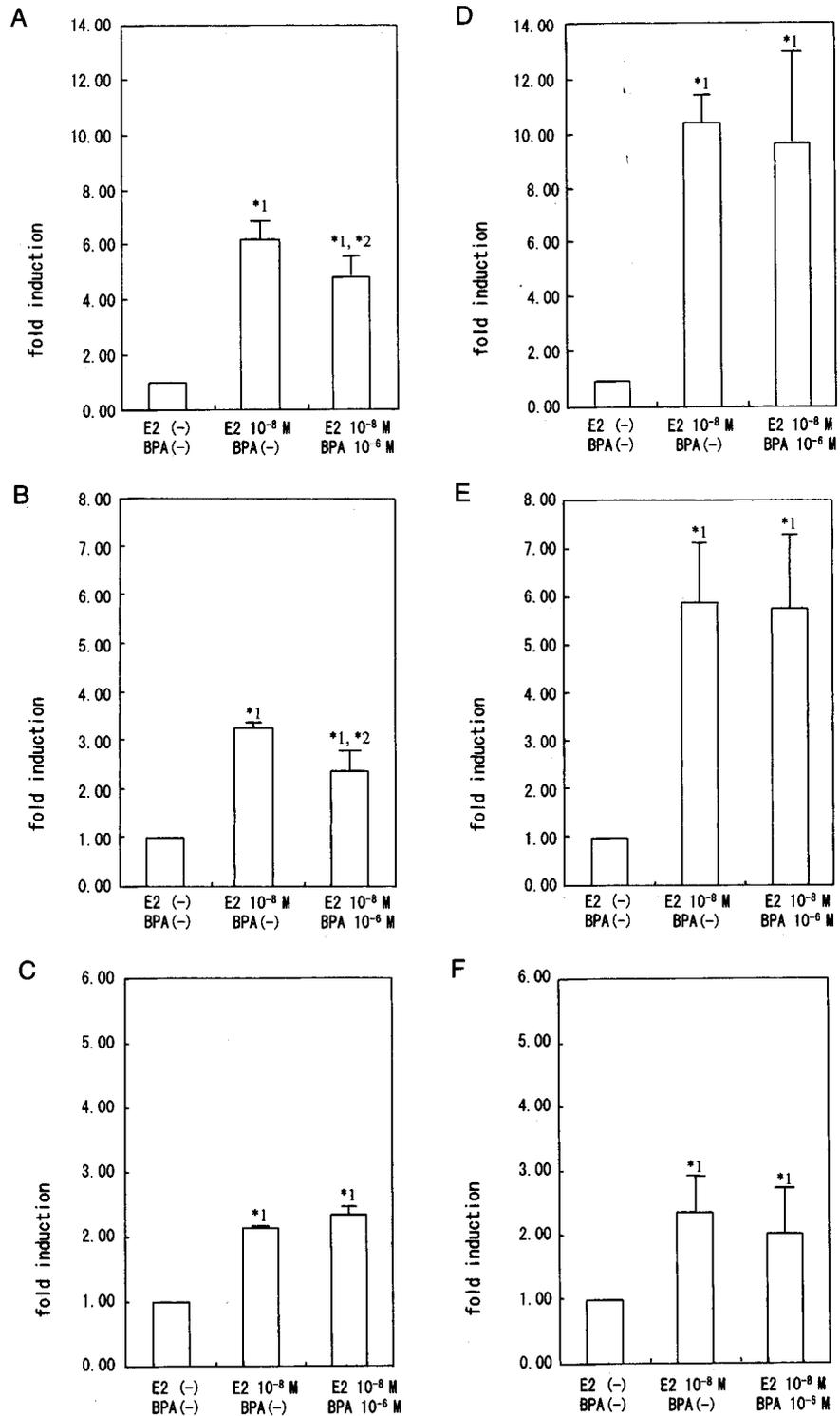
condition of  $10^{-6}$  M, BPA exhibited approximately half the estrogenic activity produced by  $10^{-8}$  M E2. It is possible that the activity of BPA via ERs may vary from tissue to tissue.

When BPA was added to the medium together with E2, it did not exert any additive effect via either ER $\alpha$  or ER $\beta$ . The antagonistic activity of BPA was only observed via ER $\alpha$  and was not detected via ER $\beta$  even when  $10^{-6}$  M BPA was added in the presence of  $10^{-8}$  M E2 using either 293T cells or Hec I cells. It is of interest to note that no antagonistic effect was detected in HOS-TE85 cells via either ER $\alpha$  or ER $\beta$  in the same setting (Fig. 2). While the reason for this difference is at present unknown, it is speculated that it might reflect the differential expression of transcriptional coactivators or differences in the stability of the receptor protein in each cell line. It is also possible that any antagonistic effect may vary depending on the concentrations of BPA and ERE-promoter context of the reporter gene. ER $\alpha$  and ER $\beta$  expression vectors have chicken  $\beta$ -actin promoter and CMV-IE enhancer, which do not contain estrogen responsive element. There is no previous report that the activity of this promoter is influenced by estrogen or BPA. It is thus assumed that the amount of ER subtype protein is almost same in each condition in the present study.

It was reported that fetal exposure to low doses BPA led to increased prostate weight in adulthood and decreased efficiency of sperm production [25–27]. Recently, it was reported that there is a promotive effect on growth and puberty by fetal exposure of BPA in mouse [28]. Several lines of evidence have implicated the influence of environmental disruptors if exposure occurs during fetal stage. For instance, the early stages of embryonic development are affected by dioxins [29] and BPA [30, 31]. The tissue distribution of ER $\alpha$  and ER $\beta$  is different; moderate to high expression of ER $\alpha$  in rat was detected in uterus, testis, pituitary, ovary, kidney, epididymis and adrenal, while moderate to high expression of ER $\beta$  in rat was detected in prostate, ovary, lung, bladder, brain, uterus and testis [13]. From the results of this report, the activity of BPA via ERs may vary from tissue to tissue. Although the estrogenic/anti-estrogenic activity of BPA may occur in tissues which express ER, the magnitude of activity is unknown because the distribution and concentration of BPA in tissues are unclear. It is possible that the antago-



**Fig. 1.** Transcriptional activity of BPA and/or 17 $\beta$ -estradiol for ER $\alpha$  (A, B, C) and ER $\beta$  (D, E, F). 293T cells (A, D), Hec-1 cells (B, E) and HOS-TE85 cells (C, F) were transfected with the luciferase reporter plasmid containing ERE, an internal control sea pansy luciferase expression plasmid and either the human ER $\alpha$  or ER $\beta$  expression plasmid. After transfection, the cells were incubated for 12 h in the absence of 10<sup>-8</sup> M 17 $\beta$ -estradiol (E2) and then incubated for another 24 h with or without BPA (10<sup>-9</sup> to 10<sup>-6</sup> M) and/or E2 10<sup>-8</sup> M. Each bar and point represents means  $\pm$  SD (n = 3). \*, P < 0.05 by Student's *t*-test compared with control.



**Fig. 2.** Anti-estrogenic activity of BPA and/or 17β-estradiol for ERα (A, B, C) and ERβ (D, E, F). 293T cells (A, D), Hec-1 cells (B, E) and HOS-TE85 cells (C, F) were transfected with the luciferase reporter plasmid containing ERE, an internal control sea pansy luciferase expression plasmid and either the human ERα or ERβ expression plasmid. After transfection, cells were incubated for 12 h in the absence of 10<sup>-8</sup> M 17β-estradiol (E2) and then incubated for another 24 h with or without BPA (10<sup>-9</sup> to 10<sup>-6</sup> M) and/or E2 10<sup>-8</sup> M. Each bar and point represents means ± SD (n = 3). \*1, P < 0.05 by Student's *t*-test compared with control. \*2, P < 0.05 by Student's *t*-test compared with 17β-estradiol treated samples.

nistic activity is being specifically detected in tissue expressing ER $\alpha$  and containing a high concentration of BPA.

It is known that ER $\alpha$  and ER $\beta$  form heterodimers, hence it is conceivable that BPA might exert its effects through the heterodimers as well. If this were the case, it is far from clear whether BPA elicits its activity in the same manner with heterodimers as homodimers. In this study, we demonstrated the ER subtype-specific BPA effects. In view of the varying ratios of ER subtypes among respective tissues along with present data, it probably safe to say that BPA exerts different effects dependent on the target tissue.

In the present study, we determined that BPA mimics the action of estrogen in a specific way, which depends on the ER subtype and cell line, as deter-

mined by an *in vitro* transcription stimulation assay. These results suggest that the modes of actions for environmentally produced estrogen-like substances are more complex than expected. At present, the biological influences of the substance remains to be clarified. The present results offer clues to future studies as to how environmental chemicals affect human health.

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