Effects of Tea Catechins on the ERE-Regulated Estrogenic Activity

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Tea catechins exert many biological effects, including anticancer and antibacterial activities. Also, it is reported that some plant flavonoids exhibit estrogenic activity. In this study, we investigated estrogenic or antiestrogenic activities of catechins in HeLa cells transiently transfected with an estrogen response element (ERE)-regulated luciferase reporter and an estrogen receptor (ER) α or ERβ expression vector. Catechins alone did not induce luciferase (luc) activity in either of the ERs. Addition of 17β-estradiol (E_2) plus epicatechin gallate (ECG) or epigallocatechin gallate (EGCG) at 5×10^{-6} M resulted in significant decreases in the ERα-mediated luc activity compared with that of E_2 alone. On the contrary, lower concentrations significantly increased the E_2-induced luc activity. Similar effects were observed with tamoxifen. The ERβ-mediated estrogenic activities were stimulated by catechins. In conclusion, some catechins, particularly EGCG, were antiestrogenic for ERα at higher doses, and co-estrogenic for ERα at lower doses and for ERβ. The lower doses were found in human plasma after tea-drinking. In addition, some catechins may be antiendocrine disruptors because they suppressed bisphenol A-induced luc activities.

Keywords: *Catechins; epigallocatechin gallate; estrogen; estrogen receptor; tamoxifen; bisphenol A*

INTRODUCTION

Many biological effects of catechins of tea (*Camellia sinensis*), including anticancer, antiinflammatory, and antibacterial activities, have been reported. Especially, epigallocatechin gallate (EGCG) contained more than 50% in green tea catechins (Hara, 1997), showed the strongest effects among tea catechins. EGCG inhibited growth of a number of tumor cell lines (Chen et al., 1998; Otsuka et al., 1998) by inducing apoptosis (Paschka et al., 1998; Yang et al., 1998b; Gupta et al., 2000). EGCG has been described as an inhibitor of telomerase activity, supposed to be one of the major mechanisms underlying the anticancer effects of tea (Naasani et al., 1998). EGCG also inhibits endothelial cell growth in vitro and angiogenesis in vivo (Cao and Cao, 1999), suggesting a potential antimetastatic activity toward cancer cells.

On the other hand, some plant flavonoids, such as daidzein, genistein, kaempferol, and naringenin, exhibit weak estrogenic activity in the competitive ligand binding, cell proliferation, and gene expression assays (Martin et al., 1978; Safe and Gaido, 1998). They could be preventive or therapeutic for estrogen-deficient diseases, such as menopausal disorders and osteoporosis, and estrogen-dependent cancers. In this study, we investigated any estrogenic or antiestrogenic activities of tea catechins, which are also flavonoids, using an estrogen response element (ERE)-regulated luciferase reporter gene (Klinge et al., 1997).

The human estrogen receptors (ERs), which are members of the nuclear receptor superfamily, have two isotypes, ERα and the recently identified ERβ (Mosselman et al., 1996; Ogawa et al., 1998). The ERα and ERβ are expressed in a tissue-specific manner. In general, whereas ERα is expressed in major female organs such as ovaries, uterus, vagina, and mammary glands, ERβ is expressed in the ovary and male organs such as testis and prostate (Mosselman et al., 1996; Muramatsu and Inoue, 2000). ERα and ERβ might play different roles in gene regulation (Paech et at., 1997). Some chemicals, including tamoxifen (Tam), the anti breast cancer drug, are found to exhibit estrogenic and antiestrogenic activity by using ERα and ERβ expression vectors/ERE-regulated luciferase reporter gene assays (Paech et al., 1997). In the present study, an ERα or ERβ expression vector was respectively transfected into HeLa cells, which are nonresponders for estrogen, to estimate activities of tea catechins to an ERα- or ERβ-mediated system. We compared the effects of tea catechins with Tam in this report.

In addition, it is known that certain chemicals in the environment such as bisphenol A (BPA) and 4-*n*-nonylphenol (NP) are estrogenic (Krishnan et al., 1993; Soto et al., 1995; Fielden et al., 1997). They seem to result in modulating the endocrine system to contribute to adverse health, reproduction, and developmental effects in humans and wildlife (Guillette et al., 1994; McLachlan et al., 1980), and, therefore, are commonly

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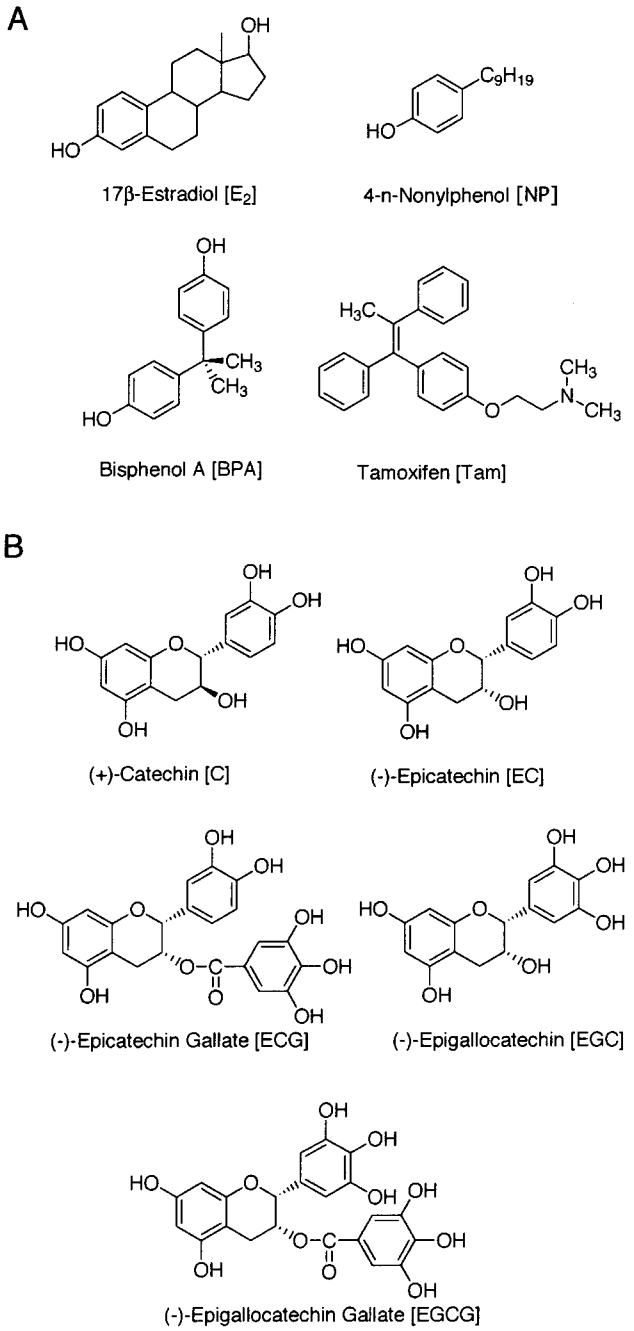

Figure 1. Structures of ER ligands (A) and tea catechins (B).

referred to as endocrine disruptors. We examined the estrogenic activity of BPA and NP in the assay and the effects of catechins against these endocrine disruptors.

MATERIALS AND METHODS

Materials. Tam, 17β-estradiol (E_2), catechin (C), epicatechin (EC), epicatechin gallate (ECG), epigallocatechin (EGC), and EGCG were obtained from Sigma (St. Louis, MO). BPA and NP were purchased from Kanto-Chemical (Tokyo, Japan). Structures of these chemicals are shown in Figure 1.

Cell Culture. HeLa S3 cells were maintained in Dulbecco's modified Eagle medium (DMEM) (Nissui, Tokyo, Japan) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Sigma) and 100 μg/mL each of sodium ampicillin and kanamycin sulfate at 37 °C under humidified 5% CO_2 /95% air.

Transfection of HeLa Cells for ERα- and ERβ-Mediated Luciferase Assay. HeLa S3 cells (5×10^5) were plated in each well of a 6-well costar plate in the assay medium [phenol red-free DMEM (Cosmo Bio, Tokyo, Japan) supplemented with 10% heat-inactivated charcoal/dextran treated FBS (Hyclone, Logan, UT)]. After 24 h, the cells were cotransfected for ERα-mediated assay with 0.9 μg of pGL3-pro-3(EREc38) (Klinge et al., 1997), 0.45 μg of pCMV5hERα (Wrenn and Katzenellenbogen, 1993), and 0.9 μg of πH3M-β-gal (Kuruto-Niwa et al., 1998) per well. For the transfection, DNA was preincubated with Tfx-20 (Promega, Madison, WI) with a ratio of 1 μg DNA/3 nmol Tfx-20 in DMEM for 15 min at room temperature (Schenborn et al., 1996; Ishimi et al., 1998). HeLa cell medium was removed and replaced with 0.9 mL of preincubated Tfx-20/DNA mixture, and cells were incubated at 37 °C for 60 min in a CO_2 incubator. At the end of the incubation period, 1.8 mL of fresh assay medium was added to the mixture. In addition, indicated amounts of estrogen and/or the test materials or an equal volume of ethanol or DMSO (solvent) as a control was added, and cells were further incubated. For ERβ-mediated assay, pCXN2-hERβ (Ogawa et al., 1998) was used instead of pCMV5hERα.

Luciferase and β-Galactosidase Assays. After 24 hours of transfection, cells were harvested and washed twice with phosphate-buffered saline (PBS). The cells were then lysed in 100 μL of lysis solution (100 mM potassium phosphate pH 7.8, 0.2% Triton X-100, 1 mM dithiothreitol). The luciferase (luc) activity was assayed using a PicaGene kit (Toyo ink, Tokyo, Japan).

β-galactosidase activity in cell extracts was measured by a Galacto-Light Plus chemiluminescent reporter assay kit (Tropix, Bedford, MA) in order to normalize the transfection efficiency in each experiment (Jain and Magrath, 1991).

Statistical Analysis. In all experiments, mean ± SEM is reported. Statistical comparisons among groups were determined using one-way analysis of variance (ANOVA). Individual comparisons were performed using Duncan's multiple test. Statistical significance was ascribed to the data when $p < 0.05$.

RESULTS

Assay for ER-Mediated Estrogenic Activity in HeLa Cells. First, we examined E_2-induced expression of a luciferase reporter gene in HeLa cells. Luciferase activity was measured in HeLa cells by transient transfection with an ERE-regulated luciferase reporter gene [pGL3 pro-3(EREc38)], which contains three tandem copies of EREc38 (a 38-bp ERE consensus sequence) at the upstream of the SV40 promoter linked to the firefly luciferase reporter gene (Klinge et al., 1997). By transfection of the ERα or ERβ expression vector, E_2 induced a dose-dependent increase in luc activity (Figure 2A and B). The ERα-mediated estrogenic activity increased up to 10^{-8} M E_2, whereas the ERβ-mediated estrogenic activity was less enhanced by E_2 than the ERα-mediated activity and reached a plateau at 10^{-10} M E_2. In contrast, no luc activity was observed in HeLa cells by E_2 without ER expression vector (Figure 2C), confirming that HeLa cells are not estrogen-responsive cells. The following experiments were performed at the lower concentration of E_2 in which the transfected HeLa cells were responsive, that is 10^{-9} M.

BPA and NP, which are known as exoestrogens, were also examined in this assay. The ERα-mediated induction by BPA was dose-dependent up to 10^{-6} M but weaker than that by E_2, whereas NP did not induce the luc activity up to 10^{-5} M (Figure 2D and E). The ERβ-mediated induction by BPA and NP was not observed at all (data not shown). Therefore, 10^{-7} M of BPA, which was a lower and effective concentration in the HeLa cells, was used in the ERα-mediated reporter system.

The ER-mediated estrogenic activities of five catechins were then assessed using a luciferase reporter

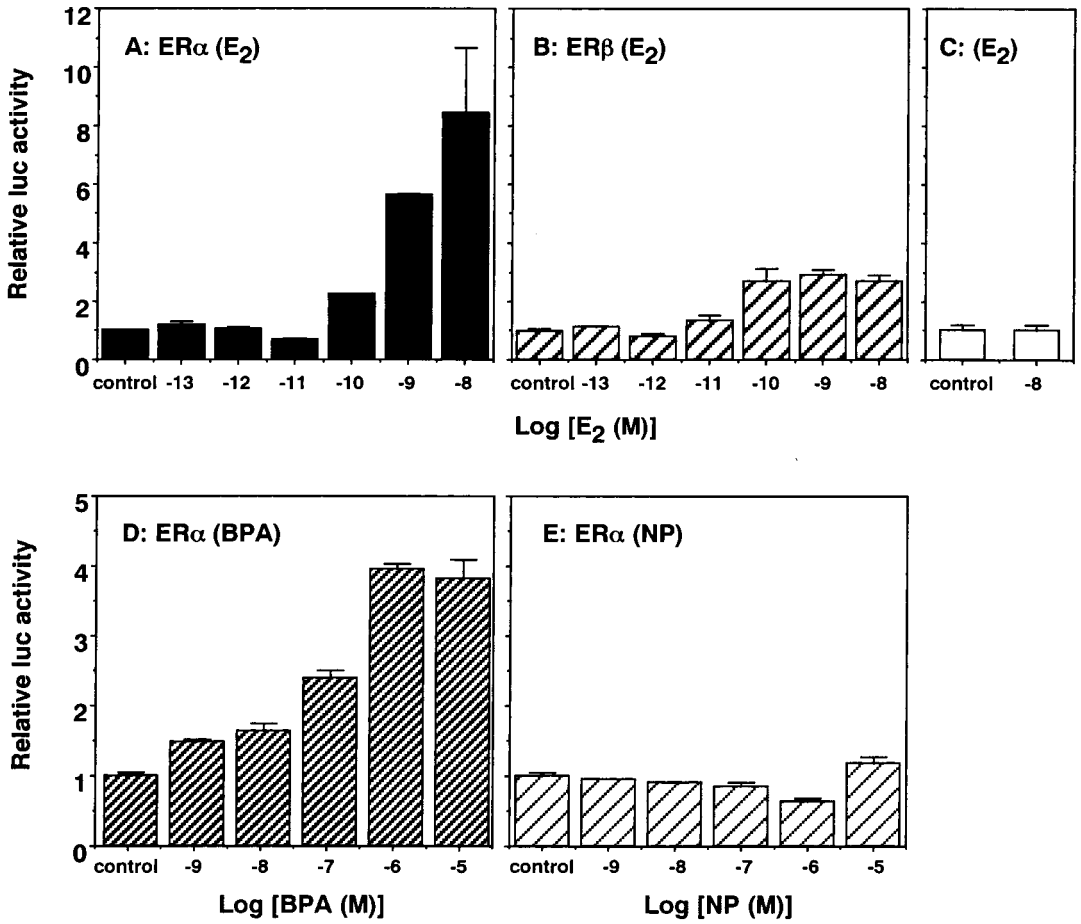

Figure 2. ER-mediated induction of the ERE-regulated luc activity by E_2, BPA, and NP. HeLa cells were transfected with pGL3-pro-3(EREc38), πH3M-β-gal, and (A, D, and E) pCMV5hERα, (B) pCXN2hERβ, or (C) none. Transfected cells were treated with the indicated concentrations of (A − C) E_2, (D) BPA, or (E) NP. The controls were treated with ethanol or DMSO at a final concentration of 0.1%. Data are the means ± SEM from two different experiments.

gene assay. No catechins, that is C, EC, ECG, EGC, and EGCG, induced luc activity at concentrations ranging from 10^{-8} to 10^{-5} M in either the ERα or ERβ assay system (data not shown).

Inhibition of E_2/ERα-Mediated Reporter Activity by Catechins. We investigated the synergistic or antagonistic response of catechins with E_2 through ERα in the ERE-regulated reporter gene assay. In Figure 3, transfected cells were treated with E_2 (10^{-9} M), catechins (5×10^{-6} M), E_2 plus catechins, Tam (5×10^{-7} M), and E_2 plus Tam. Tam, which is well-known as an estrogen antagonist, was used as a control for antagonistic activity. Co-treatment with E_2, ECG, and EGCG resulted in significant decreases in luc activity compared with the treatment with E_2 alone, which induced an approximately 10-fold induction of luciferase activity (Figure 3). However, the effects of ECG and EGCG at 5×10^{-6} M were weaker than that of 5×10^{-7} M Tam (Figure 3). In contrast, C, EC, and EGC at this concentration did not significantly affect E_2-induced luc activity.

Dose-Dependent Effects of Tam, ECG, and EGCG on E_2/ERα-Mediated Reporter Activity. Tam alone did not induce luc activity at concentrations ranging from 5×10^{-9} to 5×10^{-7} M (Figure 4A). However, Tam had an agonistic activity with 10^{-9} M Ez through ERα in the lower concentration range (5×10^{-8} and 5×10^{-9} M), in great contrast to an antagonistic activity at 5×10^{-7} M (Figure 4A).

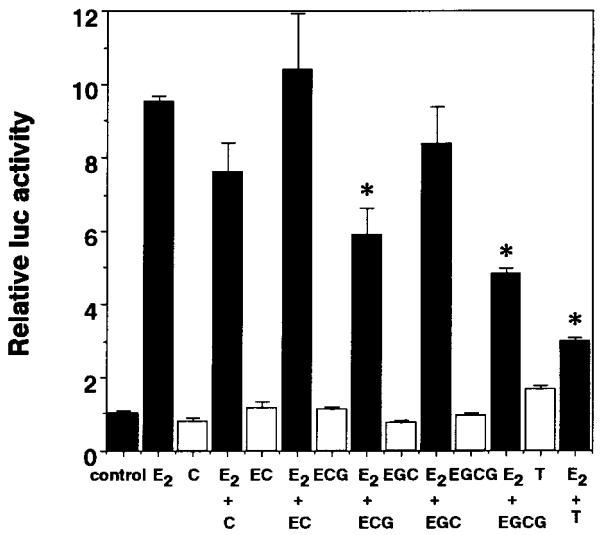

Figure 3. ERα-mediated effects of catechins on E_2-induction. HeLa cells were transfected with pGL3-pro-3(EREc38), πH3M-β-gal, and pCMV5hERα. Transfected cells were treated with E_2 (10^{-9} M) alone, catechins (5×10^{-6} M) alone, E_2 plus catechins, Tam (T) (5×10^{-7} M), or E_2 plus T. The controls were treated with ethanol at a final concentration of 0.1%. Data are the means ± SEM from three different experiments. *, significantly different from E_2 at $p < 0.05$ by Duncan's multiple test.

EGCG at 5×10^{-6} M suppressed the activity of estrogen (Figures 3 and 4C), whereas lower concentra-

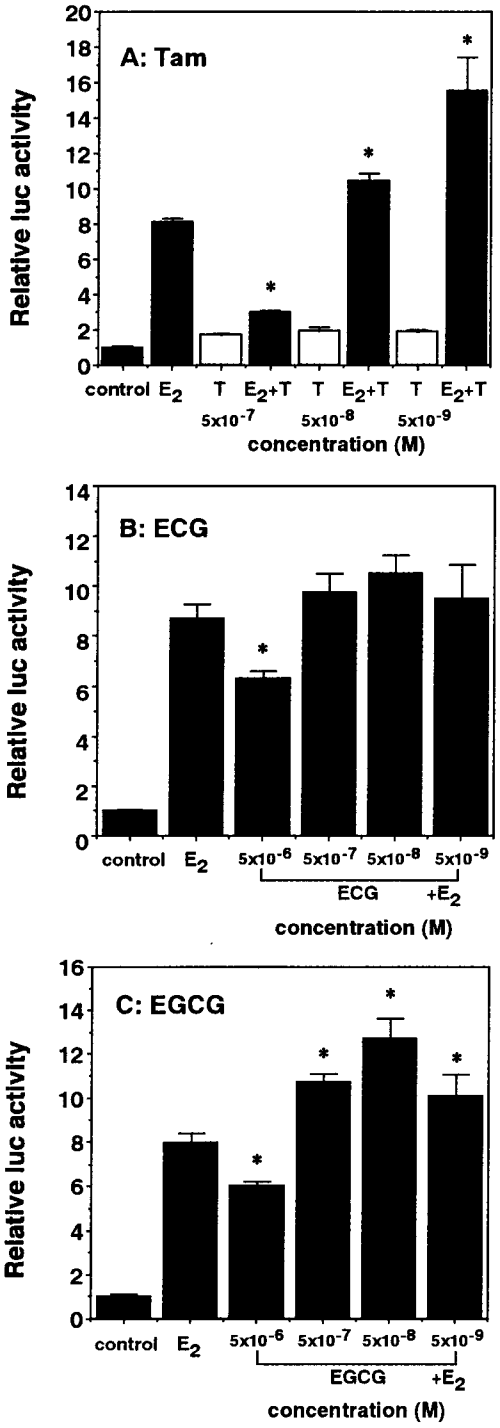

Figure 4. ERα-mediated effects of Tam, ECG, and EGCG on E_2-induction. HeLa cells were transfected with pGL3-pro-3(EREc38), πH3M-β-gal, and pCMV5hERα. Transfected cells were treated with E_2 (10^{-9} M) alone and the indicated concentrations of (A) Tam (T), (B) ECG, or (C) EGCG with or without E_2. The controls were treated with ethanol at a final concentration of 0.1%. Data are the means ± SEM from three different experiments. *, significantly different from E_2 at $p < 0.05$ by Duncan's multiple test.

tions of EGCG (5×10^{-9} to 5×10^{-7} M) resulted in significant increases in luc activity (Figure 4C). Therefore, EGCG seemed to have effects similar to those of Tam, although the effect of EGCG was approximately 10-fold less than that of Tam (Figure 4A and C). ECG at lower concentrations seemed to increase E_2-induced luc activity, although the effect was not statistically significant (Figure 4B).

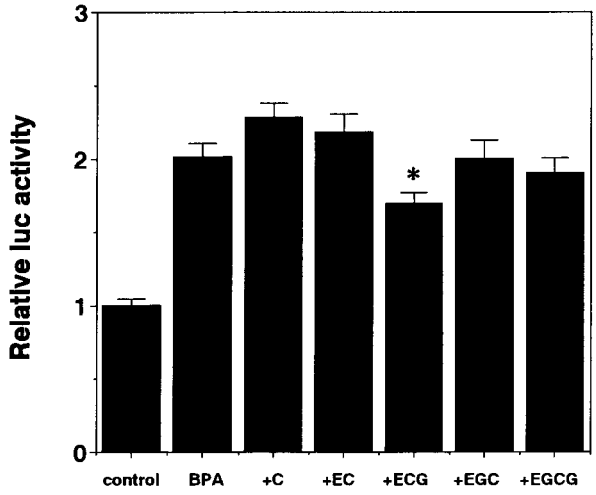

Figure 5. ERα-mediated effects of catechins on BPA-induction. HeLa cells were transfected with pGL3-pro-3(EREc38), πH3M-β-gal, and pCMV5hERα. Transfected cells were treated with BPA (10^{-7} M) alone and BPA plus catechins (5×10^{-6} M). The controls were treated with DMSO at a final concentration of 0.1%. Data are the means ± SEM from four different experiments. *, significantly different from BPA at $p < 0.05$ by Duncan's multiple test.

Effects of Catechins on BPA/ERα-Mediated Reporter Activity. Figure 5 shows the results of co-treatment with BPA plus catechins. Transfected cells were treated with BPA (10^{-7} M) alone and BPA plus catechins (5×10^{-6} M). Addition of ECG brought a significant suppression of BPA-induced luc activity (Figure 5). EGCG seemed to decrease BPA-induced luc activity, although the effect was not statistically significant. The lower concentrations of these catechins slightly increased BPA-induced luc activity (data not shown).

Effects of Catechins on E_2/ERβ-Mediated Reporter Activity. We also examined any effects of catechins on the E_2-induced activity through ERβ in the ERE-regulated reporter gene assay. Tam at 5×10^{-7} M completely inhibited the activity (Figures 6 and 7A). Co-treatment with E_2 and catechins (5×10^{-6} M) was not inhibitory, but catechins except ECG significantly increased the luc activity compared with that of E_2 alone (Figure 6).

Figure 7B−D shows that EC, EGC, and EGCG increased the luc activity in the presence of E_2 in a dose-dependent manner. The results were similar to that of EGCG at the lower concentrations in the E_2/ERα system (Figure 4C). On the contrary, lower concentrations of Tam (5×10^{-8} and 5×10^{-9} M) had no effects with E_2 (Figure 7A). This result was different from that in ERα, where Tam increased the E_2-induced activity at the lower concentrations.

DISCUSSION

We investigated the ERα or ERβ-mediated effects of five catechins, that is C, EC, ECG, EGC, and EGCG, using an ERE-regulated reporter gene assay. No catechins exhibited ER-mediated estrogenic activities at concentrations ranging from 10^{-8} to 10^{-5} M in either ERα or ERβ assay system, indicating that they did not act as agonists by themselves. Plasma levels of EGCG concentration were reported to be 1 to 3×10^{-7} M after drinking two or three cups of tea (Yang et al., 1998a).

Then, we examined the synergistic response of E_2 plus catechins through ERα or ERβ compared with Tam.

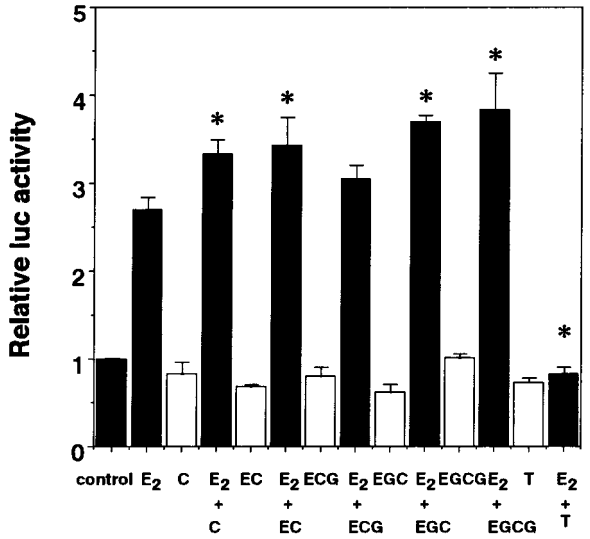

Figure 6. ERβ-mediated effects of catechins on E_2-induction. HeLa cells were transfected with pGL3-pro-3(EREc38), πH3M-β-gal, and pCXN2hERβ. Transfected cells were treated with E_2 (10^{-9} M) alone, catechins (5×10^{-6} M) alone, E_2 plus catechins, Tam (T) (5×10^{-7} M), or E_2 plus T. The controls were treated with ethanol at a final concentration of 0.1%. Data are the means ± SEM from three different experiments. *, significantly different from E_2 at $p < 0.05$ by Duncan's multiple test.

Tamoxifen is known as an antiestrogen that is used in breast cancer chemotherapy, but exhibits agonist activity in the uterus (Grainger et al., 1996; Kedar et al., 1994). With ERα, the higher amounts of ECG or EGCG (5×10^{-6} M) had inhibitory effects against estrogen (10^{-9} M) (Figure 3). However, 10^{-9} M of estrogen is excessive and not the usual level in vivo. From our results, the effects of excessive estrogen might be inhibited by high doses of ECG and EGCG. Several groups have reported that increased consumption of green tea by heavy drinking or taking green tea tablets inhibit the progress of breast cancer (Fujiki et al., 1999; Nakachi et al., 1998). Our results provide new insights for the mechanisms of action of tea catechins as a possible preventive for estrogenic cancer. Because ECG and EGCG have a gallate residue in their structures, the residue might be essential for the anti-estrogenic activity. Hashimoto et al. (1999) reported that the presence of galloyl moiety in the structures of ECG and EGCG was responsible for their high affinity for the lipid bilayers and increased intracellular uptake. This affects the membrane structure and partly accounts for the high activities in the in vitro experiments.

On the contrary, lower amounts of catechins, especially EGCG, increased the luc activity (Figure 4). These indicated that opposite effects were found at high and low doses. These contradictory effects are usually observed in endocrinological studies; low concentrations of a hormone can stimulate a tissue, but high concentrations can have the opposite effect (vom Saal et al., 1997). Tam has also the estrogenic activity through ERα in lower concentrations (5×10^{-8} to 10^{-9} M) in the presence of estrogen (Figure 4A). This enhanced induction suggested that ECG or EGCG were capable of potentiating the E_2 activity at the lower concentrations. This could be attained in human plasma after tea drinking (Yang et al., 1998a). Therefore, usual tea drinking might be helpful for the prevention of estrogen-deficient diseases such as menopausal disorders and osteoporosis.

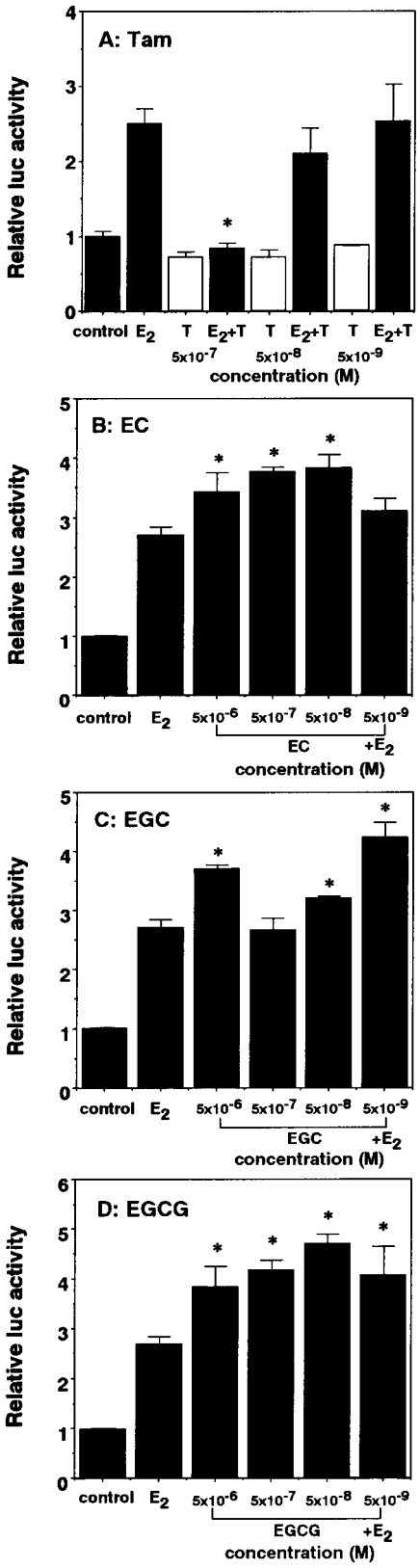

Figure 7. ERβ-mediated effects of Tam, EC, EGC, and EGCG on E_2-induction. HeLa cells were transfected with pGL3-pro-3(EREc38), πH3M-β-gal, and pCXN2hERβ. Transfected cells were treated with E_2 (10^{-9} M) alone, the indicated concentrations of (A) Tam (T), (B) EC, (C) EGC, or (D) EGCG with or without E_2. The controls were treated with ethanol at a final concentration of 0.1%. Data are the means ± SEM from three different experiments. *, significantly different from E_2 at $p < 0.05$ by Duncan's multiple test.

On the other hand, catechins were not inhibitory but increased the ERβ-mediated luc activity compared with E_2 alone at either of the concentrations tested in our experiments (Figures 6 and 7). Tam inhibited the activity at 5×10^{-7} M, but had no significant activity through ERβ in lower concentrations (5×10^{-8} and 5×10^{-9} M) in the presence of estrogen (Figure 7A), quite different from the effects through ERα. Hall et al. (1999) reported that the partial agonist activity of Tam through ERα was abolished upon coexpression of ERβ. Also, the effects of catechins on E_2 induction were sightly different between ERα and ERβ in this study. These results suggested different regulatory functions for the two ER subtypes. In fact, ERα and ERβ are differently expressed in several organs (Enmark et al., 1997; Kuiper et al., 1997). Their ultimate roles became clear by knockout mice experiments of each ER gene (Muramatsu and Inoue, 2000). Ogawa et al. (1998) reported that ERα and ERβ could interact in vivo by cross-signaling each other.

We also investigated the interaction of BPA, an estrogenic environmental contaminant exposed during plastic manufacturing, instead of E_2, through the ERα-transfected HeLa cells. Co-treatment with BPA plus ECG or EGCG resulted in little decreases in luc activity compared to the treatment with BPA alone (Figure 5). However, the concentration of BPA in this experiment, that is 10^{-7} M, is extremely high and beyond the level of environmental contamination. In fact, BPA concentrations in healthy human serum were low (0−1.6 ng/mL) (Sajiki et al., 1999). The present results suggested that some catechins might be expected to be antiendocrine disruptors.

Recently it was reported that 2 to 3% of ERα and ERβ are localized to the cell membrane, and that membrane and nuclear ERs originate from a single transcript (Razandi et al., 1999). In the present study, we investigated only the nuclear ERα- and ERβ-mediated effects using the ERE-regulated reporter gene. Catechins might affect the membrane ERs and be involved in the nongenomic signaling pathways. Because several coactivators of ER were found (Muramatsu and Inoue, 2000) and they might also interact with catechins, further studies are essential.

ABBREVIATIONS USED

EGCG, epigallocatechin gallate; ERE, estrogen response element; ER, estrogen receptor; Tam, tamoxifen; BPA, bisphenol A; NP, 4-*n*-nonylphenol; E_2, 17β-estradiol; C, catechin; EC, epicatechin; ECG, epicatechin gallate; EGC, epigallocatechin; DMEM, Dulbecco's modified Eagle medium; FBS, fetal bovine serum; luc, luciferase.

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