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Screening of Chemopreventive Tea Polyphenols Against PAH Genotoxicity in Breast Cancer Cells by a XRE-Luciferase Reporter Construct

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Abstract: *Polycyclic aromatic hydrocarbons (PAHs) are established cancer initiators that can be found in our food and environment. Some dietary plant polyphenols are strong inhibitors to PAH-induced mutagenesis, whereas others may not be as effective. To identify the chemopreventive compounds from a huge volume of dietary components, the development of an efficient screening method is required. In this study, a xenobiotic response element (XRE)-luciferase reporter plasmid was constructed to screen for some potential chemopreventive agents in tea against PAH-induced DNA damage. Tea is one of the most consumed beverages worldwide, and its beneficial effects on health have been documented. Previous studies have claimed that tea polyphenols could be protective against various cancers, and the rich database can be a source for comparison. Among the green and black tea polyphenols, the XRE-luciferase reporter assays suggested that only epigallocatechin gallate (EGCG) was effective in reducing XRE-driven luciferase assay in MCF-7 cells at the concentrations tested. Further study indicated EGCG could reduce* CYP1A1 *and* CYP1B1 *mRNA abundances and decrease the DMBA-DNA lesions. The results of DNA covalent binding of all tea polyphenols tested were consistent with the XRE-reporter assays. This study illustrated that the XRE-reporter assay was a viable screening test for dietary chemopreventive agents against PAH-initiated breast mutagenesis. It has the advantages of shorter sample processing time and producing no radioactive waste over directly measuring the CYP1A1/1B1 expressions, DNA lesion, or gel mobility shift assay.*

Introduction

Xenobiotic responsive elements (XREs) are the domains in the promoter region of some xenobiotic responsive genes. These gene expressions can be regulated through the interaction of XRE and the dimer-containing aryl hydrocarbon receptor (AHR) and AHR nuclear translocator (ARNT). AHR is a cytosolic protein that binds to polycyclic aromatic hydrocarbon (PAH). The bound AHR then translocates to the nucleus and dimerizes with ARNT (1). Cytochrome *P*-450 *1A1* and *1B1* genes that biotransform PAHs can be regulated in this pathway (2,3). The involvement of AHR and CYP1 enzymes in PAH-induced carcinogenesis has been demonstrated in some gene-targeting experiments. Compared with 70% in wild-type mice, only 7.5% of *CYP1B1* knockout mice have developed lymphomas induced by dimethylbenz[a]anthracene (DMBA) (4). On the other hand, benzo[a]pyrene (BP) fails to induce *CYP1A1* expression in AHR null mice, and no BP-induced tumor has been observed in the null mice (5).

Breast cancer is one of the most prevalent cancers in women and is responsible for 32% of the average annual rate of all female cancers from 1992 to 1998 in the United States (6). From the migration pattern and breast cancer incidence, environment has been demonstrated to be a factor in the etiology of breast cancer. PAHs are commonly found in diesel exhaust, charbroiled meat, tobacco smoke, overheated cooking oil, etc. (7,8). These toxic compounds are metabolized into DNA-attacking species in our body, and an increased amount of PAH-DNA adducts has been uncovered in normal tissues adjacent to tumor of breast cancer patients than that in breast tissues of cancer-free females (9). By contrast, Rundle et al. (10) have not detected any association between PAH-DNA adducts isolated from breast cancer and normal breast tissue and have suggested that the sampling method can be subjected to bias (11). Nevertheless, interrupting PAH metabolism may still be feasible to avoid breast cancer.

Tea is a common beverage, and tea leaves are harvested from the plant *Camellia sinensis*. Green tea contains epicatechin (EC), (–)-epigallocatechin (EGC), (–)-epicatechin gallate (ECG), and (–)-epigallocatechin gallate (EGCG), whereas black tea polyphenols are composed of theaflavin (TF1), theaflavin-3-gallate (TF2A),

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theaflavin-3-gallate (TF2B), and theaflavin-3,3-digallate (TF3). Epidemiological and experimental studies have demonstrated that both green and black tea diminished cancer risk in breast, lung, esophagus, colon, and skin (12–15), and the initiation, promotion, and progression stages of tumorigenesis are suppressed. The clinical trial on the chemopreventive effects of green tea polyphenols against breast cancer has also begun in the United States (16). In view of the great interest in tea and breast cancer, we compared the protective effects of tea polyphenols against the genotoxicity of PAH in the breast cancer cells MCF-7 by an XRE-reporter construct in the present study.

MCF-7 cells have comparable expressions of AHR, *CYP1A1*, and *CYP1B1* as the non–tumor-derived breast epithelial MCF-10A cells (17), and MCF-7 cells have the advantages of simpler subculturing conditions and shorter doubling time than MCF-10 cells. With the assumption that XRE transactivation played a pivotal role in PAH genotoxicity, MCF-7 cells are useful in identifying chemopreventive compounds as suggested by Smith et al. (18).

Materials and Methods

Chemicals

DMBA and DMSO were obtained from Sigma-Aldrich Fine Chemicals (St. Louis, MO). Theaflavins were isolated as previously described (19). All other chemicals, if not stated, were acquired from Sigma-Aldrich.

Cell Culture

MCF-7 cells (gift from Dr. Craig Jordan, Northwestern University) were cultured in RPMI B 1640 phenol red free media (Sigma-Aldrich) and 10% fetal bovine serum (Invitrogen Life Technologies, Rockville, MD) at 37°C and 5% carbon dioxide. Subconfluent cell cultures were treated with DMBA and various concentrations of tea polyphenol.

XRE-Luciferase Gene Reporter Assay

A fragment with five XRE elements from rat *cyp1a1* 5′-flanking region was amplified from rat genomic DNA as described by Backlund et al. (20). No other response elements were identified in this fragment. The polymerase chain reaction (PCR) product was digested with *SmaI* and *BamHI* and subcloned into a firefly luciferase reporter vector pTABLuc (Clontech, Palo Alto, CA).

MCF-7 cells were seeded at 10^5 cells per well in 24-well plates. After 24 h, the cells were transiently transfected with 4.0 μg of the XRE reporter plasmid and 1.0 μg of renilla luciferase control vector pRL (Promega, Madison, WI) in LipofectAmine (Invitrogen Life Technologies). The transfection efficiency was monitored by the control vector. After 16 h, the medium was removed and the cells were treated with 1 μM DMBA and various concentrations of tea polyphenol for 24 h. The amounts of these two luciferases were determined using a Dual-Luciferase Assay Kit (Promega). The luciferase bioluminescence was measured by using a FLUOstar Galaxy plate reader. The XRE transactivation activities represented by firefly luciferase light units were then normalized by that of renilla luciferase.

Measurement of Cell Viability

Cell viability was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) straining as described by Mosmann (21). Briefly, MCF-7 cells were plated in 96-well plates at 10^4 cells per well, and 1 μM DMBA and various concentrations of tea polyphenols were administered. At the end of the treatment, 50 μl of 1 mg/ml MTT was added, and the cells were incubated at 37°C for 4 h. Cell viability was determined by the absorbance at 600 nm.

Measurement of DMBA-DNA Covalent Binding

This assay was performed as previously described (22). MCF-7 cells were plated in 6-well plates at 5×10^5 cells per well and allowed to attached for 24 h. Then 0.1 μg/ml medium of [G-^{3}H]-DMBA (68.0 Ci/mmol, Amersham, Arlington Height, IL) was administered with or without tea polyphenol. After 16 h, cells were washed twice with cold phosphate-buffered saline, trypsinized and pelleted. Nuclei were separated by incubating the cells for 10 min on ice in lysis buffer A (10 mM Tris-HCL, pH 7.5, 320 mM sucrose, 5 mM magnesium chloride and 1% Triton X-100). The nuclei were collected by centrifugation at 5,000 rpm for 10 min at 4°C after the incubation. The nuclei were then lysed by 400 μl lysis buffer B (1% sodium dodecyl sulfate in 0.5 M Tris. 20 mM EDTA and 10 mM NaCl, pH 9), followed by the treatment of 20 μl 20 mg/ml Proteinase K for 2 h at 48°C. After that, the samples were allowed to cool to room temperature and the residual protein was salted out by adding 150 μl of saturated NaCl. The samples were then subjected to centrifugation at 13,000 rpm for 30 min at 4°C. Genomic DNA was isolated from supernatant by ethanol precipitation, and redissolved in autoclaved water. Absorbances at 260 nm and 280 nm were employed to determine the amount and purity of the extracted DNA. The amount of DNA recovered from each well were ranged from 1.5 to 3.0 mg. DNA samples attained a 260 nm/280 nm ratio of >1.9 were used for scintillation counting.

Semiquantitative RT-PCR Assay

A reverse transcription-polymerase chain reaction (RT-PCR) assay was used to quantify mRNA expressions. Total RNA was isolated from cells grown in six-well plates (Costar) in triplicates by a method previously described (23). One microgram of RNA was used for cDNA synthesis, and the final volume was diluted to 20 μl. Primers of *CYP1A1*,

CYP1B1, and β-actin sequences as published formerly (24) and a Perkin Elmer Thermocycler (GeneAmp PCR System 2400) were utilized to amplify the target cDNAs separately after the first strand reaction. All PCR reactions consisted of 0.2 mmol/l dNTP, 2 μl cDNA, 0.2 μmol/l of each primer, 1×PCR buffer, and 1 U of Taq polymerase (Invitrogen). The conditions were 94°C for 45 s, 65°C for 45 s, 72°C for 1 min, and a final extension period of 7 min at 72°C. The amplification cycles were 25 for *CYP1A1*, 23 for *CYP1B1*, and 19 for β-actin. The PCR products were separated on 1.8% agarose gel, stained with ethidium bromide, and photographed. A scanner equipped with Scion Image software (Scion Corporation, Frederick, MD) was used to compare the optical density of the amplified fragments. The optical density of β-actin PCR product was the control normalizing those of *CYP1A1* and *CYP1B1*. The amount of mRNA and number of cycles were determined in separate experiments to ensure the linearity of signals.

Statistical Methods

A SigmaPlot software package (Chicago, IL) was utilized for statistical analysis. The results, whenever applicable, were analyzed by one-way ANOVA followed by Bonferroni's multiple comparison test if significant differences ($P < 0.05$) were observed. *t*-test was performed for the other comparisons.

Results

XRE-Driven Luciferase Activities

DMBA treatment increased the luciferase activity by about threefold. The addition of 5, 10, or 25 μM of the tea polyphenol TF1, TF2A, TF2B, TF3, EGC, and EC could not significantly reduce DMBA-induced luciferase activities. Among those tested, TF1 was able to potentiate DMBA-induced XRE transcription (Fig. 1B). EGCG was the only tea polyphenol that could decrease XRE-driven luciferase activities at 10 μmol/l (Fig. 1A). Quercetin, which was previously demonstrated (22) as a chemopreventive agent, was included as a positive control. MTT assay did not indicate that the number of viable cells was significantly affected by the 24-h treatments (data not shown).

Effects of Tea Polyphenols on DMBA-DNA Covalent Binding

Tritiated DMBA was added to MCF-7 cells in the presence or absence of tea polyphenol. The amount of [^{3}H]-DMBA-DNA binding was measured by scintillation counting. All tea polyphenols except EGCG-cotreated cultures had similar [^{3}H]-DMBA-DNA binding per microgram of DNA compared with the control (Fig. 2A and B). On the other hand, 10 μmol/l of EGCG significantly decreased DNA lesions (Fig. 2A). Five and 10 μmol/l of quercetin also reduced the DMBA-DNA covalent binding.

EGCG and Quercetin Inhibit DMBA-Induced *CYP1A1* and *CYP1B1* mRNA Expressions

CYP1A1/*1B1* are two genes with XREs in their promotor regions. The mRNA expressions of *CYP1A1*/*1B1* were performed on selected polyphenol-treated cultures, and the gel images of RT-PCR are shown in Fig. 3. Cell cultures cotreated with DMBA and EGCG or quercetin did demonstrate reductions in *CYP1A1*/*1B1* expressions. The other treatments did not illustrate any appearance decreases (some data not shown). These results were consistent with the luciferase assays.

Discussion

In this cell culture study, we employed an XRE-luciferase assay technique and found the green tea EGCG reduced DMBA-DNA covalent binding. This finding was similar to a previous study using *Salmonella typhimurium* revertant count as the screening method for chemopreventive agents in green tea (25). Compared with some flavonoids such as quercetin and baicalein (23), EGCG was a weak DMBA-DNA binding inhibitor. Because of the correlation of XRE-luciferase activities and DMBA-DNA covalent binding, the XRE-luciferase assay could be a viable screening system for the identification of chemopreventive phytosubstances.

DMBA, like other PAHs, is metabolized to its ultimate carcinogenic form by *CYP1A1* and *1B1* enzymes (26) and is a specific breast carcinogen in rodent models. PAHs can induce the enzyme expressions by binding to AHR and transactivating the XRE domains to increase the transcription of *cyp1a1* and *cyp1b1* genes. Nonetheless, XRE transactivation may also be driven by a protein tyrosine kinase-mediated signaling pathway (20) and is independent of AHR status. Theaflavins may inhibit *CYP1A1* expression through this protein kinase-mediated route (27). The XRE-reporter gene assay used in this study could cover both the AHR-dependent and -independent transactivation pathways. The reporter construct was not full-length promoters from *cyp1a1* or *cyp1b1*. Chemopreventive agents that affect promoter-binding regions other than XRE would not be identifiable by this assay.

Among the green tea catechins, EGCG has stronger, higher antiproliferative, antineoplastic, and antimutagenic activities than EC and EGC (28–30). Oral or topical administration of EGCG inhibits 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone–induced lung tumors (31), azoxymethane-induced colon cancer (32), and DMBA-induced breast cancer (33). These results indicate that EGCG is protective against chemical-induced carcinogenesis. Other studies have demonstrated that EGCG can inhibit the biotransformation of carcinogens by

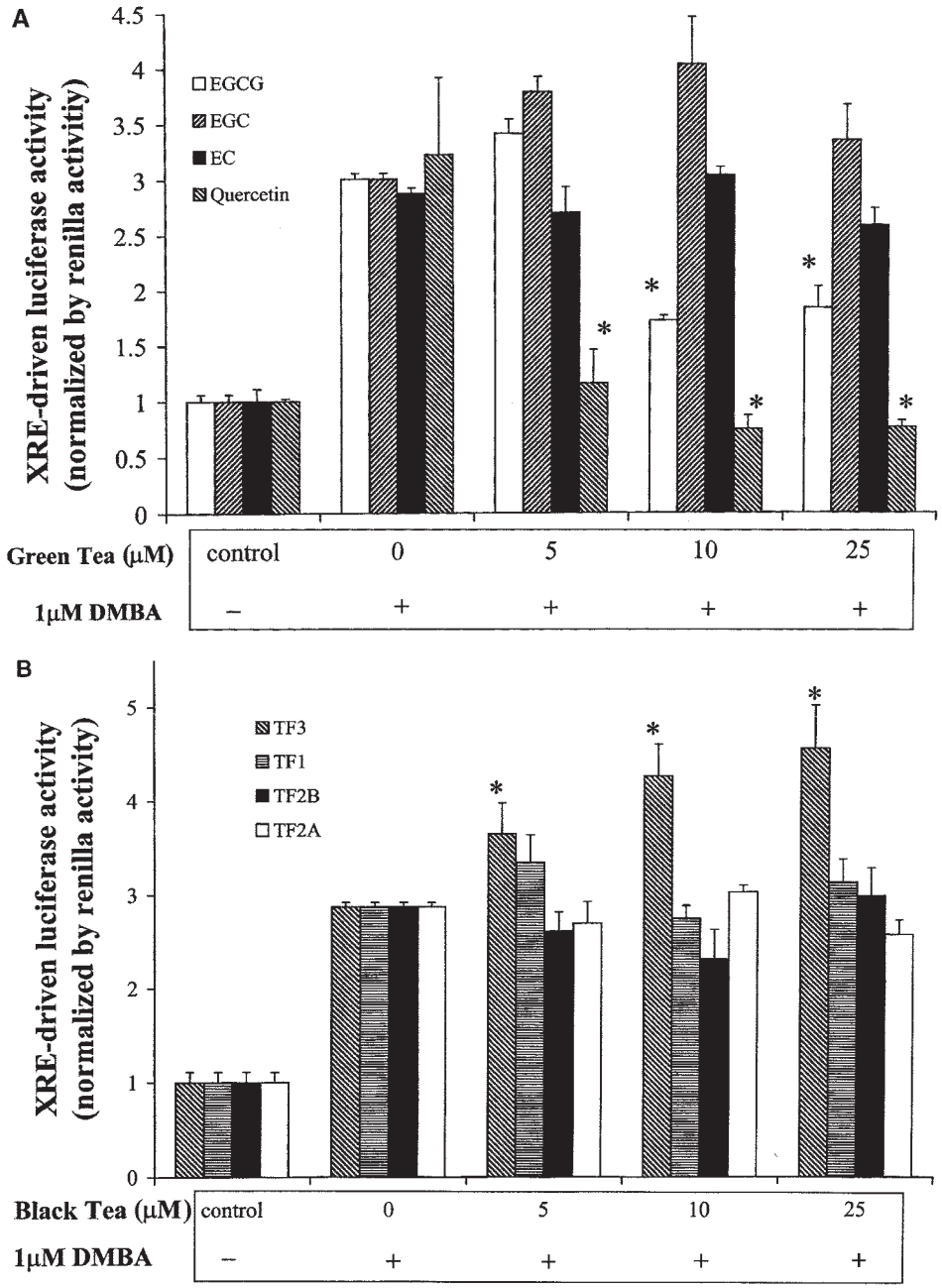

Figure 1. Effect of tea polyphenols on XRE-driven luciferase activities. MCF-7 cells were transiently transfected with a luciferase reporter gene containing XRE and a renilla luciferase control plasmid (pRL). Green (A) or black (B) tea polyphenol was co-administered with 1 μM DMBA for 24 h. The arbitrary light units of firefly luciferase were normalized by that of renilla luciferase, and their activities were measured by a Dual-Luciferase Assay Kit. Values are means ± SE, $n = 3$. Means marked with different letters significantly ($P < 0.05$) differ.

modulating some enzyme activities. It modulates *CYP1A* enzyme expression and activities and decreases the production of ultimate carcinogens (31,34). Our cell culture model was consistent with these findings.

Theaflavins also exhibit anticarcinogenesis effects in different models, and little is known about the mechanism of its chemoprevention. They inhibit neoplastic transformation in human lung cells (35), mammary cells, and rat tracheal epithelial cells (36). Similar to green tea polyphenols, they are strong antioxidants (19) and are capable of inhibiting DNA-carcinogen binding (37). Others have suggested that theaflavins may play a role in modulating carcinogen activation (38). Black tea has been demonstrated to be inhibitory in BP metabolism in a cell-free system (39) and in a hepatocarcinoma cell culture model (27). Our results did not support the notion that theaflavins extracted from black tea inhibited the bioactivation and binding of carcinogen to DNA at the cellular level. The disagreement could be explained by the lower concentrations used in our study than the former studies. Nevertheless, the dosages of tea polyphenols used in these studies including this investigation are higher than the submicromolar physiological range (40).

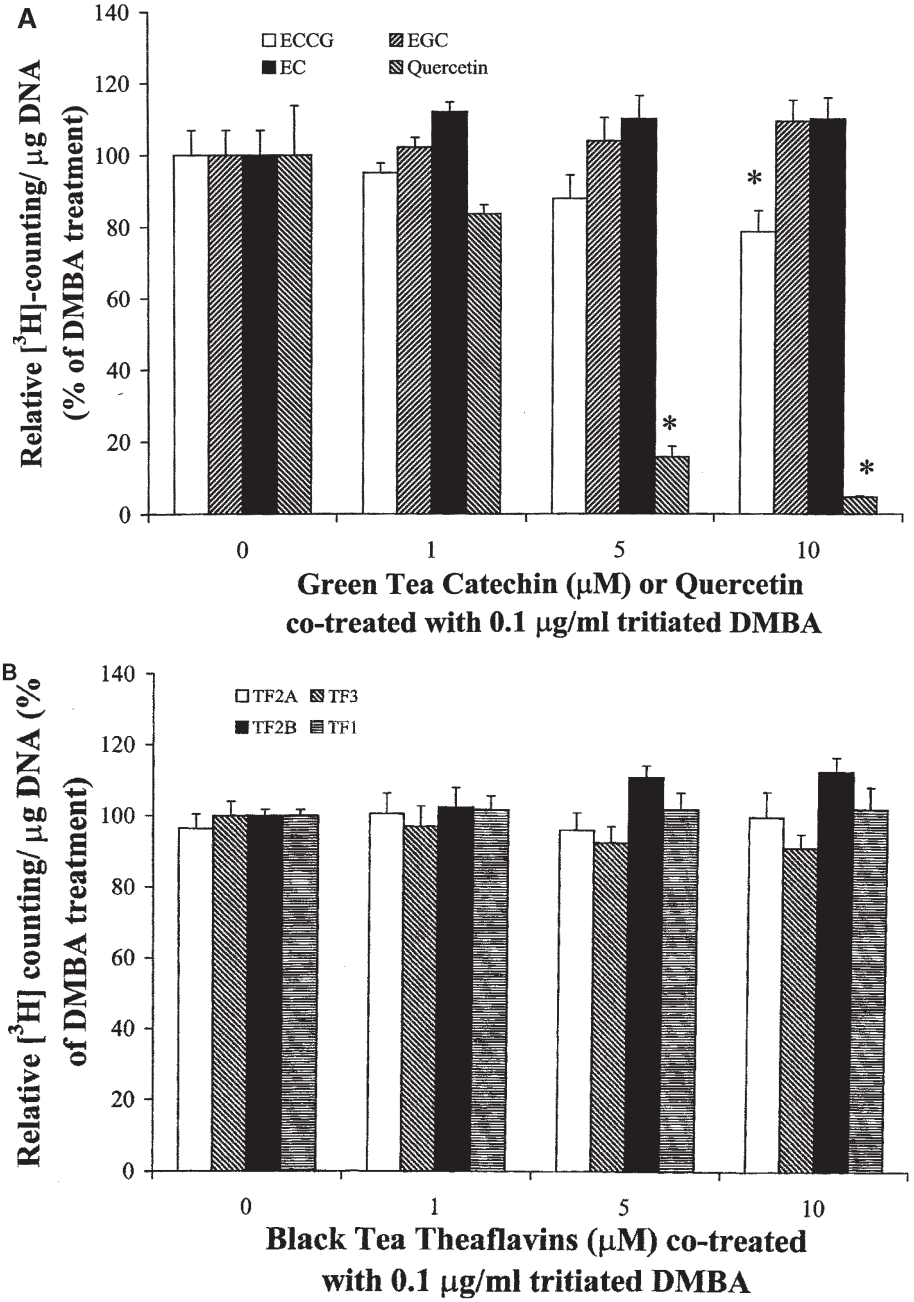

Figure 2. Effect of tea polyphenols on DMBA-DNA covalent binding. MCF-7 cells were cultured in six-well plates and treated with tritiated DMBA in 0.1 µg/ml DMSO and (A) green or (B) black tea polyphenol. After 24 h of treatment, genomic DNA was isolated, and the DMBA-DNA lesions were determined by scintillation counting. The counting represented the amount of the DMBA metabolites conjugated to the DNA samples. Values are means ±SE, $n = 3$. Means with different letters vary ($P < 0.05$).

Quercetin as demonstrated in this study was a more effective suppressor of XRE transactivation and a stronger inhibitor of DMBA-DNA binding than the catechins and theaflavins in the breast cells. Because quercetin is an agonist of AHR (22), it may compete for the binding of AHR and reduce the expressions of *CYP1A1/1B1* as illustrated in this study. The toxicity of DMBA could be reduced by the downregulation of the genes.

Black tea as well as green tea may affect the tumor promotion or progression processes (41–44). The present study did not address the effects at the promotion or the progression stage of carcinogenesis, which might partly explain the discrepancies between this and the animal studies described previously.

In summary, this study indicated a correlation between results of XRE-driven luciferase activities and DMBA-DNA lesions in MCF-7 cells, and the XRE-reporter assay could be a high-throughput screening tool for dietary chemopreventive compounds against PAH-induced genotoxicity in the breast. Compared with the alternative gel shift assay, a reporter assay does not generate radioactive waste and shorter sample-processing time.

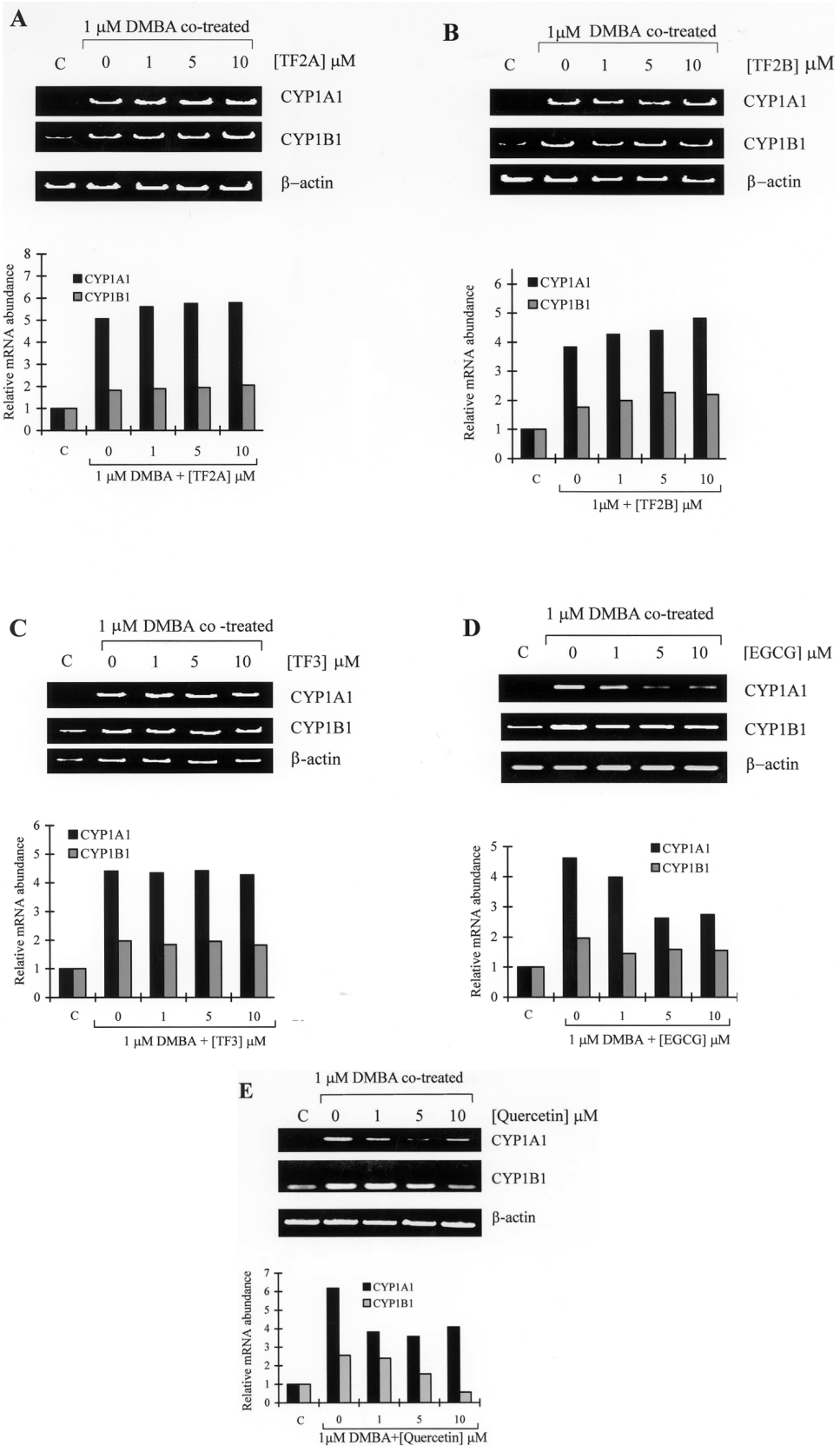

Figure 3. Effect of EGCG and quercetin on *CYP1A1/1B1* mRNA expression. MCF-7 cells were co-treated with 1 μmol/L DMBA and tea polyphenol and cultured for 24 h. mRNA expressions of *CYP1A1* and *CYP1B1* were quantified by RT-PCR as described in **Methods**. The gel images of EGCG, quercetin, and selected theaflavin treatments (A: TF2A, B: TF2B, C: TF3, D: EGCG, and E: quercetin) are shown. Lanes 0, 1, 5, and 10 are the tea polyphenol concentrations in μmol/l, C: control cultures received the carrier solvent DMSO only. The relative mRNA abundance is measured as the optical density of target PCR fragments normalized by that of β-actin. The data represent one of two experiments with similar results.

Acknowledgments and Notes

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