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Cacao Polyphenol Extract Suppresses Transformation of an Aryl Hydrocarbon Receptor in C57BL/6 Mice

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Dioxins enter the body through the diet and cause various toxicological effects through transformation of an aryl hydrocarbon receptor (AhR). Plant extracts and phytochemicals including flavonoids are reported to suppress this transformation. This paper investigates the suppression by a cacao polyphenol extract (CPE) of AhR transformation in vivo. The CPE was administered orally to C57BL/6 mice at 100 mg/kg of body weight, followed 1 h later by 3-methylcholanthrene (MC), an AhR agonist, injected intraperitoneally at 10 mg/kg of body weight. CPE suppressed the MC-induced transformation to the control level by inhibiting the formation of a heterodimer between AhR and an aryl hydrocarbon receptor nuclear translocator in the liver at 3 h postadministration. It also suppressed MC-induced cytochrome P4501A1 expression and NAD(P)H:quinone-oxidoreductase activity, whereas it increased glutathione *S*-transferase activity at 25 h. CPE constituents and their metabolites might contribute, at least in part, to the suppression of AhR transformation. The results indicate that the intake of CPE suppressed the toxicological effects of dioxins in the body.

KEYWORDS: Cacao polyphenol extract; aryl hydrocarbon receptor; cytochrome P4501A1; NAD(P)H: quinone-oxidoreductase; glutathione *S*-transferase

INTRODUCTION

Dioxins, environmental contaminants, enter the body via diet and cause various toxicological effects through aryl hydrocarbon receptor (AhR)-dependent actions (*1*−*4*). In the absence of a ligand, AhR exists in the cytoplasm as a complex with two molecules of heat shock protein 90 (hsp90), hepatitis B virus X-associated protein 2, and p23. After the binding of a ligand, the AhR dissociates from these partner proteins (*5*−*7*), translocates into the nucleus, and forms a heterodimer with an aryl hydrocarbon receptor nuclear translocator (Arnt) (*8, 9*). These sequential steps are referred to collectively as AhR transformation. The AhR/Arnt heterodimer functions as a transcription factor, which binds to the dioxin responsive element (DRE) and induces the expression of a battery of genes including those for drug-metabolizing enzymes such as cytochrome P4501A1 (CYP1A1) (*10*), NAD(P)H:quinone-oxidoreductase (QR) (*11*), glutathione *S*-transferase (GST) Ya subunit (*12*), and UDP-glucuronocyltransferase (*13*). AhR transformation is involved in the toxic effects of dioxins, because AhR-knockout mice are resistant to 2,3,7,8-tetrachrolodibenzo-*p*-dioxin (TCDD) (*14*). Therefore, suppression of AhR transformation is important to reduce the toxicological and biological effects of TCDD.

Cacao polyphenol extract (CPE), an ingredient of cocoa and chocolate, has various beneficial effects such as antioxidative effect (*15*), inhibition of diabetes-induced cataract (*16*), prevention of atherosclerosis (*17*), and reduction of hyperglycemia (*18*). CPE is rich in polyphenols including catechins and their oligomers, procyanidins (*19*). After the administration of cocoa, conjugated forms of (−)-epicatechin (EC) to glucuronide and sulfate and a methylated form, 3′-*O*-methyl-(−)-epicatechin (MEC), are detected in the plasma in addition to the intact form (*20*). EC and (−)-epicatechin-(4β-8)-(−)-epicatechin, procyanidin B2 (PB2), reach the colon without absorption or metabolism in the small intestine, where they are catabolized by colonic microflora into low molecular weight phenolic acids (*21, 22*). It was also reported that components of cacao and their metabolites were excreted into urine (*23*). These reports suggest that the beneficial effects of CPE are, at least in part, due to polyphenols and their metabolites.

Our previous studies demonstrated that green and black tea extracts suppressed AhR transformation in vitro (*24, 25*). Polyphenols derived from plant foods, such as flavonoids including catechins, curcumin, and resveratrol, are reported to suppress the transformation in vitro (*24, 26*−*28*). Recently, it was demonstrated that flavonoids also suppressed the transformation after permeating human colon adenocarcinoma Caco-2 cells, a

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model for enterocytes (*29*). These findings suggest that polyphenol-rich plant foods may suppress AhR transformation and downstream signal transduction events, such as the induction of drug-metabolizing enzymes, in vivo. Previously, we demonstrated that an intake of molokhia (*Corchorus olitorius* L.) extract inhibited the translocation of AhR into the nucleus and its transformation in the liver of SD rats (*30*). However, data demonstrating the inhibitory effect of dietary agents on AhR transformation in vivo is scarce. In this study, we examined whether the intake of CPE suppressed AhR transformation induced by 3-methylcholanthrene (MC), an agonist of the AhR, and modulated the induction of drug-metabolizing enzymes.

MATERIALS AND METHODS

Materials. MC and corn oil were purchased from Nacalai Tesque (Kyoto, Japan). TCDD was obtained from AccuStandard (New Haven, CT) and dissolved in dimethyl sulfoxide (DMSO). CPE, procyanidins, 3′-*O*-methyl-(+)-catechin (MCA), and MEC were prepared according to previously reported methods (*19*). The CPE used in this study contains 2.5% (+)-catechin, 6.6% EC, 3.8% PB2, 2.9% procyanidin C1 (PC1), and 1.8% cinnamtannin A2 (CA2) (*19*). EC and (+)-catechin were from Kurita Kogyo (Tokyo, Japan). Hippuric acid, ferulic acid (FA), caffeic acid (CA), 3,4-dihydroxyphenylacetic acid (diHPAc), and *m*-hydroxyphenylacetic acid (mHPAc) were obtained from Sigma Aldrich (St. Louis, MO). *m*-Coumaric acid (mCO) and *p*-coumaric acid (pCO) were purchased from Extrasynthèse (Genay, France). *m*-Hydroxybenzoic acid (mHBA), *m*-hydroxyphenylpropionic acid (mHPP), and *p*-hydroxyhippuric acid (pHHA) were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan), Lancaster Synthesis Ltd. (Morecambe, U.K.), and Bachem Holding AG (Bubendorf, Switzerland), respectively. All other reagents were of the highest grade available from commercial sources.

Cell-free Experiment. Cytosol (15 mg of protein/mL) from the liver of SD rats (*26*) was incubated with 1 nM TCDD or DMSO alone as a vehicle control in HEDG buffer [25 mM HEPES−NaOH, pH 7.4, 1.5 mM ethylenediaminetetraacetic acid (EDTA), 1.0 mM dithiothreitol (DTT), and 10% glycerol] and 1.15% KCl containing protease inhibitors [1 μM phenylmethanesulfonyl fluoride (PMSF), 10 mM leupeptin, and 5 mg/μL aprotinin] at 20 °C for 2 h. To estimate the suppressive effect on the DNA-binding activity of AhR, CPE and its related compounds or DMSO alone as a vehicle control was added to the cytosolic fraction 10 min prior to the addition of 1 nM TCDD. The mixture was subjected to a southwestern chemistry-based enzyme-linked immunosorbent assay (SW-ELISA) for the measurement of DNA-binding activity of AhR (*31*). Compounds used in this study are listed in **Table 1**.

Cultured Cell Experiment. An intestinal permeability system using human colon adenocarcinoma Caco-2 cells and mouse hepatoma Hepa-1c1c7 cells was constructed according to a previously reported method (*30*) with some modifications. Briefly, the differentiated Caco-2 cells on the inserts were washed with fresh medium, kept in Hanks′ balanced salt solution (HBSS) for 30 min at 37 °C, and used for transport experiments. An aliquot of 100 μL of CPE dissolved in HBSS (100 μg/mL) was added to the apical side of inserts. The same volume of HBSS containing DMSO (1 μL/mL) was used as a vehicle control. The basolateral side was filled with 600 μL of HBSS. After incubation for 2 h at 37 °C, the basolateral solution was collected. Caco-2-permeated CPE or intact CPE itself (100 μg/mL) was added to Hepa-1c1c7 cells seeded on 60 mm dishes (approximately 80% confluent) for 2 h at 37 °C. Thereafter, the cells were treated with TCDD at 0.1 nM (final concentration) or DMSO as a vehicle control for a further 2 h at 37 °C. The nuclear extract was prepared from Hepa-1c1c7 cells (*32*) and used to measure the DNA-binding activity as follows.

Animal Treatments. Animal treatment in this study conformed to the "Guidelines for the Care and Use of Experimental Animals", Rokkodai Campus, Kobe University. Twenty-four male C57BL/6 mice (6 weeks old, 20−25 g, purchased from Japan SLC, Shizuoka, Japan) were divided at random into two groups of 12 each (CPE or water group). All mice were fasted for 18 h and received orally a CPE (100 mg/kg of body weight) suspension in deionized water or vehicle alone (10 mL/kg of body weight) as a control. These mice were further divided into two subgroups of six each. One subgroup was given MC (10 mg/kg of body weight) in corn oil through an intraperitoneal injection 1 h after the administration of CPE or water, whereas the other was given corn oil (5 mL/kg of body weight) as a vehicle control. Half of the mice in each subgroup (three each) were killed 3 h after the CPE injection and the rest, 25 h after. The liver was removed and used for experiments.

Table 1. Polyphenols in CPE and Their Related Chemicals Used in the Study

compound	abbreviation
cacao extract	CPE
procyanidins	
procyanidin B2 [epicatechin-(4β-8)-epicatechin]	PB2
procyanidin B5 [epicatechin-(4β-6)-epicatechin]	PB5
procyanidin C1	PC1
cinnamtannin A2	CA2
catechins and its metabolites	
(+)-catechin	
(−)-epicatechin	EC
3′-*O*-methyl-(+)-catechin	MCA
3′-*O*-methyl-(−)-epicatechin	MEC
phenolic acids	
hippuric acid	HA
ferulic acid	FE
caffeic acid	CA
m-coumaric acid	mCO
p-coumaric acid	pCO
m-hydroxybenzoic acid	mHBA
3,4-dihydroxyphenylacetic acid	diHPAc
m-hydroxyphenylacetic acid	mHPAc
m-hydroxyphenylpropionic acid	mHPP
p-hydroxyhippuric acid	pHHA

Ex Vivo Experiment. The cytosolic fraction (12 mg of protein/mL) was prepared from the liver of mice dosed with CPE or water and incubated with TCDD at 0.1 or 1.0 nM in HEDG buffer at 20 °C for 2 h. After incubation, the mixture was subjected to SW-ELISA for measurement of the DNA-binding activity of AhR.

SW-ELISA. The DNA-binding activity of AhR was measured by SW-ELISA (*30, 31*). For the cell-free system and ex vivo experiments, the reaction mixture consisted of 10 μL of HEDG buffer containing 0.75 M KCl and 40 mL of the cytosolic fraction. For the in vivo and cultured cell experiments, the reaction mixture consisted of 12.5 μL of nuclear extract (12.5 μg of proteins/assay) from the liver of mice or Hepa-1c1c7 cells and 37.5 μL of HEDG buffer. The reaction mixture was subjected to SW-ELISA. For in vivo samples, a specific antibody against AhR (Anti-AhR, MA1-514, Affinity BioReagents, Golden, CO) was used as the primary antibody.

EMSA. EMSA was also performed to determine the DNA-binding activity of AhR (*24*). Briefly, nuclear extract (10 μg of protein) from the liver of mice was incubated with 500 ng of poly[dI-dC] and the ^{32}P-labeled DRE probe (30 kcpm, 10 fmols) in HEDG buffer for 15 min. The mixture was then loaded onto a 4% nonstacking polyacrylamide gel in TBE buffer (25 mM Tris, 22.5 mM borate, and 0.25 mM EDTA). After electrophoresis, the AhR/DRE complex was visualized by autoradiography and quantitatively analyzed by Gel-Pro Analyzer (Media Cybernetics, Bethesda, MD).

Western Blot Analysis. A postnuclear fraction (5 μg of protein) for the detection of CYP1A1 and the nuclear extract (3 μg of protein) for the AhR and Arnt were separated by 10 and 7.5% sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE), respectively. The proteins were transferred onto a polyvinylidene difluoride membrane (GE Healthcare Bio-Sciences Corp., Piscataway, NJ). This was followed by the blocking of nonspecific binding with 1% skim milk in PBST for CYP1A1 or commercial blocking buffer (Nacalai Tesque) for the others for 1 h. The membrane was incubated with primary antibody for CYP1A1 (anti-CYP1A1, 1:5000, Daiichi Pure Chemicals, Tokyo, Japan), AhR (anti-AhR, MA1-514, 1:1500), and Arnt (anti-

Arnt, C-19, 1:3000, Santa Cruz Biotechnology, Santa Cruz, CA) and horseradish peroxidase-conjugated secondary antibodies. Immune complexes were visualized using ECL plus reagent (GE Healthcare Bio-Sciences Corp.) according to the manufacturer's instructions and quantitatively analyzed by Gel-Pro Analyzer.

Immunoprecipitation. Nuclear extract was incubated with anti-Arnt antibody (Santa Cruz Biotechnology) for 1 h at 4 °C, followed by the protein A/G agarose beads (Santa Cruz Biotechnology) with rotation for 24 h at 4 °C. After centrifugation at 4500*g* for 5 min at 4 °C, the supernatant was discarded, and the bead pellet was washed 5 times with lysis buffer (20 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1.0% Triton-X 100, 0.5% deoxycholate, and 0.1% SDS) by centrifugation under the same conditions. The bead pellet was subjected to SDS-PAGE using a 7.5% gel and Western blot analysis with anti-AhR antibody to detect the AhR bound to the Arnt.

Measurement of QR and GST Activities. QR and GST activities were measured in the cytosolic fraction according to the methods of Lind et al. (*33*) and Habig et al. (*34*), respectively. Briefly, the reaction mixture for QR activity consisted of 10 μL of the cytosolic fraction (0.11−0.22 μg of protein), 50 mM Tris-HCl (pH 7.5) containing 0.08% Triton-X100, 0.5 mM NADPH, and 0.077 mM cytochrome *c*. To start the reaction, 0.1 mL of 0.3 mM menadione was added to the mixture, and the change in absorbance at 550 nm was monitored for 3 min. For the measurement of GST activity, the reaction mixture consisted of 1 μL of the cytosolic fraction (0.011−0.022 μg of protein), 2.7 mL of 111 mM potassium phosphate buffer, pH 7.5, and 0.1 mL of 30 mM glutathione (reduced form). To start the reaction, 0.1 mL of 30 mM 1-chloro-2,4-dinitrobenzen was added to the reaction mixture, and the increase in absorbance at 340 nm caused by the formation of 2,4-dinitrophenylglutathione was monitored for 3 min. The activities of QR and GST are represented as micromoles per minute per milligram of protein.

Statistical Analysis. Data are expressed as the mean ± SE of at least three independent determinations for each experiment. Statistical significance was analyzed using Student's *t* test or Duncan's method.

RESULTS

Effect of CPE and Its Components on the DNA-Binding Activity of AhR in a Cell-free System and Cultured Cells. We examined the suppressive effect of CPE on the DNA-binding activity of AhR by SW-ELISA in the cell-free system. As shown in **Figure 1A**, CPE suppressed the activity in a dose-dependent manner with a 50% inhibitory concentration (IC_{50}) value of 98 μg/mL. CPE contains procyanidins, catechins, and other polyphenols (*19*). The effect of these polyphenols on AhR transformation was measured. (+)-Catechin, PB2, PB5, and CA2 suppressed the transformation at 50 μg/mL (**Figure 1B**). Methylated catechins and phenolic acids are detected as metabolites and decomposed products of CPE, respectively (*20*−*23*). The suppressive effects of these metabolites on AhR transformation were also examined (**Figure 1C**). MCA and MEC suppressed the transformation (**Figure 1C**), and their effect was stronger than that of the corresponding original compound. Regarding the decomposed products, most of the phenolic acids tested here showed a significant effect in the cell-free system.

In cultured cells, CPE (100 μg/mL) did not have a suppressive effect on Hepa-1c1c7 cells, but it revealed a suppressive effect in Hepa-1c1c7 cells after permeation through the Caco-2 cells (**Figure 2**). This result indicated that metabolites of CPE could suppress AhR transformation.

CPE Inhibits MC-Induced AhR Transformation and Regulates Induction of Drug-Metabolizing Enzymes in the Liver of Mice. Next, we investigated the effect of CPE on AhR transformation and downstream events in vivo. After the mice were orally administered CPE at 100 mg/kg of body weight followed by an intraperitoneal injection of MC at 10 mg/kg of body weight, AhR transformation was evaluated by EMSA and

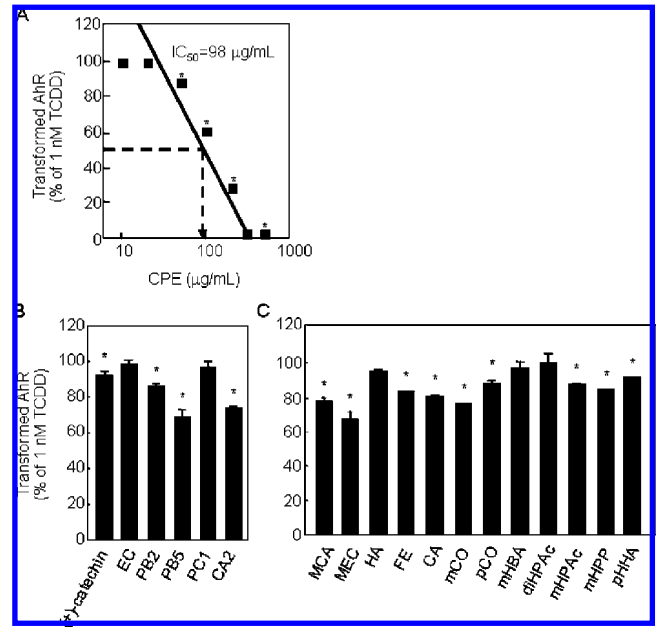

Figure 1. Suppressive effects of CPE and its components and metabolites on TCDD-induced AhR transformation in the cell-free system. The suppressive effect of (**A**) CPE at indicated concentrations, (**B**) its components at 50 μg/mL, and (**C**) metabolites at 50 μg/mL on TCDD-induced AhR transformation was evaluated by SW-ELISA. Data are shown as percent of transformed AhR induced by 1 nM TCDD and represented as the mean ± SE from three independent experiments. Asterisks indicate significant differences from the value of TCDD-induced transformation ($p < 0.05$, Student's *t* test).

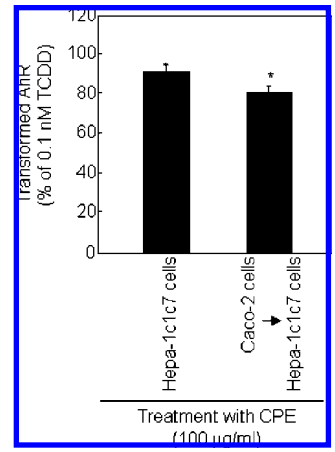

Figure 2. Suppressive effects of CPE on TCDD-induced AhR transformation in cultured cells. Hepa-1c1c7 cells were treated with 100 μg/mL of CPE or CPE permeated through Caco-2 cells 10 min prior to 0.1 nM TCDD. Nuclear extracts from cells were subjected to SW-ELISA. Data are shown as percent of transformed AhR induced by 0.1 nM TCDD and represented as the mean ± SE from three independent experiments. Asterisks indicate significant differences from the value of TCDD-induced transformation ($p < 0.05$, Student's *t* test).

SW-ELISA. EMSA results showed that MC significantly promoted the transformation as expected (**Figure 3**). CPE completely suppressed the MC-induced transformation to the control level in the liver 3 and 25 h after the injection. The suppressive effect of CPE was confirmed by SW-ELISA (**Table 2**). The results were similar to the EMSA (**Figure 3**). CPE also suppressed MC-induced CYP1A1 expression, a downstream event of AhR transformation, without a change in the constitutive level (**Figure 4A**). In the case of QR and GST, MC

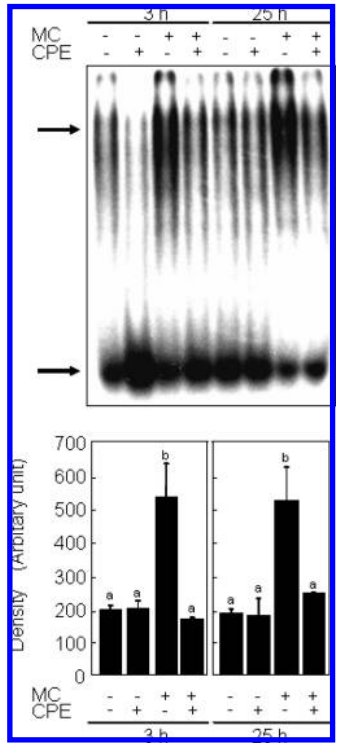

Figure 3. CPE suppresses AhR transformation induced by MC in the liver of mice. Mice were orally administered CPE at 100 mg/kg of body weight, followed by an intraperitoneal injection of MC at 10 mg/kg of body weight, and sacrificed 3 or 25 h later. A representative result of EMSA is shown in the upper panel, and the quantified density of the AhR/DRE complex is shown in the lower panel. Data are represented as the mean ± SE ($n = 3$). Different letters indicate statistically significant differences evaluated by multiple-comparison tests using Duncan's method.

Table 2. Suppressive Effects of CPE on AhR Transformation in the Liver of Mice[a]

time after MC injection	corn oil		MC	
	water	CPE	water	CPE
3 h	0.332 ± 0.012a	0.316 ± 0.017a	0.392 ± 0.013b	0.332 ± 0.031a
25 h	0.319 ± 0.006a	0.291 ± 0.019a	0.374 ± 0.008b	0.312 ± 0.013a

[a] Transformed AhR was detected by SW-ELISA as described under Materials and Methods. Data are shown as absorbance at 450 nm and represented as the mean ± SE ($n = 3$). Different letters in the same row indicate statistically significant differences evaluated by multiple-comparison tests using Duncan's method.

enhanced both enzymatic activities in the liver (**Figure 4B**,**C**). CPE significantly lowered the level of QR activity in both corn oil- and MC-dosed mice (**Figure 4B**). On the other hand, CPE enhanced GST activity in both groups additively (**Figure 4C**).

CPE Inhibits the MC-Induced Dimerization between the AhR and Arnt but Not Nuclear Translocation of the AhR into the Nucleus in the Liver. Possible inhibition of nuclear translocation of the AhR and/or heterodimerization of AhR and Arnt was investigated. As expected, MC induced the translocation of the AhR and formation of the heterodimer without affecting the Arnt level (**Figure 5**). CPE significantly inhibited the formation of the AhR/Arnt heterodimer, although it accelerated the translocation of AhR in MC-dosed mice.

CPE Suppresses AhR Transformation Ex Vivo. From the in vivo experiments, it was observed that orally dosed CPE suppressed AhR transformation by inhibiting the formation of the AhR/Arnt heterodimer in the liver. To confirm the suppressive effect of CPE, an ex vivo experiment was carried out

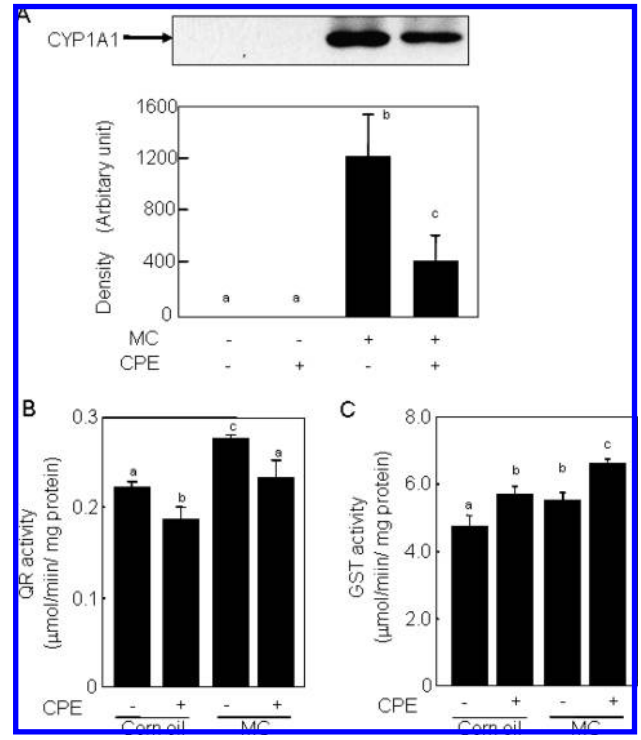

Figure 4. Effects of CPE on CYP1A1 expression, QR activity, and GST activity in the liver of mice dosed with MC. Mice were orally administered CPE at 100 mg/kg of body weight and then given an intraperitoneal injection of MC at 10 mg/kg of body weight. (**A**) CYP1A1 was detected in the liver 25 h after the CPE injection by Western blot analysis. Representative results (upper) and the quantified density (bottom) are shown. (**B**) QR and (**C**) GST activities were measured in the liver as described under Materials and Methods. Data are represented as the mean ± SE ($n = 3$). Different letters indicate statistically significant differences evaluated by multiple-comparison tests using Duncan's method.

(**Figure 6**). TCDD induced AhR transformation at 0.1 or 1.0 nM in the cytosolic fraction prepared from the liver of mice given water in a dose-dependent manner. The cytosolic fraction from the liver of CPE-injected mice showed significantly reduced AhR transformation compared with that of water-dosed mice. The suppressive effect did not disappear even 25 h after the CPE injection, suggesting that active compound(s) reached the liver and remained there, at least, by 25 h.

DISCUSSION

Dioxins enter the body mainly through the diet and accumulate in fatty tissues such as the liver and adipose tissue (*35*). The toxicological effects of dioxins are mainly induced by the transformed AhR (*14*), and the liver is one of the major organs of toxicological effects. In the present study, we demonstrated that CPE suppressed the DNA-binding activity of AhR (**Figure 3**) and subsequent induction of CYP1A1 expression (**Figure 4A**) by inhibiting the formation of a heterodimer between the AhR and Arnt (**Figure 5**) in vivo. The result from ex vivo experiments confirmed that the liver from the CPE-dosed mice tolerated the TCDD-increased DNA-binding activity of AhR (**Figure 6**). Therefore, certain active compound(s) in CPE would be, at least in part, absorbed in the body and incorporated into hepatocytes and reveal suppressive effects.

Many previous papers have demonstrated that foodstuffs such as green tea, black tea, and vegetable extracts suppressed AhR

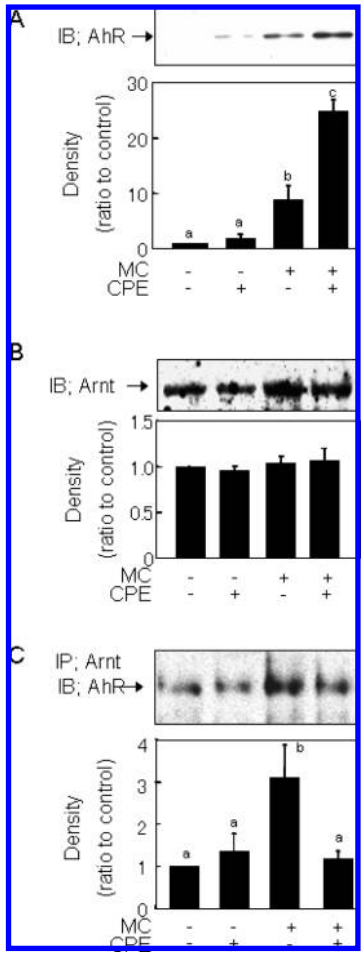

Figure 5. CPE suppresses the formation of AhR/Arnt heterodimer but not AhR nuclear translocation in the liver of mice dosed with MC. A hepatic nuclear fraction was prepared and (**A**) AhR and (**B**) Arnt were examined by Western blot analysis. (**C**) The hepatic nuclear fraction was immunoprecipitated with anti-Arnt antibody, and the AhR/Arnt heterodimer was determined by immunoblot using anti-AhR antibody. Representative blotting results are shown (upper), and quantified density was calculated as a ratio to the control and represented (bottom) as the mean ± SE ($n = 3$). Different letters indicate statistically significant differences evaluated by multiple-comparison tests using Duncan's method.

transformation and/or CYP1A1 expression in vitro (*24, 25, 30*), but in vivo experiments are scarce and inconclusive. We reported that an orally administered molokhia extract suppressed AhR transformation by inhibiting the nuclear translocation of the AhR in the liver of rats (*30*). In the present study, the administration of CPE suppressed AhR transformation by inhibiting formation of the AhR/Arnt heterodimer in the liver of mice, although CPE accelerated the nuclear translocation of AhR (**Figure 5**). The mechanism of action of CPE is different from that of molokhia, possibly due to a difference in active component(s): CPE contains polyphenols abundantly (*19*), and these polyphenols and their putative metabolites exhibited a suppressive effect in the cell-free system (**Figure 1**). In the case of molokhia, lutein and chlorophylls are identified as the active compounds (*36*). Recently, curcumin, a polyphenol in turmeric, was reported to inhibit formation of the AhR/Arnt heterodimer by inhibiting phosphorylation of the AhR and Arnt in Hepa-1c1c7 cells (*37*). In the same paper, curcumin accelerated the nuclear translocation of AhR the same as CPE in this study. Therefore, polyphenols and/or their metabolites are candidates for the active compounds in CPE.

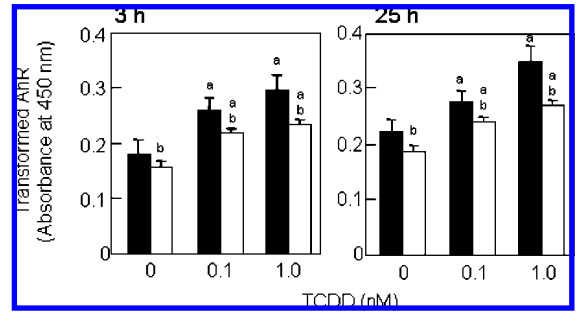

Figure 6. CPE suppresses AhR transformation ex vivo. A hepatic cytosolic fraction prepared from mice dosed with CPE at 100 mg/kg of body weight or water was subjected to an evaluation of AhR transformation induced by 0.1 or 1.0 nM TCDD ex vivo. The level of transformed AhR in the liver (**A**) 3 h and (**B**) 25 h after the CPE injection was determined by SW-ELISA as described under Materials and Methods. Data are shown as absorbance at 450 nm that reflects the transformed AhR and represented as the mean ± SE from three independent triplicate experiments ($n = 3$). a, significant difference from each negative control (vehicle alone); b, significant difference from the corresponding water group ($p < 0.05$, Student's t test).

The CPE used in this study contains 2.5% (+)-catechin, 6.6% EC, 3.8% PB2, 2.9% PC1, and 1.8% CA2 (*19*). These compounds showed suppressive effects on AhR transformation in the cell-free system (**Figure 1B**). The bioavailability of catechins and procyanidins has been described as follows: Polyphenols undergo glucuronidation by UDP-glucuronosyl transferase, sulfation by phenyl sulfotransferase, and/or methylation by catechol-*O*-methyltransferase in enterocytes and/or hepatocytes (*23, 38−40*). When cacao powder was administered orally to rats at 50 mg/kg of body weight, 0.1 μM MEC was detected in plasma (*41*), in addition to the detection of the glucuronide and/or sulfate form of catechin in urine (*20*). Moreover, over 1 μM methylated catechin was detected in human plasma after the intake of cocoa powder (*42*). In the case of aglycone forms, EC and PB2 were detected at 0.1−1.1 and 0.5 μM, respectively, in rat serum after the oral administration of cacao powder at 50 mg/kg of body weight (*41*), 0.05−0.34 μM EC was detected in plasma 1 h after the oral administration of cocoa powder to rats at 150−1500 mg/kg of body weight (*20*), and 1.13 μM EC and 5.23 μM (+)-catechin were detected in rat urine 24 h after the po injection of these catechins at 172 μmol/kg of body weight each (*23*). These reports indicate that polyphenols in CPE, at least in part, are incorporated into the body. Methylated catechins are candidates for the active compounds in CPE, because their antagonistic effect was stronger than that of aglycones (**Figure 1**). Procyanidins, which reach the large intestine without being absorbed in the small intestine, undergo biotransformation to phenolic acids by microflora (*21, 22*). After the consumption of a polyphenol-rich meal, FE was detected in plasma, and other phenolic acids such as mHPP and pHHA were present in urine (*21*). In this study, these phenolic acids also suppressed AhR transformation in the cell-free system (**Figure 1C**). Moreover, CPE had a significant suppressive effect on the DNA-binding activity of AhR after its permeation through Caco-2 cells (**Figure 2**). These results suggest that metabolites of polyphenols in CPE contributed to the suppression of AhR transformation.

The structure−activity relationships of polyphenols and phenolic acids tested in this study are as follows. Regarding the catechins and procyanidins, the activity of procyanidins is stronger than that of catechins (**Figure 1B**), and the activity of CPE fractions separated by molecular size is tetramer > trimer

> dimer > monomer fraction (data not shown). These results suggest that the higher degree of polymerization reveals the stronger suppressive effect on the DNA-binding activity of AhR. As to the phenolic acids, phenol propanoid derivatives with the less hydroxyl group in phenolic moiety show stronger activity, and binding of a hydroxyl group to the meta position is also important to reveal the activity. In addition, propenoic acid has stronger activity than propanoic acid.

After ligand binding, the AhR translocates into the nucleus and forms a heterodimer with Arnt (*8, 9*). Phosphorylation of AhR and/or Arnt was considered to be essential for the transcriptional activity of the heterodimer (*43*−*45*). In a previous study, curcumin inhibited the phosphorylation of AhR and Arnt (*37*). It was also reported that (−)-epigallocatechin-3-gallate suppressed the DNA-binding activity of AhR by interacting with hsp90 (*46*) without inhibiting the nuclear translocation of AhR. It is, therefore, suggested that one or more active components in CPE interact with partner proteins of the AhR and/or inhibit certain kinase activity, which is involved in AhR transformation. Regarding the nuclear translocation, CPE accelerated nuclear translocation of the AhR in the liver of MC-dosed mice (**Figure 5**). We speculate the following reason on this point: After the conformational change of AhR by MC (*47*), the active component in CPE interacts with the transactivation domain in the AhR, resulting in acceleration of nuclear translocation, because the transactivation domain is reported to regulate subcelluer localization of the AhR without agonist (*48*). Further study is needed to clarify the mechanism of action of CPE and its components in detail.

Regarding drug-metabolizing enzymes, CYP1A1 protein, a phase I enzyme, is recognized as a typical gene product of AhR transformation (*49*). The AhR also regulates the expression of QR and GST Ya (*11, 12*). In this study, CPE suppressed MC-induced CYP1A1 expression and QR activity, whereas it promoted GST activity in the liver. GST and QR expression are regulated by not only the AhR/DRE but also nuclear factor erythroid 2-related factor 2 (Nrf2)/antioxidant response element (ARE) (*50*). Previous studies demonstrated that polyphenols enhanced GST activity in vivo (*51, 52*). Therefore, the active component in CPE has a possibility to regulate expression of GST by Nrf2/ARE, whereas it may regulate expression of QR by AhR/DRE. Further experiments are needed to investigate the effect of CPE on the Nrf2/ARE pathway. Because GST plays an important role in the conjugation of certain chemicals including carcinogens (*53*), the induction of GST activity by CPE, in addition to the suppression of MC-induced CYP1A1 expression, may prevent the toxicological effects, including carcinogenicity, of polyaromatic hydrocarbons.

ABBREVIATIONS USED

AhR, aryl hydrocarbon receptor; CPE, cacao polyphenol extract; MC, 3-methylcholanthrene; hsp90, heat shock protein 90; Arnt, aryl hydrocarbon receptor nuclear translocator; DRE, dioxin responsive element; CYP1A1, cytochrome P4501A1; QR, NAD(P)H:quinone-oxidoreductase; GST, glutathione *S*-transferase; TCDD, 2,3,7,8-tetrachrolodibenzo-*p*-dioxin; EC, (−)-epicatechin; MEC, 3′-*O*-methyl-(−)-epicatechin; PB2, procyanidin B2; PB5, procyanidin B5; DMSO, dimethyl sulfoxide; MCA, 3′-*O*-methyl-(+)-catechin; PC1, procyanidin C1; CA2, cinnamtannin A2; SW-ELISA, southwestern chemistry-based enzyme-linked immunosorbent assay; HBSS, Hanks′ balanced salt solution.

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