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A Combination of Tea *(Camellia senensis)* Catechins Is Required for Optimal Inhibition of Induced *CYP1A* Expression by Green Tea Extract

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It was previously demonstrated that the commercial green tea extract Polyphenon 100 (P100), and to a lesser extent (−)-epigallocatechin-3-gallate (EGCG), partially antagonizes 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD)-induced transcription of human *CYP1A1* (Williams, S. N.; Shih, H.; Guenette, D. K.; Brackney, W.; Denison, M. S.; Pickwell, G. V.; Quattrochi, L. C. *Chem.−Biol. Interact.* **2000**, *128*, 211−229). Here, P100 is compared to a reconstituted mixture of the four major tea catechins (referred to as P100R) to determine whether inhibition was due to additional polyphenols in the extract or from synergistic interactions among the tea catechins. It was found that cotreatment of cells with TCDD and either P100 or P100R inhibited TCDD-induced *CYP1A* promoter-driven luciferase reporter activity (HepG2 cells) and *CYP1A* expression (HepG2 and primary human hepatocytes), similarly. These results indicate that modulation of human *CYP1A* expression by P100 can be attributed entirely to the combination of the four tea catechins. These findings may be important in the evaluation of future chemoprevention strategies using green tea and single catechin agents.

KEYWORDS: Green tea; *Camellia senensis*; polyphenols; catechins; cytochrome P450; *CYP1A*; human hepatocytes

INTRODUCTION

Interest in green tea as a cancer preventive agent in humans has increased dramatically over the past 15 years. Epidemiological studies, although inconclusive, suggest a protective effect of tea consumption on certain human cancers (reviewed in ref *1*). Extensive animal studies have demonstrated that green tea polyphenols inhibit carcinogen-induced tumors in numerous tissues, inhibit tumor promotion, and decrease tumor size and/or multiplicity [reviewed in refs *2* and *3*). Collectively, research has warranted a further look into the effects of green tea on cancer.

Numerous animal models have demonstrated that green tea protects against various polycyclic aromatic hydrocarbon (PAH)-induced cancers (*4*, *5*). Protection against PAH-induced cancers may be due to the inhibition of PAH metabolism by green tea (*6*). One mechanism by which green tea modulates carcinogenesis may be through its ability to inhibit cytochrome (CYP) P4501A (CYP1A1 and CYP1A2) enzyme activity (*6*, *7*) and/or expression (*8*). CYP1A enzymes play a key role in the metabolism of carcinogens (e.g., PAH) and other foreign chemicals, and their expression is also induced by many of these agents (*9*). The induction of the CYP1A enzymes by PAH and their halogenated derivatives, such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), occurs at the level of transcription and is mediated by the cytosolic aryl hydrocarbon receptor (AhR). After binding xenobiotic ligands such as TCDD, the AhR migrates to the nucleus and associates with the AhR nuclear translocator protein, and the dimer so formed activates transcription of several genes, including the *CYP1A1* and *CYP1A2* genes (reviewed in ref *10*).

Previously, we demonstrated that the commercial green tea extract Polyphenon 100 (P100), and to a lesser extent the tea catechin (−)-epigallocatechin-3-gallate (EGCG), partially antagonizes TCDD-induced binding of the AhR to DNA and inhibits subsequent transcription of human *CYP1A1* (*8*). P100 is a complex mixture; however, the most abundant components are the four tea catechins, EGCG, (−)-epigallocatechin (EGC), (−)-epicatechin gallate (ECG), and (−)-epicatechin (EC) (**Figure 1**). These tea catechins constitute ∼90% of the total phenolic compounds present in the P100 extract with EGCG being the most abundant (*11*). Because EGCG was unable to recapitulate the effects of P100 extract on induced *CYP1A1* gene expression, we postulated either that P100 contains other polyphenolic compounds capable of mediating this effect or that the four major catechins interacted in a synergistic manner to inhibit the AhR-mediated pathway. Here, we demonstrate the ability of a mixture of the four tea catechins to inhibit the induced expression of human *CYP1A1* and *CYP1A2*.

MATERIALS AND METHODS

Materials and Reagents. Fetal bovine serum was purchased from HyClone (Logan, UT). α-Modified essential medium (α-MEM),

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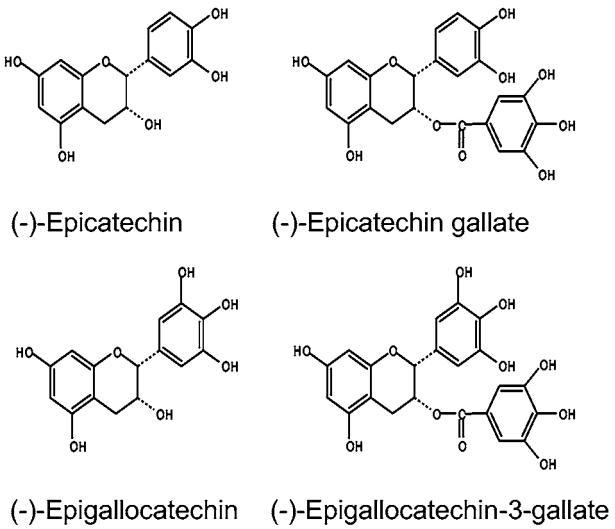

Figure 1. Structures of green tea catechins.

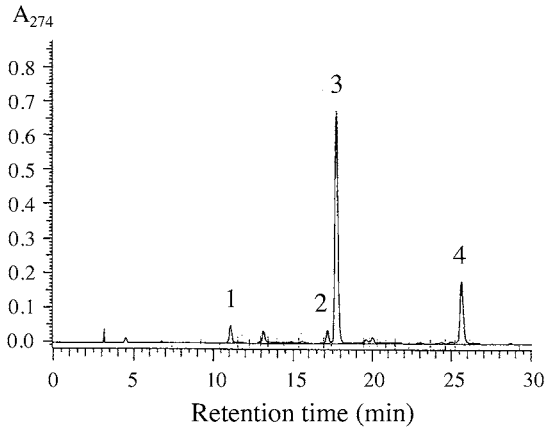

Figure 2. Representative HPLC chromatogram of catechins in green tea extract (274 nm). Peak assignments: (1) EGC; (2) EC; (3) EGCG; (4) ECG.

Table 1. Composition of Polyphenon 100[a]

catechin	% by wt
(–)-epigallocatechin gallate (EGCG)	50.5
(–)-epigallocatechin (EGC)	26.5
(–)-epicatechin gallate (ECG)	11.5
(–)-epicatechin (EC)	5.9
total catechin	94.4

[a] As determined by HPLC.

penicillin/streptomycin, and trypsin/EDTA were purchased from Life Technologies, Gibco-BRL (Grand Island, NY). All other tissue culture reagents were of the highest grade commercially available from Sigma Chemical Co. (St. Louis, MO). The green tea extract Polyphenon 100 (P100, >90% green tea catechins) and the individual catechins EGCG, EGC, ECG, and EC were purchased from Sigma Chemical; the same lot number of each respective chemical was used in all experiments. Prior to performance of the experiments described here, a sample of Polyphenon 100 was sent to Hauser Laboratories Inc. (Boulder, CO) for analysis of catechin composition. Analysis was done in duplicate by high-performance liquid chromatography (HPLC) using individual standards for each of the four major catechins present in the sample (**Figure 2**; **Table 1**). Results were similar to those reported by Weisburger et al. (*11*). P100 and catechins were reconstituted with dimethyl sulfoxide (DMSO) to final concentrations of 0.1 g/mL for P100 and 50 mM for individual catechins; stocks were stored under argon gas at −80 °C to prevent oxidation. TCDD was from Chemsyn Science Laboratories (Lenexa, KS) and β-naphthoflavone from Sigma Chemical.

Table 2. Equivalent Micromolar Amount of Catechins Found in P100 μg/mL Treatments

P100 (μg/mL)	EGCG (μM)	EGC (μM)	ECG (μM)	EC (μM)	total catechin
3	3.4	2.7	0.8	0.6	7.5
6	6.9	5.4	1.6	1.3	15.2
12	13.8	10.8	3.2	2.6	30.4
25	27.6	21.6	6.5	5.1	60.8
50	55.1	43.3	13.0	10.2	121.6
75	82.6	64.9	19.4	15.2	182.1
100	110.2	86.5	25.9	20.3	242.9

Cell Culture. HepG2 cells were grown as monolayers in α-MEM supplemented with 10% fetal bovine serum, 1 mM sodium pyruvate, 100 units/mL penicillin, and 0.1 mg/mL streptomycin. The stable cell line HepG2-101L (referred to as 101L cells in text) was maintained as above except with the addition of 0.4 mg/mL of G418 (Sigma Chemical) as a selection agent. Cells were maintained at 37 °C under a humidified atmosphere containing 5% CO_2. Unless stated otherwise, cells were treated simultaneously (cotreated) in complete medium with 1 nM TCDD and the amount of P100 (μg/mL) or reconstituted P100 (μg/mL) as indicated in the figures for 6 h at 37 °C. "Reconstituted P100", referred to as P100R in the text, was generated by combining the individual catechins in micromolar amounts based on analysis of the catechin content of P100 (**Table 1**). For example, treatment with 100 μg/mL P100R was accomplished by adding the four individual catechins (50 mM stocks) to medium to the following final concentrations: EGCG = 110.2 μM, EGC = 86.5 μM, ECG = 25.9 μM, and EC = 20.3 μM (**Table 2**). Serial dilution with medium was carried out to achieve the lower concentrations used in the studies. The solvent for all agents, DMSO, was added to control cultures at a concentration of 0.1% (v/v) or at a volume equal to treatment volumes in P100R experiments (maximum 0.5% v/v).

Cytotoxicity assays were performed in HepG2 cells cultured in 96-well plates using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) (*12*). Compounds were added in complete medium, and cells were treated for 24 h and then allowed to recover for 72 h before they were assayed as described previously (*8*). In individual experiments, six wells were used per treatment group. The mean concentration that inhibited 50% of cell growth (IC_{50}) was calculated by averaging the individual values from two to three separate experiments performed for each compound.

Luciferase Assays. All luciferase assays utilized the recombinant human hepatoma cell line HepG2-101L that was engineered to contain a stably integrated human *CYP1A1* promoter-driven firefly luciferase reporter gene plasmid (*13*). 101L experiments were performed as described previously (*8*), except 24-well plates were used. Briefly, cells were plated at 125000 cells per well in 24-well plates, allowed to recover for 48 h, and then treated for 6 h as described above. Cells were assayed for luciferase activity and normalized as relative light units (RLU) per microgram of protein.

Statistics. Statistical differences between values were determined by a one-way ANOVA, followed by the Dunnett multiple-comparisons test. Statistics were performed using InStat Instant Statistics (GraphPad Software, San Diego, CA). For the graphs, data were expressed as "percent of TCDD" as described in the figure legends. In these experiments, the range of values for DMSO-treated controls was 618−876 RLU/μg of protein.

Northern and Western Blot Analyses. HepG2 cells were treated for 6 h as described above, and total RNA was isolated (*14*). Northern blot analysis using 10 μg of RNA was performed as described previously in detail (*8*). For western blot analysis, HepG2 cells (~75% confluency) were treated as described above for 24 h to induce CYP1A1 protein. Microsomal proteins were isolated, and western blot analysis using 10 μg of protein per sample was performed as described previously (*8*). As a positive control, each blot was run with one lane

containing recombinant CYP1A1 (*15*), which was generously provided by Dr. Jerome Lasker (Hackensack University Medical Center, Hackensack, NJ).

Primary cultures of human hepatocytes from three donors (HL1−3), were treated for 24 h, and microsomes were isolated and analyzed by western blotting as described previously (*8*), with the exception of the electrophoresis step, which was modified to resolve CYP1A1 and CYP1A2. To achieve such separation, microsomal proteins were separated by the Novex NuPage Bis-Tris electrophoresis system using the Bis-Tris-HCl-buffered polyacrylamide gel (with 10% acrylamide) as described by the manufacturer (Novex, San Diego, CA). As controls to confirm specific bands as CYP1A1 or CYP1A2, each blot was run with one lane containing recombinant CYP1A1 and one containing microsomal CYP1A2 standard (Gentest, Woburn, MA). In the figures, these lanes are designated "rCYP" for recombinant CYP. Because CYP1A1 protein, but not CYP1A2, is expressed in HepG2, we also ran on each blot an equal amount of TCDD-induced HepG2 cell microsomes (10 μg) for comparative purposes. The antibody for detection of CYP1A1 and CYP1A2 was from Puracyp (La Jolla, CA); this antibody was raised against hamster CYP1A2 and recognizes both human CYP1A1 and CYP1A2 proteins. Mouse anti-actin monoclonal antibody (Chemicon, Temecula, CA), which recognizes all isoforms of actin, was used to normalize the amount of protein loaded in each lane. Immunochemical staining and visualization were performed as described previously (*8*).

Translocation Studies: Yellow Fluorescent Protein-Tagged Mouse AhR (AhR-YFP). The AhR-YFP was provided by Dr. Gary Perdew (The Pennsylvania State University, State College, PA). In situ visualization of AhR-YFP in COS-1 cells was performed as described previously (*16*). Briefly, cells at ~50% confluency in 35 mm tissue culture dishes were transfected for 5 h using LipofectAMINE (Life Technologies, Inc., Manassas, VA) per the manufacturer's instructions. After 24 h of recovery, cells were treated for 1 h in serum-free medium with either 0.1% DMSO, 10 nM TCDD, individual catechins at concentrations of 1−200 μM, or 50 μg/mL P100 or P100R. Fluorescent images were digitally captured using a MicroMax 5 MHz camera (Princeton Instruments, Trenton, NJ) and Image Pro Plus software (Media Cybernetics, Silver Spring, MD). For analysis, a minimum of 200 cells was examined in each treatment group. The number of cells in which nuclear fluorescence exceeded cytoplasmic fluorescence was determined and represented as a percent of the total number of cells counted. In all experiments, 1 h of TCDD treatment caused nuclear translocation of the AhR-YFP in >85% of the cells.

Human Hepatocytes. Human hepatocytes were obtained from the Liver Tissue Procurement and Distribution System (LTPADS, University of Minnesota, Minneapolis, MN). The hepatocytes were isolated by collagenase digestion and then plated onto rat tail collagen in serum-supplemented Williams' E medium as described in ref *17*. Hepatocytes isolated from three different human livers were received and are designated human liver 1−3 (HL1−HL3). Hepatocytes arrived in the laboratory in T-25 flasks (~2.5×10^6 cells/flask) ~24−48 h after plating and maintained their viability throughout the total 5 days in culture. Upon arrival, the medium was replaced with a modified serum-free Waymouth MB-752 medium containing 0.1 μM insulin and 100 units/mL penicillin as previously described (*18*); 1 mg of soluble Matrigel in 4 mL of medium was added to each flask. Matrigel was prepared from the Engelbreth−Holm−Swarm tumor provided by Dr. Hynda Kleinman, NIDR, NIH, Bethesda, MD. Fresh medium was added every 24 h. Cells were treated with green tea components after ~24−36 h of acclimation. The limited number of hepatocytes from each individual subject precluded the analysis of cytotoxicity; however, after addition of treatments, no reduction in cell density or other morphological changes in the cultures were observed.

RESULTS

Cytotoxicity Studies. We first evaluated the toxicity of tea catechins in HepG2 cells using the MTT cytotoxicity assay to determine the IC_{50} values for each catechin (**Figure 3**). The order of toxicity (highest to lowest) of the compounds in HepG2 cells was ECG > EGCG > EGC > EC. ECG and EGCG displayed steep toxicity curves, such that progression from no toxicity to almost complete cell death was observed over a single log dose range. The slope of the toxicity curve for EGC was markedly less, whereas EC was nontoxic to cells at all concentrations tested (up to 200 μM).

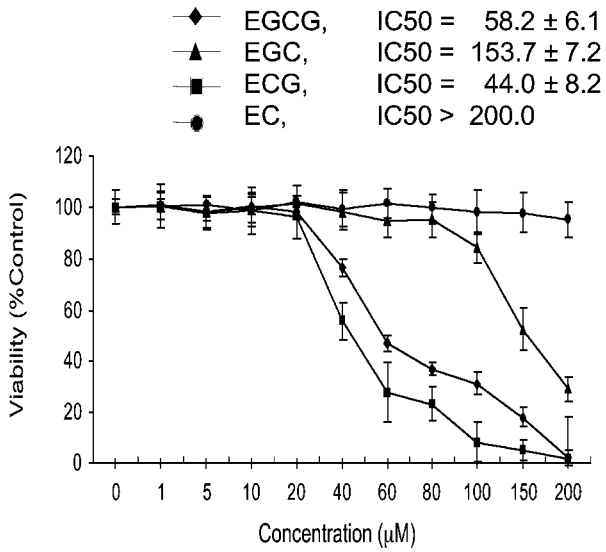

Figure 3. Cytotoxicity of tea catechins in HepG2 cells. HepG2 cells were treated in 96-well tissue culture plates with concentrations of catechins varying from 0.1 to 200 μM, allowed to recover for 72 h, and assayed for the inhibition of mitochondrial function using the MTT assay as described under Materials and Methods. IC_{50} values for growth inhibition were calculated from cytotoxicity data and are presented as mean ± standard error for $n = 18$.

Inhibition of Reporter Gene Activity. Previous studies from our laboratory demonstrated that P100 inhibited TCDD-mediated binding of the AhR to DNA, thereby inhibiting *CYP1A1* expression (*8*). Additionally, we found that of the four catechins tested, only EGCG was able to inhibit the TCDD response; however, this inhibition averaged only 22% compared to the ~50% inhibition seen with 100 μg/mL P100 in the same experiments. On the basis of recent studies demonstrating that a combination of flavonoids may be more effective than the individual flavonoids alone on some biochemical processes (*19*, *20*), we hypothesized that it might be the particular mixture of catechins in P100 exerting an inhibitory effect. Accordingly, we "reconstituted" P100 by combining the four individual catechins into a mixture, termed P100R, that contained concentrations mirroring those found in the P100 treatments (see Materials and Methods for details). We first evaluated the toxicity of P100R in HepG2 cells using the MTT cytotoxicity assay. We found that the IC_{50} of P100R, 0.19 ± 0.01 mg/mL, was similar to that of P100, which was 0.21 ± 0.02 mg/mL (data not shown). We then evaluated the effects of P100R on *CYP1A1* promoter activity. First, the ability of P100R to inhibit TCDD-induced *CYP1A1* expression was examined and compared to those of P100 and EGCG. Treatment of 101L cells with 1 nM TCDD for 6 h resulted in a 32−39-fold induction of luciferase activity over DMSO-treated controls. We found that cotreatment of cells with TCDD and P100R resulted in the same degree of inhibition as did P100, ~50% (**Figure 4A** and ref *8*). A concentration-dependent inhibition of TCDD-mediated *CYP1A1* promoter activity was observed in cells cotreated with various concentrations of P100R and 1 nM TCDD (**Figure 4B**).

Inhibition of *CYP1A1* Message and Protein. To confirm the effects of P100R on gene transcription, HepG2 cells were treated as described above, and then total RNA was isolated

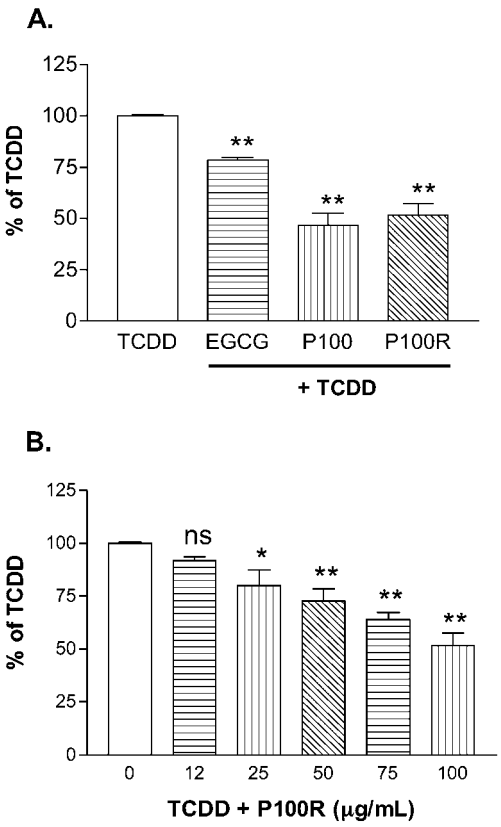

Figure 4. Effect of P100R on *CYP1A1* promoter activity: (A) 101L cells were treated for 6 h with 1 nM TCDD alone or in the presence of EGCG (10 μM), P100 (100 μg/mL), or P100R (100 μg/mL); (B) 101L cells were treated for 6 h with 1 nM TCDD alone or in the presence of P100R at the indicated concentrations. Luciferase activity in cell lysates was determined as described under Materials and Methods. Data are expressed normalized to TCDD controls (percent of TCDD). The standard deviation (SD) of TCDD control values among experiments was <10%. Data points in (A) and (B) represent the means ± SD of three independent experiments performed in triplicate. Values are significantly different from the TCDD-treated sample at $p < 0.05$ (*) or $p < 0.01$ (**) by ANOVA. ns, not statistically significant.

for northern blot analysis of *CYP1A1* mRNA. Treatment of cells for 6 h with 1 nM TCDD resulted in a robust induction of *CYP1A1* mRNA (**Figure 5A**). Addition of increasing amounts of P100R with TCDD resulted in a concentration-dependent inhibition of TCDD-induced *CYP1A1* mRNA. Cotreatment with 100 μg/mL P100R and TCDD resulted in a 66% inhibition of *CYP1A1* mRNA compared to cells treated with TCDD only. Western blot analysis of microsomal proteins isolated from HepG2 cells indicated the same trend of inhibition. CYP1A1 protein was not detected in DMSO control cells, but after 24 h of treatment with TCDD, CYP1A1 protein was induced (**Figure 5B**). We found that CYP1A1 protein expression was dose-dependently inhibited by cotreatment of cells with P100R and TCDD (**Figure 5B**). At the highest concentration of P100R examined, TCDD-induced CYP1A1 protein expression was inhibited by ~60%.

Inhibition of Induced CYP1A in Primary Cultures of Human Hepatocytes. The levels of CYP1A1 and CYP1A2 proteins were next investigated in primary cultures of human hepatocytes from three different donors (HL1−3) following cotreatment with TCDD and either P100, EGCG, or P100R. Considerable interindividual variability was observed in the magnitude of CYP1A1 and CYP1A2 induction by treatment

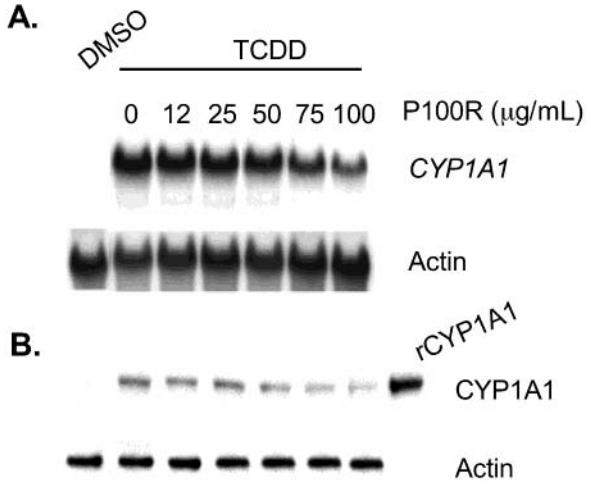

Figure 5. Effect of P100R on *CYP1A1* expression in HepG2 cells: (A) inhibition of TCDD-induced *CYP1A1* mRNA expression (cells were treated for 6 h with 1 nM TCDD alone or in the presence of P100R at the indicated concentrations, and mRNA accumulation was measured; total RNA was isolated and analyzed for *CYP1A1* and 18S mRNAs by northern blot as described under Materials and Methods); (B) inhibition of TCDD-induced expression of CYP1A1 protein (cells were treated with DMSO and 1 nM TCDD or cotreated with 1 nM TCDD and P100R at the indicated concentration for 24 h; microsomes were isolated, and western blot analysis of CYP1A1 and actin was performed using 10 μg of protein as described under Materials and Methods). Recombinant CYP1A1 was used as a positive control (designated rCYP1A1).

with 1 nM TCDD alone (**Figure 6**). However, our findings are consistent with the report that TCDD induces predominantly CYP1A2 in human hepatocytes at the level of both mRNA and protein (*21*), whereas the PAH β-naphthoflavone induced both CYP1A1 and CYP1A2 similarly (**Figure 6B**).

Cells from donor HL1 were cotreated with TCDD and P100 or EGCG. CYP1A2 was highly induced by TCDD in HL1. Interestingly, this donor was the only smoker. Although CYP1A1 was also induced by TCDD, the level of induced expression was relatively lower than that of induced CYP1A2 (**Figure 6A**). When cells were cotreated with TCDD and increasing amounts of P100, a concentration-dependent inhibition of CYP1A2 was observed. At the highest concentration of P100 examined (50 μg/mL), the amount of TCDD-induced CYP1A2 protein was reduced to a level similar to that observed in DMSO control cells; furthermore, at concentrations of P100 exceeding 6 μg/mL, the induction of CYP1A1 was completely inhibited (**Figure 6A**, left panel). The effect of EGCG on CYP1A expression was also examined in HL1 cells (**Figure 6A**, right panel). We found that 10 μM EGCG inhibited TCDD-induced CYP1A2 expression by ~30%. Inhibition of TCDD-induced CYP1A1 was not as dramatic as that observed for CYP1A2, and a significant effect was observed only at the highest concentration used, 10 μM EGCG.

We next examined P100R for its effect on CYP1A expression in cells from donor HL2 and HL3. A concentration-dependent inhibition of CYP1A2 was observed when HL2 cells were cotreated with TCDD and increasing amounts of P100 (**Figure 6B**). The induction of CYP1A1 by P100 was weak and observable only at longer exposure times (see TCDD lane in the lower panel of **Figure 6B**). Generally, the effects of P100R were similar to those seen with P100 treatment. A concentration-dependent trend of inhibition of CYP1A2 was seen in HL2 cells cotreated with TCDD and increasing amounts of P100R; similar results were observed in the HL3 cells (**Figure 6B**,**C**). One

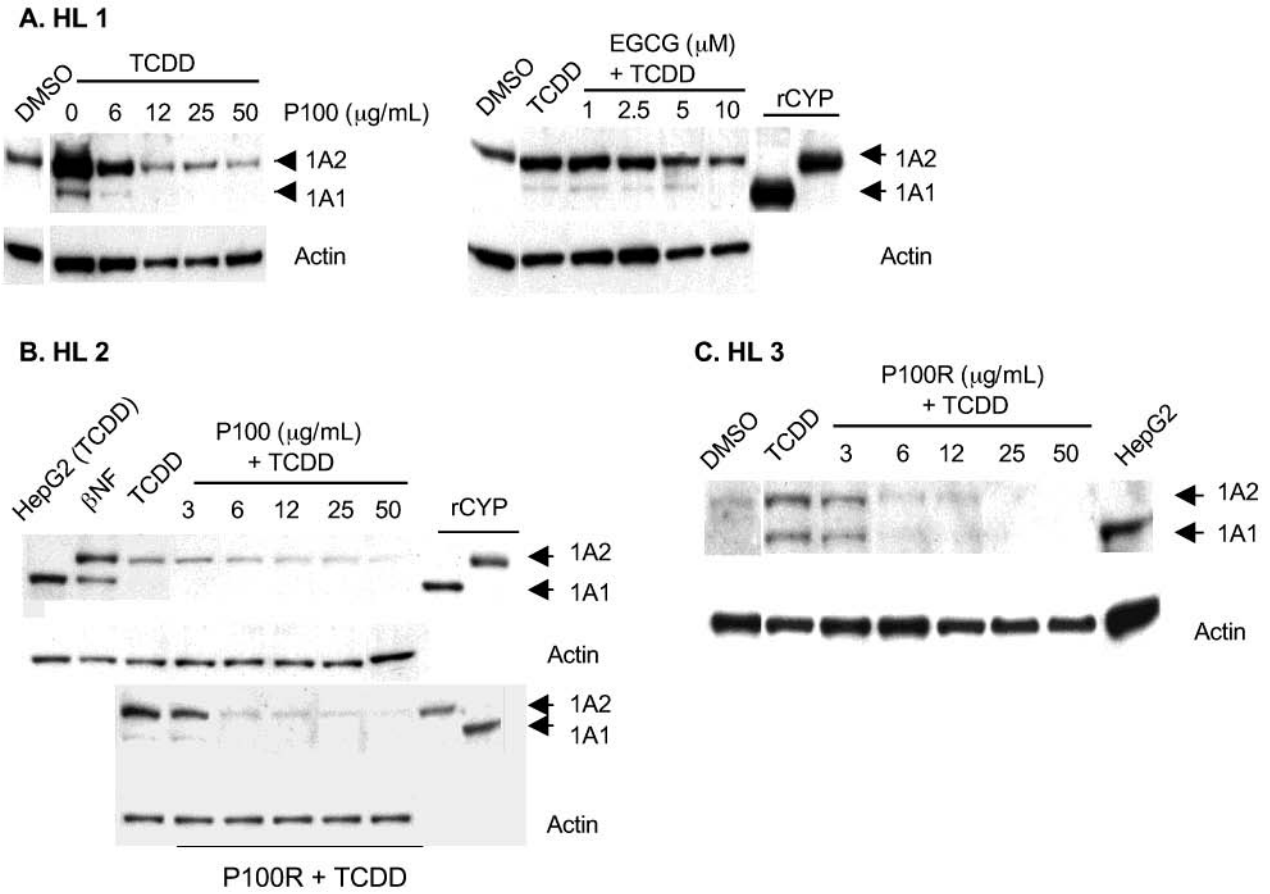

Figure 6. Effect of P100 and P100R on CYP1A protein levels in primary cultures of human hepatocytes. Primary cultures of hepatocytes were treated for 24 h with DMSO and 1 nM TCDD or cotreated with 1 nM TCDD and P100, EGCG, or P100R at the indicated concentrations for 24 h. Western blot analysis of CYP1A1, CYP1A2, and actin proteins was performed using 10 μg of microsomal protein as described under Materials and Methods. Recombinant CYP1A1 and CYP1A2 were used as positive controls (designated rCYP).

difference between P100 and P100R treatments was that P100R appeared to exhibit a steeper inhibition curve with regard to the effect of TCDD on CYP1A2. As shown in **Figure 6B**,**C**, cotreatment with 3 μg/mL P100R and TCDD produced only a marginal effect when compared to TCDD control; however, a marked inhibition of the TCDD response was observed at the next highest concentration of P100R examined (6 μg/mL), and complete inhibition of the TCDD response occurred with P100R at 50 μg/mL treatment. The difference between P100R and P100 treatments could possibly be accounted for by the presence in the P100 extract of polyphenolic compounds with AhR agonist activity, thereby diminishing the antagonistic effects of the catechins. However, studies analyzing additional human hepatocyte cultures would be required to address this issue.

Nuclear Translocation of AhR-YFP. Transiently expressed fluorescent-tagged AhR has been used to examine AhR nuclear uptake in response to various stimuli (*16*). To examine whether one mechanism by which green tea inhibits CYP1A transcription is through blocking the TCDD-mediated nuclear uptake of the AhR, we focused on the ability of green tea extract and components to induce nuclear translocation of the AhR. The AhR-YFP fusion protein was transiently expressed in COS-1 cells and in cells treated with TCDD, EGCG, P100, or P100R. Nuclear uptake was measured by in situ visualization of AhR-YFP in COS-1 cells using fluorescent microscopy. As shown in **Figure 7**, fluorescence in DMSO-treated cells was primarily cytosolic. After treatment for 1 h with 10 nM TCDD, nuclear localization of the AhR-YFP protein was observed. Treatment with P100 or P100R at a concentration of 25 or 50 μg/mL induced AhR translocation in a manner similar to that of TCDD. EGCG at concentrations of either 20 or 30 μM also induced AhR translocation in a manner similar to that of TCDD, whereas 10 μM EGCG caused translocation to a lesser degree. EGC, ECG, or EC treatments did not induce the translocation of the AhR-YFP above control levels (data not shown). Nuclear fluorescence exceeded cytoplasmic fluorescence in 7% of control cells, 92% of TCDD-treated cells, 51 and 86% of EGCG-treated cells (10 and 30 μM, respectively), 88% of P100-treated cells (50 μg/mL), and 79 and 87% of P100R-treated cells (25 and 50 μg/mL, respectively).

DISCUSSION

Our results confirm and extend data we previously reported on the ability of green tea extract to exert antagonistic effects on the AhR and to inhibit TCDD-induced *CYP1A1* gene expression. Previously, we demonstrated that of the four major catechins, only EGCG had inhibitory actions similar to the extract, but to a lesser degree (*8*). These results suggested the possibility that other polyphenolic compounds possessing antagonistic activities on the AhR-mediated pathway were present in the extract. Alternatively, synergistic activity among the catechins or between EGCG and other polyphenols present could account for the inhibition of induced *CYP1A1* expression. Synergistic actions have been described between tea catechins (*20*), as well as between green tea and other agents (*19*, *20*, *22*−*24*). In light of these published studies, we reasoned that the four major catechins in P100 could interact in some manner

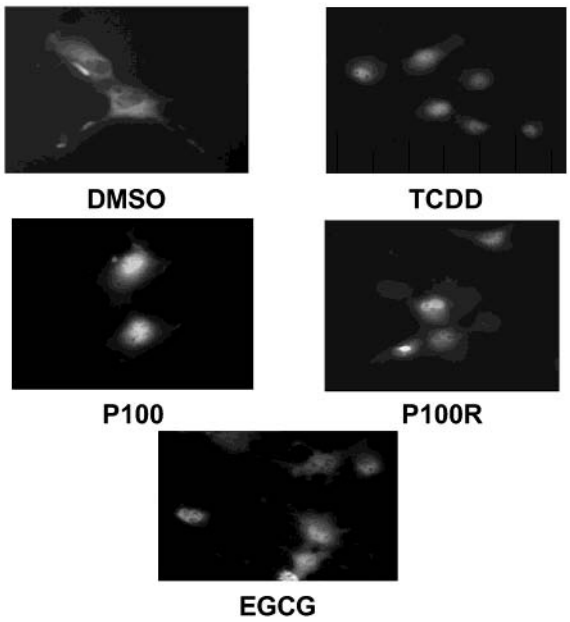

Figure 7. Nuclear translocation of transiently expressed AhR-YFP. COS-1 cells were transiently transfected with the AhR-YFP plasmid as described under Materials and Methods. Cells were treated for 1 h with DMSO, 10 nM TCDD, 30 μM EGCG, or 50 μg/mL of P100 or P100R, as indicated, prior to visualization. Representative cells at 20× magnification are shown in each panel.

that results in the observed inhibitory effect on ligand-induced *CYP1A* expression. To eliminate the possibility that a non-catechin component was exerting antagonistic effects and to investigate the potential synergy among the catechins, we examined the effects of a "reconstitution" of P100 (P100R) that contained amounts of individual catechins mirroring those found in the P100 treatments.

We found that P100R did indeed exert effects similar to those of P100 itself. We showed that P100R (i) blocks induced reporter gene activity in HepG2 cells, (ii) blocks the induction of *CYP1A* mRNA and protein in HepG2 and human hepatocytes, and (iii) activates the AhR so that it migrates to the nucleus. Thus, the mixture of four catechins at concentrations found in the P100 extract gave the same antagonist profile as the extract. These results indicate that other non-catechin constituents in the P100 extract were not responsible for the observations and that synergistic interactions of the catechins could possibly account for the full inhibitory effect (i.e., ~50% inhibition by P100 or P100R versus ~20% by EGCG). Many studies have implicated EGCG as the active component of green tea because it is the most abundant catechin and because cancer inhibitory activity of EGCG has been recognized (reviewed in ref *2*). In the experiments reported here, we demonstrated that although EGCG had some inhibitory activity and is likely an important component in inhibiting TCDD-induced *CYP1A* expression, it alone was not responsible for the full effect of green tea extract.

Our findings do reveal differences between HepG2 cells and primary human hepatocytes in response to cotreatments with TCDD and P100R. We found that cotreatment of HepG2 cells with TCDD and 12 μg/mL P100R (containing 13.8 μM EGCG, see **Table 2**) did not produce a significant inhibition of luciferase activity or *CYP1A1* mRNA or protein (**Figures 4** and **5**). These results conflict with our previous studies showing that cotreatment of HepG2 with TCDD and 10 μM EGCG consistently resulted in ~20% inhibition of TCDD-induced luciferase activity (*8*). Indeed, we found in the present study that equivalent inhibition occurred only when P100R contained ~28 μM EGCG. Although we do not presently understand this discrepancy, we can conclude that the mixture of the four catechins does have an effect on the AhR pathway similar to that of the commercial extract P100 and that other polyphenolic agents do not contribute to the antagonistic activity on this pathway.

Results of our studies in human hepatocytes are more consistent with the possibility that synergistic interactions of tea catechins were responsible for the effect on the AhR pathway. Indeed, we found that the inhibition of induced *CYP1A1* and *CYP1A2* expression was achieved at significantly lower concentrations of P100 or P100R in primary human hepatocytes than in HepG2 cells (**Figures 5** and **6**). Concentrations of 6 and 12 μg/mL of either P100 or P100R produced significant inhibition (~70−85%) of CYP1A2 expression, even though these treatments contained only 6−14 μM EGCG, whereas cotreatment with 10 μM EGCG inhibited TCDD-induced CYP1A2 expression by only 30% (**Figure 6A**, right panel). Although we have not identified the mechanism for the observed difference between HepG2 and primary cells, one possibility is that hepatocytes maintain a differentiated phenotype [especially cultured on Matrigel (*25*)]; thus, biochemical pathways may be present in these cells that are absent or altered in HepG2. For example, EGCG and green tea extract have been shown to inhibit protein kinase C (PKC) activity (*26*, *27*), and PKC has been demonstrated to affect AhR function (*28*, *29*). Green tea polyphenols are also known to modulate signal transduction pathways by inhibiting the activities of, for example, protein kinase A, mitogen-activated protein kinase, and vascular endothelial growth factor receptor (reviewed in ref *30*).

The synergistic interactions underlying the effectiveness of a number of phytomedicines, including green tea and its components, have been extensively documented. Curcumin (*19*), the antineoplastic drug doxorubicin (*22*), tamoxifen (*20*), phytic acid (*23*), and sulindac (*24*) have all been reported to exert synergistic activities with green tea or EGCG. The possible synergy observed in primary human hepatocytes on the AhR pathway and on the inhibition of CYP1A gene transcription most likely involves numerous biochemical targets because green tea catechins produce such a variety of biological effects. Nevertheless, examples from the literature may shed some light on potential mechanisms. Suganuma et al. (*20*) reported that EC, and not EGC or ECG, enhanced the incorporation of EGCG, EGC, and ECG into the human lung cancer cell line PC-9 and that cotreatment with a combination of EC and any of the other tea catechins synergistically induced apoptosis in PC-9 cells and inhibited TNFα release in BALB/c-3T3 cells. Because EC alone had no effect on these two parameters, it was concluded that the EC-enhanced incorporation of tea catechins into the cell was responsible for the observed synergy. Indeed, our results do suggest that the ability to achieve higher concentrations of EGCG and other catechins when the catechins are administered as a mixture in P100R makes it possible for more of an antagonistic effect to be exerted on the AhR. The estimated IC_{50} values of P100R and P100 were similar, 0.19 ± 0.01 and 0.21 ± 0.02 mg/mL, respectively. A striking observation was that the cytotoxic potential of the individual catechins, especially that of EGCG, was dramatically decreased when given together in the reconstitution mixture. Although the IC_{50} of EGCG alone was ~58 μM, treatment of cells with concentrations of up to 100 μg/mL P100R or P100 (*8*) was nontoxic, even though this concentration contains ~110 μM EGCG. Our studies suggest that the mixture of the four catechins in P100 and P100R causes

activation or suppression of unknown cellular pathways such that an overall protective effect is exerted on cell survival, at least in HepG2 cells. This will most likely differ depending on cell type and treatment regimen. Although these findings are intriguing, it remains to be determined what factors are important in altering the toxicity of individual catechins compared to P100 or P100R in HepG2 cells.

Our studies also demonstrated the effects of green tea extract on *CYP1A2* expression in primary human hepatocytes. Our findings regarding CYP1A2 are important in evaluating the chemoprotective effects of green tea against various cancers, for example, as in the prevention of aflatoxin B_1 (AFB_1)-dependent hepatocellular carcinoma in humans. CYP1A2 has been shown to activate AFB_1 to the electrophilic *exo*-aflatoxin 8,9-epoxide (*31*). Although CYP3A4 also plays a role in this metabolic activation, CYP1A2 has been shown to have a higher activation potential when AFB_1 is at low concentrations, which are more representative of dietary exposures (*31*). Oltipraz is a synthetic derivative of the natural compound 1,2-dithiole-3-thione that has been shown to inhibit aryl hydrocarbon-induced CYP1A2 in primary human hepatocytes, resulting in a reduction of AFB_1 metabolism (*32*). Furthermore, Oltipraz has proven to be useful in the chemoprevention of hepatocellular carcinoma in at-risk human populations by decreasing the bioactivation of AFB_1 (reviewed in ref *33*). Because P100 and P100R were potent inhibitors of induced *CYP1A2*, our findings suggest that green tea may also be useful in such at-risk populations. Although the effective concentrations in primary hepatocytes (3−6 μg of green tea extract/mL) are ∼25−75-fold higher than plasma levels of EGCG measured after consumption of green tea extracts (*34*, *35*), our findings suggest that in vitro cell culture models utilizing primary cells may be more relevant to humans than the use of transformed cell lines.

In summary, our findings demonstrate that the effect of the mixture of tea catechins found in P100 differs from the effect of individual tea catechins on the modulation of human *CYP1A* expression. The underlying mechanisms leading to increased effectiveness of green tea as a whole and P100R compared to the single catechins remain to be elucidated. Although our studies did not examine synergy directly, they do suggest the possibility that the four major tea catechins in combination may be more effective than individual catechins with respect to chemoprevention.

ABBREVIATIONS USED

AhR, aryl hydrocarbon receptor; AhR-YFP, yellow fluorescent protein-tagged mouse AhR; EC, (−)-epicatechin; ECG, (−)-epicatechin gallate; EGC, (−)-epigallocatechin; EGCG, (−)-epigallocatechin-3-gallate; P100, Polyphenon 100; P100R, reconstituted P100; PAH, polycyclic aromatic hydrocarbon; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin

SAFETY

Precautions for the safe handling of TCDD in the laboratory based on guidelines from Dow Chemical, USA, CRE B-600-134-79, were followed.

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