TCDD-Induced CYP1A1 Expression, an Index of Dioxin Toxicity, Is Suppressed by Flavonoids Permeating the Human Intestinal Caco-2 Cell Monolayers

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Since the toxicological effects of dioxins are mainly mediated by the aryl hydrocarbon receptor (AhR), an in vitro assessment system for AhR activity was used in this study to search for flavonoids that attenuated dioxin toxicity through the intestinal epithelial monolayer. When AhR transformation in Hepa-1c1c7 cells was examined by southwestern ELISA, nine flavonoids among 34 kinds of flavonoids inhibited the transformation by more than one-half. When each flavonoid with 2,3,7,8-tetrachloro-dibenzo-*p*-dioxin (TCDD) was added to dioxin-responsive HepG2 cells, seven flavonoids significantly restrained the TCDD-induced transcriptional activity of the CYP1A1 promoter. Furthermore, those seven flavonoids that had permeated the Caco-2 cell monolayers demonstrated an inhibitory effect on both the AhR transformation and on the transcriptional activity of the CYP1A1 promoter. The expression level of the CYP1A1 mRNA and protein induced by TCDD was suppressed by flavone, galangin, and tangeretin. It is proposed from these results that some flavonoids have the ability to suppress dioxin-induced AhR activity after permeating the human intestinal epithelial cell monolayer.

KEYWORDS: TCDD; flavonoid; aryl hydrocarbon receptor; CYP1A1; Caco-2

INTRODUCTION

Polycyclic aromatic hydrocarbons such as polychlorodibenzo-*p*-dioxins and polychlorodibenzofurans and halogenated aromatic hydrocarbons are released as industrial compounds or as industrial byproducts through the process of combustion and cause serious problems as environmental contaminants. Their notorious action as xenobiotics has such toxic consequences as endocrine disruption, hepatotoxicity, dermatological disease, and carcinogenicity (*1−3*).

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) is known as the most toxic compound among dioxins and has been extensively investigated as a potent ligand of the aryl hydrocarbon receptor (AhR) (*4*, *5*). Previous studies have demonstrated that AhR knockout mice were resistant to the toxicity of TCDD (*6*), implying that the toxicological effects of dioxins are mainly mediated by AhR. In binding to TCDD, AhR releases hsp90, cochaperone p23, and immunophilin-like protein XAP2 (AIP/ARA9) (*7*, *8*), this being followed by nuclear translocation and dimerization with the AhR nuclear translocator (Arnt) (*9*).

Transactivation of a battery of AhR-responsive genes is inspired by binding this heterodimer to xenobiotic-responsive

elements (XREs) that are located in the regulatory regions of their promoter. Several xenobiotic-metabolizing enzymes, as typified by cytochrome P450 1A1 (CYP1A1), are induced by these means, and they then activate latent endogenous or exogenous carcinogens (*10*).

Although it has been accepted that most dioxins are orally absorbed through contaminated food (*11*, *12*), it is difficult and expensive to remove dioxins from food. It is therefore important to search for a food factor that offers protection from dioxin toxicity. Flavonoids in many kinds of vegetable and fruits are good dietary candidates for suppressing AhR transformation because they have a suitable structure for binding to the AhR ligand-binding pockets. It has been reported from the results of an electrophoretic mobility shift assay that such flavonoids as quercetin, rutin, and luteolin antagonistically inhibited AhR transformation in the rat hepatic cytosol (*13*). The southwestern chemistry-based enzyme-linked immunosorbent assay (SW-ELISA) is also applicable for screening antagonists among natural flavonoids (*14*). This SW-ELISA system can quantify the transformed AhR protein by using an XRE oligonucleotide probe. When the antagonistic effects of nine flavonoids (apigenin, galangin, hesperetin, kaempferol, myricetin, naringenin, quercetin, rutin, and tangeretin) were estimated in the rat hepatic fraction, the results of SW-ELISA closely matched those of a gel retardation assay with a significant correlation coefficient ($r = 0.935$) (*14*). Furthermore, several studies regarding the

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regulatory effects of flavonoids on the AhR-dependent CYP1A expression have already been reported (*15, 16*).

However, it has not yet been considered whether these flavonoids are able to be absorbed and maintain their suppressive effect on dioxin-induced toxicity even after passing across the epithelial cell monolayer. In relation to these omissions, a method for evaluating the transcriptional activity of CYP1A1 by a luciferase assay has been reported (*17*). We have also constructed an in vitro system for evaluating intestinal dioxin permeability by using the human intestinal Caco-2 cell monolayer, which is widely used as a model for the intestinal epithelial monolayer, and a reporter assay using the luciferase vector containing 3 × XRE elements (*18*).

The aim of the present study was to test whether the flavonoids are able to be absorbed and maintain their suppressive effect on AhR-dependent CYP1A expression even after passing across the intestinal cells because flavonoids would possibly be metabolized in the intestinal epithelium, changing their structure and functions. We have examined in the present study the suppressive effect of flavonoids that permeate the human intestinal Caco-2 cell monolayers on TCDD-induced CYP1A1 expression by using two methods, a cell-free (SW-ELISA) assay and a cell-based (reporter analysis) bioassay.

MATERIALS AND METHODS

Materials. The Caco-2 cell line (derived from human colonic cancer tissue) and HepG2 cell line (derived from human hepatic cancer tissue) were obtained from the American Type Culture Collection (Rockville, MD). Dulbecco's modified Eagle's medium (DMEM) was purchased from Sigma (St. Louis, MO). Penicillin−streptomycin (10 000 U/mL and 10 mg/mL in 0.9% sodium chloride, respectively) and nonessential amino acids were purchased from Gibco (Gaithersburg, MD). Fetal bovine serum was purchased from Asahi Technoglass (Chiba, Japan). Twelve-well Transwell inserts and 24-well plates were purchased from Corning-Coster Japan (Tokyo, Japan). G418 disulfate was purchased from Nacalai Tesque (Kyoto, Japan), and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) was purchased from Wako Pure Chemical Industries (Osaka, Japan). The Dual-Luciferase reporter assay system was purchased from Promega (Madison, WI), and QuantiTect SYBR Green for real-time PCR was from Qiagen (Tokyo, Japan).

Flavonoids. Apigenin and hesperetin were obtained from Sigma (St. Louis, MO). Baicalein, daidzein, eriodictyol, galangin, isorhamnetin, kaempferol, luteolin, luteolin-7,3′-*O*-bis-glucoside, tamarixetin, and tangeretin were from Extrasynthese (Genay, France). Chrysin and myricetin were from Sigma-Aldrich (Tokyo, Japan). Fisetin, flavanone, genistein, quercetin, and rutin were from Wako Pure Chemicals (Tokyo, Japan). Flavone, morin, naringenin, and quercitrin were from Nacalai Tesque (Kyoto, Japan); flavonol and naringin were from Tokyo Kasei Kogyo (Tokyo, Japan); and puerarin was from Funakoshi (Tokyo, Japan). The catechins, (+)-catechin, (3)-gallocatechin, (3)-catechin gallate, (3)-gallocatechin gallate, (3)-epicatechin, (3)-epigallocatechin, (3)-epicatechin gallate, and (3)-epigallocatechin gallate, were purchased from Kurita Kogyo (Tokyo, Japan).

Cell Culture. Caco-2 cells were cultured at 37 °C in a humidified atmosphere of 5% CO_2 in air with a culture medium consisting of DMEM, 10% fetal bovine serum, 1% nonessential amino acids, 200 U/mL penicillin and 200 μg/mL streptomycin. TCDD-responsive HepG2 cells stably transfected with the dioxin-responsive plasmid (pLUC1A1) were established as described previously (*18*). Nonessential amino acids were excluded from the culture of these stably transfected HepG2 cells (HepG2-LUC), and G418 was added to the same medium as that used for culturing the Caco-2 cells. Mouse hepatoma Hepa-1c1c7 cells were maintained in the respective α-minimum essential medium (Nissui Pharmaceutical Co., Tokyo, Japan) supplemented with 10% (v/v) fetal bovine serum (Sigma-Aldrich Co.), 4 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin. The HepG2-LUC cells were seeded at 1×10^5 cells/well in a 24-well plate that had been

precoated with collagen and were used after 1 day of culture. The Caco-2 cells used in this study were between passages 40 and 65.

Transepithelial Transport Experiments. To form the Caco-2 cell monolayers, the cells were seeded at 4×10^5 cells/well in a 12-well Transwell insert that had been precoated with collagen and were cultured for 2 weeks to obtain an integrated cell monolayer with a transepithelial electrical resistance (TER) of more than 150 Ω cm². A flavonoid sample was added to the apical chamber (500 μL) of the Caco-2 cell monolayer to give a final flavonoid concentration of 5−100 μM. The basal solution (1500 μL) was recovered after incubating for 24 h.

Southwestern Chemistry-Based Enzyme-Linked Immunosorbent Assay (SW-ELISA). To evaluate the suppressive effect of each flavonoid, Hepa-1c1c7 cells seeded on 60 mm dishes (approximately 80% confluent at a density of 2.0×10^6 cells/dish) were pretreated with a flavonoid (10 μM) for 10 min at 37 °C and then treated with 500 pM TCDD for 1 h at 37 °C. The cells were harvested with a lysis buffer (20 mM HEPES at pH 7.6, 20% (v/v) glycerol, 10 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, and 1 mM DTT) containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride (PMSF), 20 μg/mL leupeptin, and 5 μg/mL aprotinin), stood on ice for 15 min with occasional mixing, and then centrifuged at 1000*g* for 10 min at 4 °C. The precipitate was suspended in an extraction buffer (20 mM HEPES at pH 7.6, with 20% (v/v) glycerol, 500 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 1 mM DTT, and 0.1% (v/v) NP-40) containing the same protease inhibitors as the lysis buffer and rotated for 1 h at 4 °C. The suspension was centrifuged at 10 000*g* for 20 min at 4 °C, and the resulting supernatant was used as a nuclear extract for measuring transformed AhR by SW-ELISA.

SW-ELISA was performed according to the method in ref *14*. Briefly, a 96-well microtiter plate (Maxisorp; Nalge Nunc International, Tokyo, Japan) was coated overnight at 4 °C with 100 μL of 0.46 μg/mL anti-FITC antibody in 50 mM sodium bicarbonate (pH 9.6). After washing, the plate was blocked with a casein-based blocking buffer (Nacalai Tesque, Kyoto, Japan), and 100 L of a 250 fmol/mL FITC-labeled XRE probe was added to the plate. A reaction mixture containing transformed AhR was next plated into each XRE probe-bound well and incubated for 2 h at room temperature, before 100 μL of the specific antibody against Arnt or AhR was added to each well. The bound specific antibody was detected by the labeled streptavidin biotin method. The biotinylated antigoat-IgG antibody (Jackson Immuno Research Lab., West Grove, PA) and peroxidase-conjugated streptavidin (DakoCytomation) were used. Bound peroxidase activity was visualized with tetramethylbenzidine (DakoCytomation), and the color was developed with tetramethylbenzidine. The result was measured with a Wallac ARVO sx multi-label counter (Perkin−Elmer Life Sciences, Boston, MA) at a wavelength of 450 nm.

Luciferase Assay. A luciferase assay was carried out to search for those flavonoids that inhibited the TCDD-induced transcriptional activity of CYP1A1 in the TCDD-responsive HepG2-LUC cells. Sample solutions each containing a flavonoid were added to each well of a 24-well plate in which stably integrated HepG2-LUC cells had been seeded. The luciferase assay was conducted after 24 h of incubation according to the instruction manual for the Dual-Luciferase reporter assay (Promega). The 24-well plate was washed twice with PBS, and the contents dissolved in a 1 × passive lysis buffer were served for the luciferase assay.

Isolation of Total RNA and Real-Time PCR. After incubating with the Caco-2 cell basal solution containing TCDD, total RNA was extracted from the HepG2 cells by using Isogen according to the manufacturer's recommendation. The cDNA was prepared from 1 μg of the total RNA. A real-time polymerase chain reaction (PCR) was performed with SYBR Green I. After denaturing at 95 °C for 15 min, PCR was performed for 40 cycles, each of which consisted of denaturing at 95 °C for 15 s, annealing at 56 °C for 15 s, and extension at 72 °C for 10 s. The following PCR primers for CYP1A1, CYP1A2, and β-actin were used: human CYP1A1 sense, 5′-AGATGGTCAAG-GAGGACTACA-3′; human CYP1A1 antisense, 5′-CTGGATATTG-GCGTTCTCAT-3′; human CYP1A2 sense, 5′-TCCCACAGGAGAA-GATTGTC-3′; human CYP1A2 antisense, 5′-CCTTCTGGATCTTC-CTCTGT-3′; human β-actin sense, 5′-GCGAGAAGATGACCCAGAT-
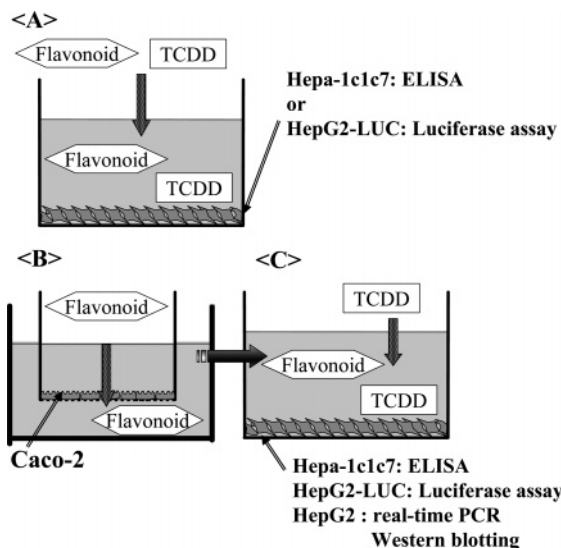
Figure 1. Experimental systems used the analyses. (**A**) System to evaluate direct effects of flavonoids on TCDD-induced AhR activation in hepatic cells; this being used for SW-ELISA with Hepa-1c1c7 cells (**Figure 2**) and luciferase assay with HepG2-LUC cells (**Figure 3**). (**B** and **C**) System to evaluate the effects of flavonoids permeated through Caco-2 cell monolayers; this being used for SW-ELISA with Hepa-1c1c7 cells (**Table 1**), luciferase assay with HepG2-LUC cells (**Figure 4**), real-time PCR (**Figures 5** and **6**), and Western blot analysis (**Figure 7**) with HepG2 cells.

CATGTT-3′; and human β-actin antisense, 5′-GCTTCTCCTTAAT-GTCACGCACGAT-3′.

The effect of the TCDD treatment on β-actin mRNA expression was not significant at any stage, indicating that β-actin could be used as a stable housekeeping gene throughout the experiment.

Western Blot Analysis. After incubating with the Caco-2 cell basal solution containing TCDD, HepG2 cells cultured in six-well plates for a day were washed twice with ice-cold PBS. The cells were scraped off and then suspended in 1 mL of PBS. The precipitate obtained by centrifugation at 1000*g* for 5 min at 4 °C was homogenized with 0.15 mL of PBS containing 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, and 10% glycerol. The homogenate was centrifuged at 10 000*g* for 5 min at 4 °C, and the resulting supernatant was dissolved in a loading buffer containing 0.125 M Tris−HCl (pH 6.8), 14% glycerol, 4% SDS, 0.05% bromophenol blue, and 10% β-mercaptoethenol at 100 °C for 5 min. Twenty micrograms of protein was fractionated by SDS−PAGE (12.5% polyacrylamide gel) and transferred to a PVDF membrane (Millipore). The membrane was blocked overnight at 4 °C by PBS-T containing 5% BSA. The membrane was then incubated for 1 h with the anti-rat CYP1A1 antibody (1:1000 dilution). The blot was washed in PBS-T and incubated for 1 h with anti-goat IgG-HRP (1:1000 dilution). A chemiluminescent substrate (ECL; Amersham Biosciences) was used for detection.

Experimental Systems Used. Experimental systems used in this study are summarized in **Figure 1**. Direct effects of flavonoids on hepatic cells were measured by SW-ELISA and luciferase assay as shown in **Figure 1A**. Effects of flavonoids permeated through the intestinal Caco-2 cell monolayers were measured as in **Figure 1B**,**C**. The basal solution was taken (**Figure 1B**) and then was served for SW-ELISA and the luciferase assay using hepatic cells (**Figure 1C**). Real-time PCR and Western blot analysis were also carried out to investigate the response of hepatic cells to TCDD in the presence of flavonoids that had permeated the Caco-2 monolayers (**Figure 1C**).

RESULTS

**Antagonistic Effects of Flavonoids on TCDD-Induced AhR Transformation by using SW-ELISA.** The toxicological effects of dioxins are mainly mediated by AhR. The effects of flavonoids on mouse AhR transformation induced by TCDD were investigated (**Figure 1A**). AhR transformation was inhibited by more than half by nine flavonoids: flavone, luteolin, tangeretin, flavonol, galangin, fisetin, tamarixetin, isorhamnetin, and (−)epigallocatechin (**Figure 2**). The TCDD-induced AhR transformation was respectively decreased to 5, 24, 17, 35, and 24% in the presence of flavone, flavonol, galangin, tamarixetin, and (−)epigallocatechin.

Effect of Flavonoids on the TCDD-Induced Transcriptional Activity of CYP1A1 by using the Stably Transfected HepG2 Cell Line. A luciferase assay using dioxin-responsive HepG2 cells (*18*) was also conducted to examine whether the flavonoids could inhibit the TCDD-induced transcriptional activity of CYP1A1. TCDD (0.5 nM) and a flavonoid (10 μM) were added to a 24-well plate in which the dioxin-responsive HepG2-LUC cells had been seeded, and the luciferase assay was conducted 24 h later (**Figure 1A**). A significant inhibitory effect was observed with seven flavonoids: flavone, apigenin, luteolin, tangeretin, flavonol, galangin, and flavanone (**Figure 3**). The TCDD-induced increase of the CYP1A1 promoter activity was substantially decreased to 15, 22, 7, 10, 7, 3, and 10% in the respective presence of flavone, apigenin, luteolin, tangeretin, flavonol, galangin, and flavanone. Many of these flavonoids that showed antagonistic activity are categorized as flavones. On the other hand, such isoflavones as daidzein and genistein showed agonistic activity, while glucosides (luteolin-7,3′-*O*-bis-glucoside and puerarin) had no effect.

These results demonstrated that flavone, luteolin, tangeretin, flavonol, and galangin had an antagonistic effect on both human AhR and mouse AhR. In contrast, fisetin, tamarixetin, isorhamnetin, and (−)epigallocatechin suppressed only mouse AhR transformation, not human AhR.

Antagonistic Effect on TCDD-Induced AhR of the Flavonoids that Permeated the Caco-2 Cell Monolayer by using SW-ELISA. We tested the effects of the flavonoids that permeated the Caco-2 cell monolayer to evaluate their absorption and metabolism in intestinal cells. The seven flavonoids (flavone, apigenin, luteolin, tangeretin, flavonol, galangin, and flavanone) that exhibited an inhibitory effect on the TCDD-induced toxicity shown in **Figures 2** and **3** were each added to the apical side of the Caco-2 cell monolayer and incubated for 24 h (**Figure 1B**). The basal medium of the Caco-2 cell monolayer was utilized by SW-ELISA (**Figure 1C**). Each of these flavonoids showed an inhibitory effect on the mouse AhR transformation induced by TCDD (**Table 1**). The inhibitory effects of flavone and tangeretin were the strongest, while there was little significant difference in effect among the others.

Inhibitory Effect of the Flavonoids that Permeated the Caco-2 Cell Monolayers on the TCDD-Induced Transcriptional Activity of CYP1A1 in HepG2-LUC Cell Line. The effects of the seven flavonoids just described (flavone, apigenin, luteolin, tangeretin, flavonol, galangin, and flavanone) were also analyzed by a luciferase assay. Among the samples tested, apigenin, luteolin, flavonol, galangin, and flavanone (0−100 μM) had an inhibitory effect in a dose-dependent manner (**Figure 4B**,**C**,**E**−**G**). As shown in **Figure 3D**, tangeretin showed the strongest inhibitory effect in a low concentration range (0−20 μM). The inhibitory effect did not change in the concentration range of 20−100 μM (data not shown). The lactate dehydrogenase assay showed that these flavonoids, including tangeretin, were not cytotoxic at the concentrations of 100 μM or lower (data not shown). Interestingly, although flavone at 10 μM had a strong inhibitory effect, this effect decreased in a dose-dependent manner (**Figure 4A**). For the following experi-
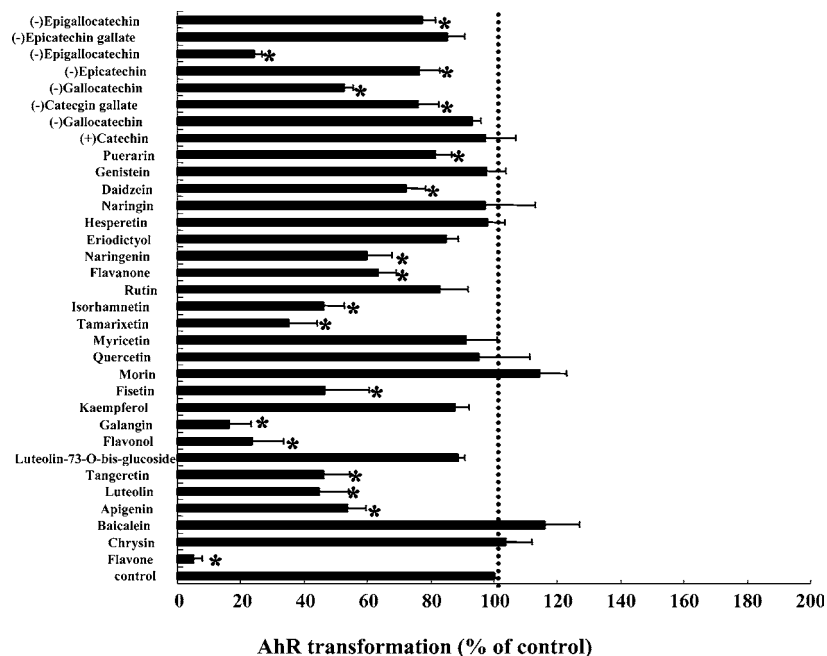
Figure 2. Antagonistic activity of 34 flavonoids toward TCDD-induced AhR transformation evaluated by SW-ELISA. Hepa-1c1c7 cells were pretreated with a flavonoid for 10 min and then treated with 0.5 nM TCDD for 1 h. A nuclear extract was used for measuring transformed AhR by SW-ELISA. The control shows the treatment with TCDD only. Each value is the mean ± SE ($n = 3$). Statistically significant differences from this value were analyzed by the t-test and are each indicated by an asterisk (*, $p < 0.05$).
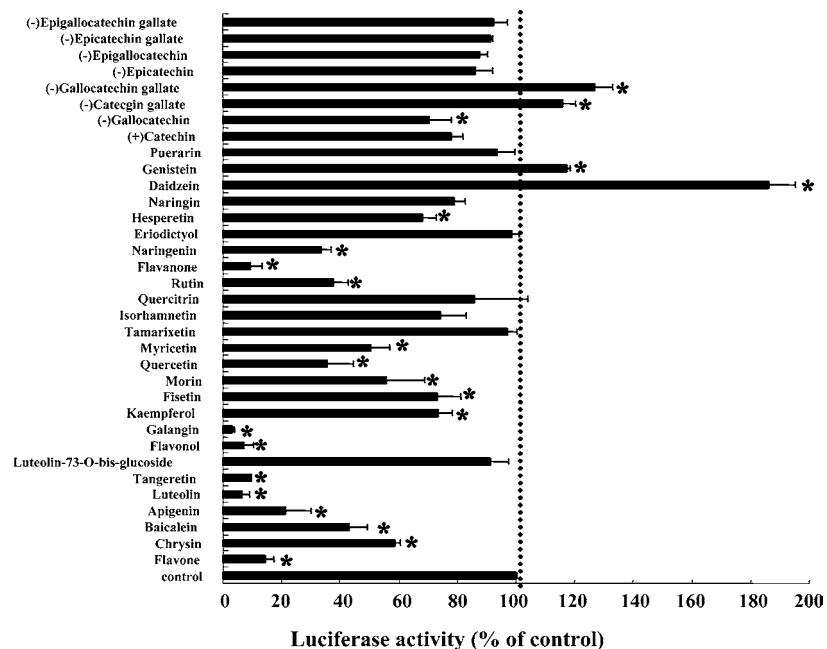
Figure 3. Inhibitory effect of 34 flavonoids on TCDD-induced CYP1A1 transcriptional activity evaluated by a luciferase assay. TCDD (0.5 nM) and a flavonoid (10 μM) were added to the dioxin-responsive stable HepG2 cells, and the luciferase assay was conducted 24 h later. The control shows the treatment with TCDD only. Each value is the mean ± SE ($n = 4$). Statistically significant differences from this value were analyzed by the t-test and are each indicated by an asterisk (*, $p < 0.05$).

ments, we chose three of the seven flavonoids. Those are tangeretin having the strongest inhibitory effect, flavone showing a unique concentration-dependence, and galangin as a typical flavonol.

Inhibitory Effect of Flavone, Galangin, and Tangeretin that Permeated the Caco-2 Cell Monolayers on TCDD-Induced CYP1A1 and CYP1A2 mRNA Expression. The mRNA expression of CYP1A1 and CYP1A2 in HepG2 cells was examined with flavone, galangin, and tangeretin permeating the Caco-2 cell monolayer to determine whether permeation through Caco-2 cells affected the inhibitory activity of those

flavonoids. The mRNA expression of CYP1A1 and CYP1A2 was induced by 1 nM TCDD in a time-dependent manner (data not shown). After 12 h of the 1-nM TCDD treatment, the respective mRNA levels of CYP1A1 and CYP1A2 had increased 20- and 7-fold, as compared to that of HepG2 cells incubated under the control condition. As shown in **Figure 5A**, 50 μM flavone inhibited the mRNA expression to 36%, whereas 100 μM flavone had a weaker effect. The CYP1A1 mRNA expression induced by TCDD was suppressed in a dose-dependent manner in the presence of galangin and tangeretin (**Figure 5B,C**).

Table 1. Antagonistic Effects of Flavonoids that Had Permeated the Caco-2 Cell Monolayer Evaluated by SW-ELISA[a]

	% of control						
concentration (μM)	flavone	apigenin	luteolin	tangeretin	flavonol	galangin	flavanone
5				92.4 ± 4.2*			
10	104.0 ± 5.7	83.0 ± 3.0*	91.6 ± 1.8*	80.5 ± 5.5*	90.0 ± 1.8	95.8 ± 1.8	101.1 ± 5.9
20				85.2 ± 4.3*			
50	61.6 ± 5.9*	91.6 ± 0.4*	76.4 ± 3.8*		78.4 ± 2.9*	85.3 ± 3.4*	88.6 ± 7.1*
100	43.6 ± 4.5*	80.6 ± 3.2*	79.2 ± 1.6*		89.6 ± 6.7*	83.8 ± 2.1*	70.4 ± 10.4*

[a] Hepa-1c1c7 cells were pretreated for 10 min with each flavonoid that had permeated the Caco-2 monolayer and then treated with 0.5 nM TCDD for 1 h. A nuclear extract was used for measuring transformed AhR by SW-ELISA. The control shows the treatment with TCDD only, the value for the control being 100 ± 1.23 (n = 14). Each value is the mean ± SE (*n* = 3). Statistically significant differences from this value were analyzed by the *t*-test and are each indicated by an asterisk (*, *p* < 0.05).
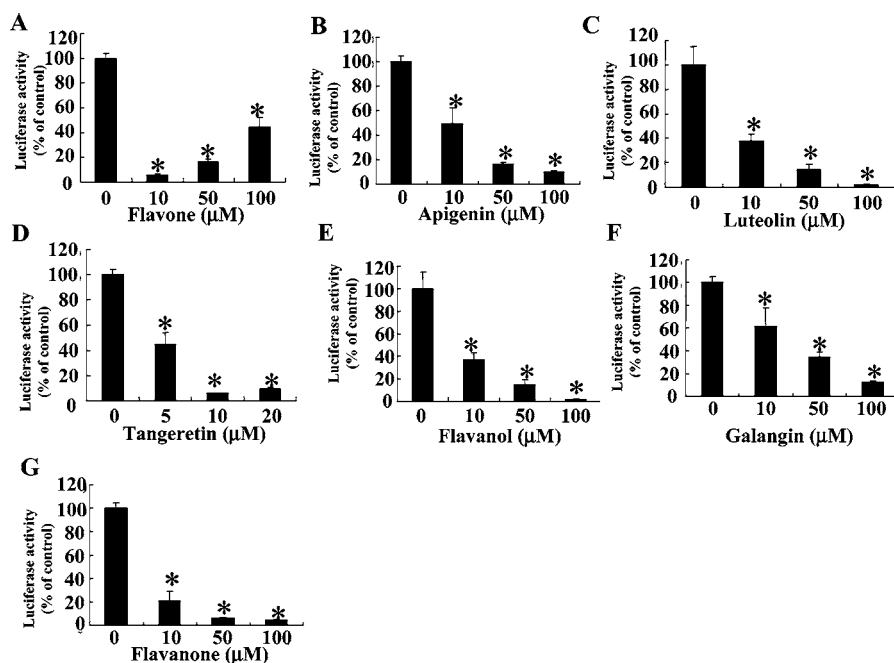
Figure 4. Effect of flavonoids that had permeated the Caco-2 cell monolayer on the TCDD-induced CYP1A1 transcriptional activity evaluated by a luciferase assay. TCDD (0.08 nM) and a flavonoid that had permeated the Caco-2 cell monolayer were added to the dioxin-responsive HepG2-LUC cells, and the luciferase assay was conducted 24 h later. (**A**) Flavone, (**B**) apigenin, (**C**) luteolin, (**D**) tangeretin, (**E**) flavonol, (**F**) galangin, and (**G**) flavanone. The control shows the treatment with TCDD only. Each value is the mean ± SE (*n* = 3). Statistically significant differences from this value were analyzed by the *t*-test and are each indicated by an asterisk (*, *p* < 0.05).

We also examined the effect of these flavonoids on the TCDD-induced increase in CYP1A2 mRNA. Fifty micromolar flavone inhibited the TCDD-induced mRNA expression of CYP1A2 to 20% of the control value, but 100 M flavone had a weaker effect, like the case of CYP1A1 (**Figure 6A**). Galangin and tangeretin dose-dependently inhibited the mRNA increase of CYP1A2 by the TCDD treatment (**Figure 6B,C**).

Suppression of TCDD-Induced CYP1A1 Expression by Flavone, Galangin, and Tangeretin that Permeated the Caco-2 Cell Monolayers. The three flavonoids that had an inhibitory effect on TCDD-induced CYP1A1 and CYP1A2 mRNA expression (flavone, galangin, and tangeretin) were investigated for their protein expression also being inhibited in HepG2 cells. After 24 h of the TCDD treatment, the CYP1A1 protein was induced in a dose-dependent manner, the protein level reaching a plateau with about 1 nM TCDD (data not shown). Flavone, galangin, and tangeretin permeating the Caco-2 cell monolayer were added to HepG2 cells with 0.08 nM TCDD, and the CYP1A1 protein level was analyzed by a Western blot analysis. The HepG2 cells treated with galangin or tangeretin showed a decrease in the CYP1A1 protein level in a dose-dependent manner (**Figure 7B,C**). Interestingly, the suppressive effect of flavone decreased in a dose-dependent manner,

although the 10 μM flavone had a strong inhibitory effect (**Figure 7A**).

DISCUSSION

More than 90% of dioxins absorbed by the body enter orally via food (*11*, *12*). Their lipophilicity makes them easily absorbed at the intestinal epithelium and accumulated in fatty tissues (*19*). However, it is difficult and costly to eliminate dioxins from food. One solution to protect the human body from oral exposure to dioxins would be to inhibit the intestinal dioxin absorption. Some food materials have been reported to be useful for this purpose. It has been reported that chlorophyll derived from *chlorella* inhibited dioxin absorption in the gastrointestinal tract and accelerated dioxin excretion in rats (*20*). Several types of dietary fiber have also been reported to enhance the fecal excretion of dioxin isomers in mice (*21*). These substances are likely to inhibit dioxin absorption by physically adsorbing dioxins to dietary fiber. We have established a convenient and relatively concise in vitro method for assessing dioxin absorption across the human intestinal epithelial monolayer (*18*, *22*) and have used this method to reproduce the effects of chlorophyll and dietary fiber.
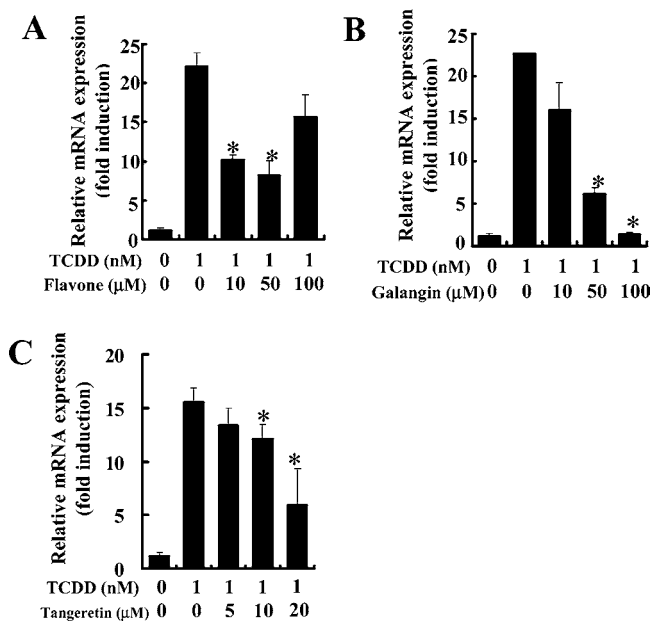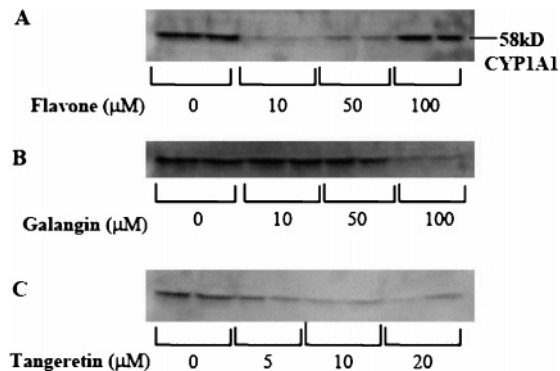
Figure 5. Inhibitory effects of flavone, galangin, and tangeretin on TCDD-induced CYP1A1 mRNA expression. Total RNA was extracted from the HepG2 cells after incubating with TCDD and a flavonoid that had permeated the Caco-2 cell monolayer. The mRNA expression of CYP1A1 was detected by real-time PCR. (**A**) Flavone, (**B**) galangin, and (**C**) tangeretin. The control shows treatment with the medium only. Each value is the mean ± SE ($n = 3$). Statistically significant differences from this value were analyzed by the *t*-test and are each indicated by an asterisk (*, $p < 0.05$).
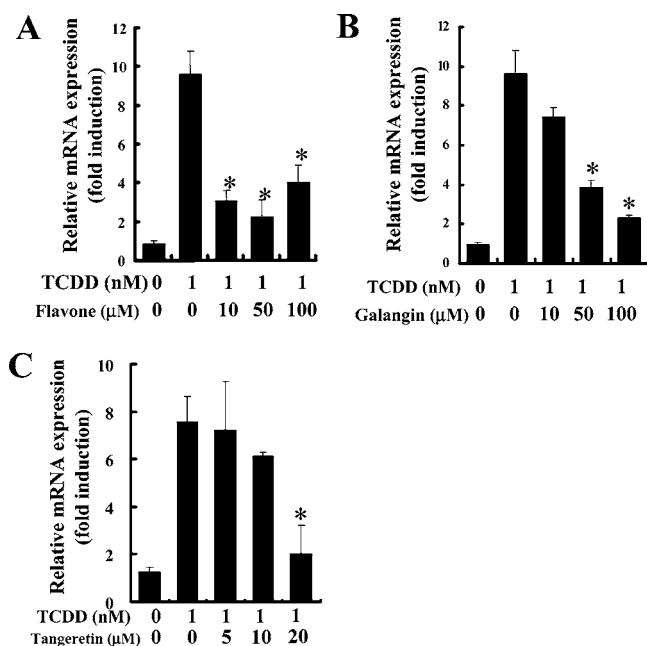
Figure 6. Inhibitory effects of flavone, galangin, and tangeretin on TCDD-induced CYP1A2 mRNA expression. Total RNA was extracted from the HepG2 cells after incubating with TCDD and a flavonoid that had permeated the Caco-2 monolayer. The mRNA expression of CYP1A2 was detected by real-time PCR. (**A**) Flavone, (**B**) galangin, and (**C**) tangeretin. The control shows treatment with the medium only. Each value is the mean ± SE ($n = 3$). Statistically significant differences from this value were analyzed by the *t*-test and are each indicated by an asterisk (*, $p < 0.05$).

An alternative solution would be the inhibition of dioxin toxicity by food substances. Recent studies have revealed the

Figure 7. Inhibitory effects of flavone, galangin, and tangeretin on TCDD-induced CYP1A1 protein expression. Flavone, galangin, or tangeretin that had permeated the Caco-2 cell monolayer and 0.08 nM TCDD were added to HepG2 cells for a day and analyzed by Western blotting. (**A**) Flavone, (**B**) galangin, and (**C**) tangeretin.

cellular mechanism for dioxin toxicity that enables us to deduce how to detoxicate dioxins in the body (*23*). We have examined in the present study the effect of flavonoids on the toxicity of TCDD by using two different evaluation methods: measuring the AhR transformation by SW-ELISA and measuring the transcriptional activity of the CYP1A1 promoter by a luciferase assay. The absorption of flavonoids through the intestinal epithelium was also taken into consideration by introducing a human intestinal epithelial Caco-2 cell model to the evaluation system. This study has demonstrated some of the flavonoids to have a suppressive effect on TCDD-induced CYP1A expression.

It is well-known that flavonoids are absorbed and metabolized in the intestines. The potential activity of flavonoids in vivo is also dependent on intestinal absorption, this being followed by metabolic reactions and subsequent interaction with the target tissues. The intestinal absorption, metabolism, distribution, and excretion of flavonoids have therefore been extensively studied. It has been reported that 11 kinds of cytochrome P450 and 37 kinds of phase II drug-metabolizing enzymes were expressed in Caco-2 cells after culturing for 16 days (*24*). These drug-metabolizing enzymes would be involved in the intestinal metabolism of flavonoids. It has been reported that chrysin and apigenin, a class of flavones, were catalyzed mainly by a conjugation pathway in Caco-2 cells, with both sulfated and glucuronidated forms being produced (*25*). Furthermore, it has been shown that the green tea catechins, including epicatechin, epigallocatechin, epicatechin gallate, and epigallocatechin gallate, were metabolized into sulfate conjugates, methylated sulfate conjugates, glucuronidated conjugates, and methylated glucuronidated conjugates in Caco-2 cell monolayers plated on a semipermeable membrane (*26*).

We investigated whether flavonoids permeating the Caco-2 cell monolayer could maintain their inhibitory effects on the TCDD-induced AhR transformation and transcriptional activity of the CYP promoter. Seven flavonoids were found to have a marked suppressive effect on dioxin toxicity, they also being effective after permeating the human intestinal epithelial Caco-2 monolayer. Galangin, flavone, and tangeretin, in particular, each showed a marked suppressive effect on the TCDD-induced increase of CYP1A1/CYP1A2 mRNAs and CYP1A1 protein, although the quantity of these flavonoids and structures of their metabolites after permeating the Caco-2 cell monolayer remain to be elucidated. Assessing the quantity and structure of metabolites of these flavonoids is in progress by using LC-MS/MS.

Some of the flavonoids that inhibited the TCDD-induced AhR activation acted differently depending on their structural characteristics; for example, tangeretin showed a strong inhibitory effect in a low range of concentrations ($0-20$ μM). Tangeretin has five methoxyl groups. Because of its lipophilic property, tangeretin will permeate the Caco-2 cell monolayer more easily than other flavonoids, thus expressing stronger inhibitory activity. Although flavone at a concentration of 10 μM had a strong inhibitory effect on the TCDD-induced AhR activation, its effect decreased in a dose-dependent manner (**Figure 4**). This result corresponds with the data indicating flavone to have an agonistic effect, although the ED_{50} value was higher by 1 or 2 orders of magnitude than the antagonistic IC_{50} value in the rat hepatic cytosol (*13*). In our preliminary experiments, the agonisitic activity of flavone was also observed in a dose-dependent manner when the dioxin-responsive cells were incubated with flavone for 24 h in the absence of TCDD (data not shown). It is suggested that flavone interacted with AhR in a manner different from that of other flavonoids. Kostelac et al. (*27*) have investigated the effects of such dietary phytoestrogens as genistein, daizein, and its metabolite, equol, on the binding rate of estrogen receptors α and β to the estrogen response element (ERE) by a surface plasmon resonance (SPR) analysis. The SPR analysis, which detects the direct interaction between flavone and AhR, would also provide valuable information on the complex regulatory mechanism of AhR transformation by flavone.

As shown in **Figures 2** and **3**, conflicting results were obtained by SW-ELISA and the luciferase assay. Apigenin and flavanone only had a strong inhibitory effect on the TCDD-induced luciferase activity in HepG2 cells, while fisetin, tamarixetin, isorhamnetin, and (−)epigallocatechin only suppressed the TCDD-induced mouse AhR transformation. Although the physiochemical properties of AhR are well-conserved among a variety of species (*28*), there are several reports of significant species-specific differences being found in TCDD responsiveness (*29−31*). It has reported that several flavonoids showed AhR-agonistic activity, inducing AhR transformation up to 50% of the maximum TCDD response in guinea pig cells, whereas no significant agonistic activity was apparent in mouse cells at the same concentrations based on luciferase activity and protein and mRNA expression (*32*). These results suggest that the different behavior of the flavonoids shown between **Figures 2** and **3** was due to species-specific AhR conformation.

It has been reported that TCDD and 3-methylcholanthrene (3-MC) induced such drug-metabolizing enzymes as CYP1A1, CYP1A2, glutathione *S*-transferase, NAD(P)H quinine oxidoreductase 1, UDP-glucuronosyltransferase 1A6 (UGT1A6), and UGT1A1 (*33−36*). We examined the induction of these genes by TCDD in HepG2 cells at the mRNA level. After 48 h of the 10 nM TCDD treatment, the respective mRNA expression of CYP1A1 and CYP1A2 was 90- and 162-fold more than that of HepG2 cells cultured under the control conditions (data not shown). On the other hand, the expression level of UGT1A1 and UGT1A6 mRNA was only about 2-fold, but no suppressive effect of the flavonoids was apparent (data not shown). It has been reported that chrysin and quercetin induced UGTs (*37*). Sugatani et al. (*38*) described the induction of UGT by flavonoids and also suggested that UGT was induced by a multicomponent enhancer containing CAR, PXR, and AhR. The induction of UGT by flavonoids without AhR mediation may probably negate the inhibitory effect on TCDD-induced UGT expression.

The effects of the flavonoids on TCDD toxicity were examined in the present investigation by two different types of assays, cell-free (SW-ELISA) and cell-based (reporter analysis). In addition, using the permeation effect through the intestinal epithelial Caco-2 monolayer enabled us to recreate the absorption of flavonoids in the intestines. Using the two different types of assays in combination with the in vitro intestinal cell monolayer model enabled us to find that the TCDD-induced AhR activation was suppressed by seven flavonoids, with three of these also being likely to exert their inhibitory effects in vivo. It has been reported that 3′-methoxy-4′-nitroflavone and LY294002 (2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one) inhibited TCDD-mediated reporter gene activation and CYP1A1 induction in vivo (*39*, *40*). It is now being considered whether the flavonoids that showed AhR-antagonistic activity in this study would also have an inhibitory effect on TCDD-induced AhR transformation in vivo. These studies will lead to more valuable information about the protection offered by food factors against toxic chemicals such as dioxin.

ABBREVIATIONS USED

AhR, aryl hydrocarbon receptor; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; CYP1A1, cytochrome P450 1A1; SW-ELISA, southwestern chemistry-based enzyme-linked immunosorbent assay; Arnt, AhR nuclear translocator; XREs, xenobiotic-responsive elements; DMEM, Dulbecco's modified Eagle's medium; TER, transepithelial electrical resistance; PBS, phosphate buffered saline; EDTA, ethylenediamine tetraacetic acid; DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; PCR, polymerase chain reaction; SDS−PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; LC-MS/MS, high-performance liquid chromatograph-tandem mass spectrometry; ED_{50}, median effective dose; IC_{50}, median inhibitory concentration; 3-MC, 3-methylcholanthrene; UGT, UDP-glucuronosyltransferase

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