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Studies on Suppression of the Dioxin Toxicity by Food Factors

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Studies on Suppression of the Dioxin Toxicity by Food Factors

食品成分によるダイオキシン毒性の抑制に関する研究

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CONTENTS

ABBREVIATIONS

AhR, aryl hydrocarbon receptor

Arnt, AhR nuclear translocator

CALUX, chemically activated luciferase expression

CYP, cytochrome P450

DMSO, dimethylsulfoxide

DRE, dioxin responsive element

DTT, dithiothreitol

EC_{50}, median effective concentration

EDTA, ethylenediaminetetraacetic acid

EGCg, (−)-epigallocatechin gallate

EMSA, electrophoretic mobility shift assay (gel retardation assay)

EROD, 7-ethoxycoumarine-*O*-deethylase

FITC, fluorescein isothiocyanate

GRAB assay, gel retardation of AhR binding assay

GST, glutathione *S*-transferase

HAH, halogenated aromatic hydrocarbon

HPLC, high-performance liquid chromatography

HRGC/MS, High-resolution gas chromatography/mass spectrometry

Hsp90, heat shock protein 90

IC_{50}, median inhibitory concentration

LC-MS/MS, high-performance liquid chromatograph-tandem mass spectrometer

LSAB, labeled-streptavidin biotin

MC, 3-methylcholanthrene

MDL, minimum detection limit

PAH, polyciclic aromatic hydrocarbon

PBST, phosphate bufferd saline containing 0.05 % Tween 20

SD, Sprague-Dawley

SW-ELISA, southwestern chemistry-based enzyme-linked immunosorbent assay

TBST, Tris buffered saline containing 0.05% Tween 20

TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin

TEF, toxic equivalency factor

Tf, theaflavin

Tf3g, theaflavin-3-gallate

Tf3′g, theaflavin-3′-gallate

Tfdg, theaflavin-3,3′-digallate

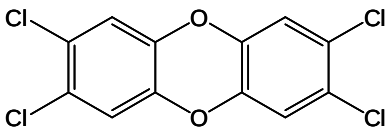
CHAPTER 1

General Introduction

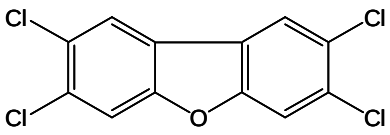
1.1. Dioxins

1.1.1. Characteristics

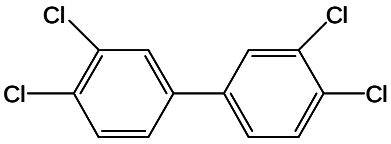
Halogenated aromatic hydrocarbons (HAHs) and polycyclic aromatic hydrocarbons (PAHs) are resistant to decomposition by acids, basics, and heat, and they cause serious problems as environmental contaminants [reviewed in 1, 2]. Dioxins are the toxic members of HAHs and PAHs, and consisted of 75 polychlorinated dibenzo-*p*-dioxins (PCDDs), 135 polychlorinated dibenzofurans (PCDFs), and 209 polychlorobiphenyls (PCBs) including co-planar PCBs (Co-PCBs) congeners, (Fig. 1.1). Of these, 7 PCDDs, 10 PCDFs, and 12 Co-PCBs congeners exhibit toxicological effects, and are collectively referred to as 'dioxin-like compounds' [3].



2,3,7,8-Tetrachlorodibenzo-*p*-dioxin



2,3,7,8-Tetrachlorodibenzofuran



3,4,3',4'-Tetrachlorobiphenyl

Fig. 1.1. Typical chemical structures of dioxins.

Dioxins are unexpected by-products of waste combustion, bleaching process in pulp industries, pesticides or other chemicals. They are released into the environment, and found in air, soil, water, and sediment. Dioxins are lipophilic compounds and accumulate in fatty organs in animals. One typical route of exposure is as follows: Dioxins contaminate the microorganisms in the river, and are magnified in fish through the food chain, and finally caused human exposure. Indeed, over 90% of

human exposure to dioxins is estimated to occur through the diet for adults [4]. For breast fed babies, the daily intake of dioxins is 1-2 orders of magnitude higher on a per body weight basis compared to adults [4]. Accidental or occupational exposure also occurs; e.g., an accident at a chemical factory in Seveso, Italy in 1976; contaminations in the herbicide during the Vietnam War; and contaminations in food in southern part of the USA in 1997.

1.1.2. Biological effects

The biological effects of dioxins are well-studied in numerous reports using 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), the most toxic compound among dioxin congeners, in experimental animals. The toxicity of TCDD is acute, sub-chronic, and chronic, and varies in species, strains, and sex. An acute toxicity of TCDD is indicated as 50% lethal dose (LD_{50}) values (Table 1.1). The LD_{50} values differ over 8000-fold between male guinea pig and male hamster. Besides lethality, dioxins express various adverse effects such as body weight loss, cancer promotion, immunosuppression, and birth defects in animals [reviewed in 5]. With regard to humans, chloracne, increasing risk of cancers, neurodevelopmental delays are reported as the toxicological effects of dioxins in cohort studies of accidental or occupational exposure [4].

Table 1.1. LD_{50} values of animals against TCDD

Species	Strains	Sex	LD_{50} value (μg/kg BW)	Reference
Guinea pig	Hartley	Male	0.6	6
Rabbit	New Zealand albino	Mixed	115	6
Rat	Long-Evans	Male	17.7	7
Rat	Long-Evans	Female	9.8	7
Rat	Han/Wistar	Mixed	>7200	7
Mouse	C57BL/6J	Male	182	8
Mouse	DBA/2J	Male	2570	8
Hamster	Golden Syrian	Male	5051	9

These toxicological effects of dioxins are mainly mediated by the aryl hydrocarbon (dioxin) receptor (AhR) [10-12]. Previous reports have demonstrated that the AhR knockout mice are resistant to the toxicity of TCDD [13, 14]. The AhR is a ligand-activated transcription factor of the basic helix-loop-helix/Per-Arnt-Sim (bHLH-PAS) protein family, and has been found to favor lipophilic compounds which

have van der Waals dimensions of 14 × 12 × 5 Å, as it is observed that TCDD is the most typical ligand [15-18]. The difference in sensitivity of animal strains against TCDD is considered to be due to the difference in the affinity of ligands to the AhR or its downstream event; i.e., the AhR in TCDD-resistant DBA/2J mouse and Han/Wistar rat has a point mutation in the ligand binding domain and the transactivation domain, respectively [19, 20].

Since the AhR is an orphan receptor, many researchers addressed to search for its endogenous ligands [reviewed in 18]. From the studies demonstrating that AhR knockout mice have growth and developmental defects [21-23], it is suggested that the AhR and/or its transformation is required to the developmental stage. As the candidates for endogenous ligands, indoles (e.g., indigo and indirubin), metabolites of arachidonic acid (e.g.,lipoxin A4, a lipoxygenase product of arachidonic acid) , and tetrapyroles (bilirubin and biliverdin) are reported as the possible ones [18]. Although it is not clear yet what the role of these possible endogenous ligand(s) *in vivo* is, the AhR is expressed in various tissues and its original function is suggested to be a developmental regulatory gene [24].

The unliganded AhR exists in the cytosol as a complex with two molecules of heat shock protein 90 (Hsp90), the X-associated protein 2 (XAP2), and p23. Following binding of agonists including dioxins to the AhR complex, the AhR translocates into the nucleus, dissociates the complex, heterodimerizes with a bHLH-PAS protein partner, AhR nuclear translocator (Arnt), and binds to a specific DNA sequence called dioxin or xenobiotic responsive element (DRE or XRE) [25-28], 5′-TNGCGTG-3′, resulting in transcriptional activation of adjacent genes [18, 25, 27, 28]. These sequential actions of the AhR are called as ‘transformation (activation)’ and lead the expression of various proteins including drug metabolizing enzymes such as cytochrome P4501A1 (CYP1A1), glutathione *S*-transferase (GST), and NAD(P)H:quinone-oxidoreductase (NQO1) [29-31]. Transformed AhR also disrupts intracellular signal transduction by changing the phosphorylation state of several regulatory proteins [32]. The signal transduction induced by dioxins is summarized in Fig. 1.2.

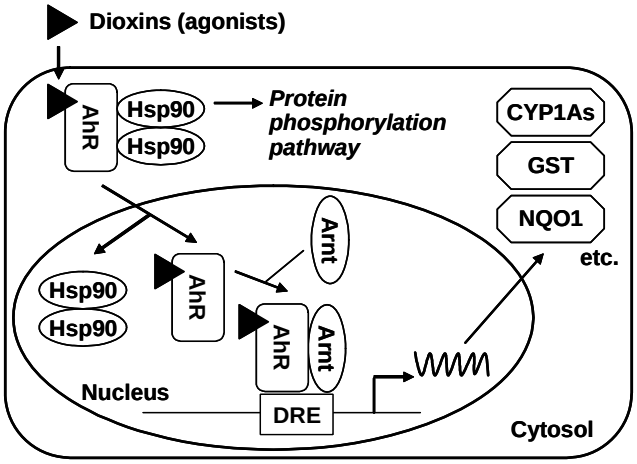


Fig. 1.2. The signal transduction induced by dioxins via AhR.

1.2. Strategy for protection from dioxin toxicity

1.2.1. Suppression of AhR transformation by food factors

Because AhR transformation is the initial step in development of dioxin toxicity, suppressing the transformation would protect against the toxicity. Since dioxins invade our bodies mainly through the diet as mentioned above, it is difficult to suppress AhR transformation by certain synthetic drugs such as 3′,4′dimethoxyflavone [33] and LY294002 [2-(4-morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one] [34], and important to search for natural antagonists. Flavonoids, as the food factors, are the good dietary candidates for suppressing AhR transformation, because they have the suitable structure for the AhR binding pocket. In Chapter 2, the author examined whether flavonoids can suppress AhR transformation in the cell-free system using rat liver cytosol. The author and others have demonstrated that flavonoids [35-38], resveratrol [39] and curcumin [40], and certain vegetable constituents [41, 42] act as antagonists of AhR. These results indicate a possibility that plant foods contain novel antagonists of AhR. In Chapter 4 and 5, the author isolated and identified lutein and chlorophylls from green tealeaves [43], and theaflavins (Tfs) from black tealeaves. Regarding these novel antagonists shown in Chapters 2, 4, and 5, those activities were estimated by the gel retardation of AhR binding (GRAB) assay using a radioisotope, however, more rapid, low-cost, and safety screening methods are desirable.

1.2.2. Development of enzyme-linked immunosorbent assay (ELISA) for detection of AhR transformation

To estimate the biological/toxic potency of dioxins, numerous *in vitro* bioassays have been developed; for example, cell culture-based risk assessment bioassay and noncell-based hazard assessment bioassay [reviewed in 44]. These bioassays are based on the AhR-dependent mechanism to measure AhR ligand binding, AhR transformation, or its downstream events, including enzyme induction. Of these bioassays, cell culture-based bioassays with recombinant cell lines are extremely sensitive and suitable for a high-throughput screening for the assessment of AhR-dependent potentials of a variety of pure compounds and extracts from environmental and biologic matrices [45-51]. However, these assays require equipments for cell cultures and luminometer/fluorometer for a multiple-well microtiter plate. Furthermore, there is no applicability to tissues or primary cultured cells isolated from experimental animals.

Regarding noncell-based bioassays, GRAB assay is certainly one of the excellent methods for measuring the ability of a compound or extracts to stimulate AhR transformation [48, 49, 52, 53], but this method requires handling and disposal of radioactive compounds to prepare radio-labeled DRE probe. One approach has been done that AhR transformation can be estimated by a dot blot immunoassay with an eluate from DRE probe-conjugated affinity column [54]. Although this assay does not require live animals, cell culture, and radioactive compounds, its sensitivity is relatively low and not suitable for analysis of a large number of samples. In Chapter 3, the author developed a new ELISA system based on southwestern chemical technique (SW-ELISA), which can quantify the transformed AhR protein using DRE oligonucleotide probe. This new method is also applicable for the screening of antagonists such as natural flavonoids [55].

1.2.3. Suppression of the dioxin toxicity by foods

Previous reports [35-43] demonstrated that food factors can act as antagonists for the AhR in the cell-free system and hepatocytes; however, it is unclear whether these

natural antagonists can show the effects when they are ingested as 'foods' *in vivo*. Tea, which is consumed worldwide, has beneficial effects on human health, such as antioxidative, anti-carcinogenic, and anti-mutagenic activities [56, 57]. These effects are considered to be exhibited by polyphenols present in tea, such as catechins, Tfs, and thearubigins. The author has indicated that these polyphenols are also good candidates for suppressing AhR transformation in the cell-free system in Chapters 4 and 5. Then, in Chapter 6, the author demonstrated an evidence for the preventive effects of tea on the dioxin toxicity *in vivo*; i.e., drinking tea suppresses intraperitoneally injected 3-methylcholanthrene (MC)-induced AhR transformation, expression of CYP1A1 and its enzymatic activity in the rat liver. Findings in Chapter 6 indicate that drinking tea elevates the physiological levels of polyphenols in the serum, which would show antagonistic effects on the AhR in the liver. The natural antagonists for the AhR are abundantly present not only in tea leaves but in vegetables and fruits. Therefore, intake of plant foods leads to an increase in the physiological levels of natural antagonists and contributes to prevention of the dioxin toxicity.

In Chapters 2-5, the author demonstrated that food factors, such as flavonoids, catechins, Tfs, lutein, and chlorophylls, work as antagonists for the AhR by suppressing its transformation *in vitro*. To reveal the mechanistic action of these natural antagonists on suppressing AhR transformation, the author examined whether flavonoids including (−)-epigallocatechin gallate (EGCg) bind to the AhR *in vitro*. In Chapter 7, the inhibitory effects of flavonoids on the binding of MC to the AhR was evaluated by the method using [^{3}H]MC and hydroxylapatite. The interaction between the AhR and EGCg was also demonstrated by using surface plasmon resonance analysis.

The findings in this dissertation indicate that certain natural antagonists reveal the inhibitory effects on the binding of dioxins to the AhR, the initial step of development of dioxin toxicity. Intake of plant foods, which abundantly contain the natural antagonists, leads to suppressing AhR transformation and its downstream events *in vivo*. These findings provide that the plant foods have a potential to suppress the dioxin toxicity through the suppressing AhR-dependent pathway.

CHAPTER 2

Flavones and Flavonols at Dietary Levels Inhibit a Transformation of Aryl Hydrocarbon Receptor Induced by Dioxin

2.1. Introduction

Dietary exposure to the environmental pollutants dioxins is a serious health concern. Dioxins cause various adverse effects through the aryl hydrocarbon receptor (AhR) transformation [17, 58, 59]. As the transformation of AhR is a primary and key step in the development of dioxin toxicity, an inhibitor of the transformation would protect against the toxicity. The inhibitors should ideally be factors in our daily diet, because dioxins enter the body mainly through the diet. However, the only inhibitors identified to date are synthetic drugs [60, 61]. AhR has been found to favor compounds that are hydrophobic and have van der Waals dimensions of 14 × 12 × 5 Å [15, 16, 61]. Flavonoids have these chemical characteristics, and are abundant in plant foods [62, 63]. The subclass flavones and flavonols are usually ingested from vegetables, fruits, teas and red wine, and flavanones, isoflavones and catechins from citrus fruits, beans and teas, respectively [64, 65]. The daily intake is estimated to be 23 mg per capita in The Netherlands, 170 mg in the USA and 70 mg in Japan [66]. In addition, recent information suggests that several flavonoids act as inhibitors of the AhR [67]. Therefore, the author considered that the flavonoids are the good dietary candidates for preventing dioxin toxicity. The purpose of the study in this chapter is to investigate the inhibitory IC_{50} values of flavonoids for the transformation of AhR induced by 1 nM of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), and thereby to understand which flavonoids are available as inhibitors by comparing their IC_{50} values to the physiological levels [35].

2.2. Materials and methods

2.2.1. Materials

The flavonoids apigenin and hesperetin were obtained from Sigma Chemical Co. (St. Louis, MO). Baicalein, daidzein, eriodictyol, galangin, isorhamnetin, kaempferol, luteolin, luteolin-3′,7-di-*O*-glucoside, tamarixetin, and tangeretin were from Extrasynthèse (Genay, France). Chrysin and myricetin were from Aldrich (Tokyo, Japan). Fisetin, flavanone, genistein, quercetin, and rutin were from Wako Pure Chemical (Osaka, Japan). Flavone, morin, naringenin, and quercitrin were from Nacalai Tesque (Kyoto, Japan). Flavonol and naringin were from Tokyo Kasei Kogyo (Tokyo, Japan). Puerarin was from Funakoshi (Tokyo, Japan). The catechins (+)-catechin, (−)-gallocatechin, (−)-catechin gallate, (−)-gallocatechin gallate, (−)-epicatechin, (−)-epigallocatechin, (−)-epicatechin gallate, and (−)-epigallocatechin gallate were purchased from Kurita Kogyo (Tokyo, Japan). The purity of each chemical was confirmed by a nuclear magnetic resonance spectral analysis with a Bruker

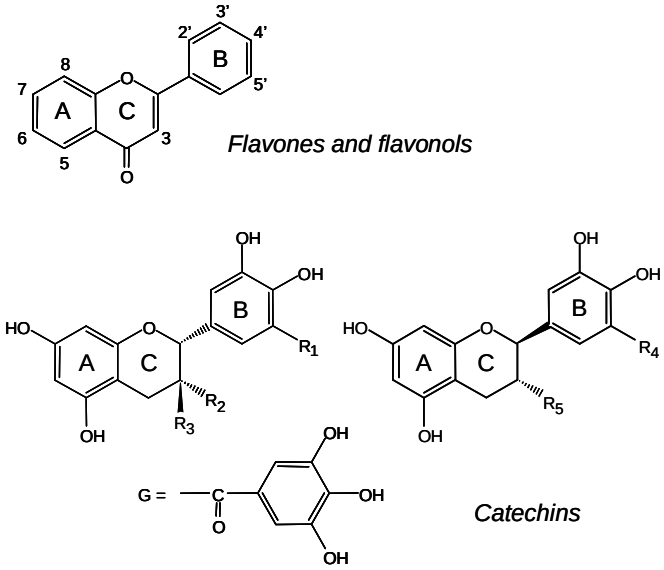


Fig. 2.1. The basic structures of flavonoids.

AC-250 (Bruker Analytik GmbH). α-Naphthoflavone, β-naphthoflavone, 4,7-phenanthrolin, and ellipticine were obtained from Sigma Chemical Co. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) and 3-methylcholanthrene (MC) were purchased from AccuStandard (New Haven, CT) and Nacalai Tesque, respectively. All

other reagents used were of the highest grade available from commercial source.

2.2.2. Preparation of rat hepatic cytosol

Rat hepatic cytosol was used as a source of AhR to determine the transformation in the cell-free system. Animal treatments in the present study conformed to *The Guidelines for the Care and Use of Experimental Animals, in Rokkodai Campus, Kobe University*. Livers from male Sprague-Dawley rats (6 weeks old, 140-170 g, obtained from Japan SLC, Shizuoka, Japan) were perfused with an ice-cold phosphate-buffered saline and homogenized in a double volume of HEDG buffer (25 mM HEPES of pH 7.4, 1.5 mM EDTA, 1.0 mM dithiothreitol, 10% glycerol). The homogenate was centrifuged at 10,000 × g for 20 min, and the supernatant was recentrifuged at 105,000 × g for 70 min, and the obtained supernatant was referred to as cytosol. After measuring the protein contents [68], the cytosolic fraction was used for estimation of the antagonistic effects of flavonoids on AhR transformation.

2.2.3. Transformation of the AhR

The cytosol (4.0 mg protein/ml) was incubated with TCDD or MC in dimethylsulfoxide (DMSO) in HEDG buffer at 20^{o}C for 2 h, and with DMSO (10 μl/ml) alone as a control. In the antagonism test, flavonoid or catechin at various concentrations was added to the mixture 10 min prior to the incubation. The resultant mixture was subjected to an electrophoretic mobility shift assay in the manner described in the next section.

2.2.4. Determination of AhR transformation

Transformation of AhR in the cytosol was evaluated by an electrophoretic mobility shift assay [69], using a prepared oligonucleotide for DRE. The DRE probe corresponded to the 26-bp AhR binding site [17]; 5′-GAT CTG GCT CTT CTC ACG CAA CTC CG-3′ (non-coding) and 5′-GAT CCG GAG *T*T*G CGT G*AG AAG AGC CA-3′ (coding) was synthesized at Hokkaido System Science (Sapparo, Japan). The prepared double-strand oligonucleotide was 5′-end labeled with T4-polynucleotide kinase (Takara Biomedicals, Otsu, Japan) and [γ-^{32}P]ATP (Amersham Pharmacia Biotech,

Buckinghamshire, England). Free nucleotides were removed from the labeled DRE probe on a Sephadex G-25 spin column (Roche Diagnostics, Co., Indianapolis, IN). The reaction mixture for the binding of AhR/DRE consisted of 10 μg of cytosolic protein, 250 ng of poly[dI-dC] (Roche Diagnostics, Co.) in 12 μL of HEDG buffer containing 150 mM KCl, and was incubated for 15 min at room temperature. ^{32}P-Labeled DRE probe (30 kcpm, 10 fmol) was added, and the mixture was incubated for a further 15 min at room temperature. The entire volume of the mixture was loaded onto a 4% nonstacking native polyacrylamide gel containing 0.25 × TBE buffer (25 mM Tris, 22.5 mM borate, 0.25 mM EDTA) and was electrophoresed in the same buffer at 60 V for 30 min before loading and for 90 min after loading. After electrophoresis, the gels were dried and exposed to X-ray films. The AhR/DRE complex was identified by competition assay with a 50-fold excess of non-labeled DRE probe and determined by autoradiography with a Digital Imaging System Is-1000 (Alpha Innotech, San Leandro, CA).

2.3. Results

2.3.1. Antagonism of flavonoid on AhR transformation

The basic chemical structures of flavonoids were shown in Fig. 2.1. On incubating AhR with 1 nM TCDD, effects of flavonoid on the complex of AhR/DRE were determined by gel mobility shift assay. Fig. 2.2.A shows that additions of flavonoid prior to TCDD suppressed the formation of the AhR/DRE complex while additions after AhR had been transformed by TCDD were ineffective against the suppression. Thus, flavonoids inhibited the transformation of AhR competing with the agonist TCDD, i.e. antagonistically. Fig. 2.2.B, C shows typical results for the antagonistic action of flavonoid. Quercetin suppressed TCDD-induced transformation dose-dependently (Fig. 2.2.B). Luteolin similarly suppressed the transformation induced by 5 nM of MC (Fig. 2.2.C), which is another strong agonist [70].

2.3.2. Antagonistic IC_{50} and agonistic ED_{50} values of flavonoids

The author determined the IC_{50} values by plotting a log of the dose concentration against the ratio of transformation. As shown in Fig. 2.3, using both a positive control (1 nM TCDD, 100%) and negative one (vehicle alone, 0%) at each time, the suppressing activity of flavonoid was plotted as % of transformation from independent triplicate assays. On the other hand, some flavonoids have been reported to exhibit agonistic

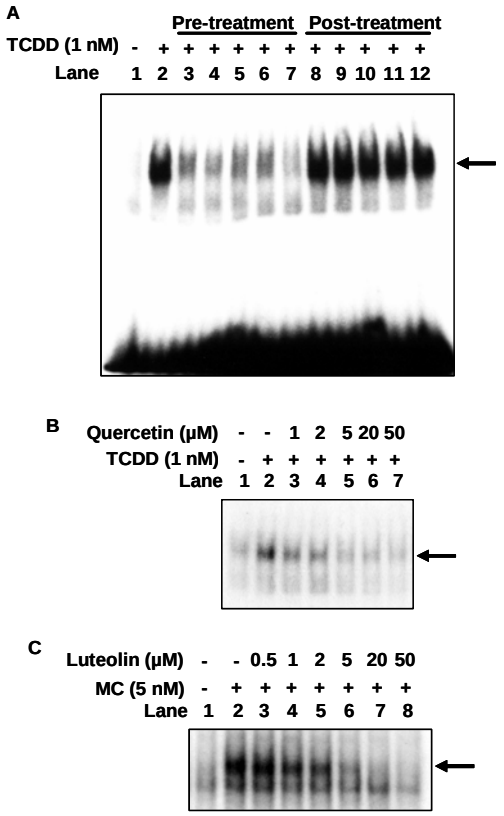


Fig. 2.2. Inhibitory effect of flavonoids on TCDD or MC-induced transformation of AhR. A: A typical gel-retardation assay for the effect of flavones and flavonols. Arrows indicate AhR/DRE complex. Rat hepatic cytosol was incubated with 5 μM flavonoids before the transformation of AhR by 1 nM TCDD (pre-treatment, lanes 3-6) or after (post-treatment, lanes 7-10). Lane 1, the vehicle DMSO alone; 2, 1 nM TCDD alone; 3 and 8, flavone; 4 and 9, chrysin; 5 and 10, flavonol; 6 and 11, galangin; and 7 and 12, flavanone. B: A dose-dependent effect of flavonoids on the transformation. Quercetin was added to the cytosol at a concentration of 0-20 μM before the treatment with 1 nM TCDD. C: An inhibitory effect of flavonoids on the MC-induced system. Luteolin was added to the cytosol at 0-50 μM before treatment with 5 nM MC.

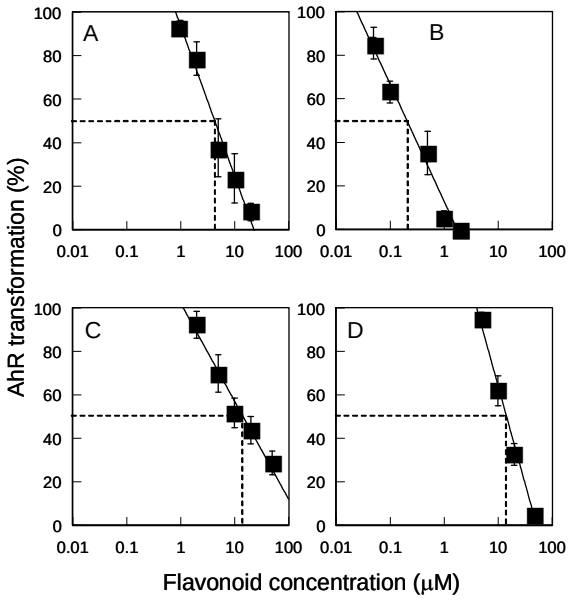


Fig. 2.3. Determination of IC_{50} values of flavonoids for suppressing the AhR transformation. Rat hepatic cytosol was incubated with various concentrations of flavonoids; tangeretin (A), galangin (B), eriodictyol (C), and rutin (D), before treating with 1 nM TCDD, and the transformation of AhR was determined as mentioned in Fig. 2.2. The ratio of transformation was compared to those of both positive control (1 nM TCDD) and negative one (vehicle alone) and plotted as % of transformation against a log of the dose concentration. The determination was carried out three times independently, and then the amount requiring 50% suppression was calculated.

action and produce a toxicity [67, 71]. Then, the author also determined the agonistic ED_{50} values. Table 2.1 lists the IC_{50} values for 34 natural flavonoids and four known antagonistic drugs, along with the agonistic effects for comparison. Almost all of the natural flavonoids showed antagonism without agonism. Flavone and tamarixetin had agonistic effects, but the ED_{50} values were higher by one or two orders of magnitude than the corresponding antagonistic IC_{50} values. Chrysin, flavonol, quercetin, flavanone, and daidzein at 100 μM had a weak agonistic effect. This means that natural flavonoids at low concentrations inhibit the dioxin action and are not in themselves toxic.

2.3.3. Relation between the structure and the antagonistic activity

In antagonistic action, flavones, flavonols and flavanones showed low IC_{50} values, and non-polar or less polar molecules were stronger. Subsequently, flavone, flavonol, galangin, and flavanone gave values similar to that of a known antagonistic drug, α-naphthoflavone. The results indicated that hydrophobicity and molecular size were important for AhR. AhR also favors a stereo coplaner structure like the strongest agonist TCDD [15, 16, 71]. Flavones and flavonols have a plane benzopyranone skeleton, and flavanones a less plane dihydrobenzopyranone skeleton, but catechins are not plane benzodihydropyrans. Subsequently, in antagonistic action the flavonoids ranked in the order of flavones and flavonols > flavanones > catechins. Isoflavones having almost no effect differed markedly in stereo structure from the above flavonoids in that the B-ring was bound to the 3-position.

Luteolin-3′,7-di-*O*-glucoside, naringenin-7-*O*-rutinoside and isorhamnetin were weaker than the corresponding aglycones (skeleton without sugar), though a 3-substitute such as quercetin-3-*O*-rhamnoside was not. These results reveal a region of the flavonoid with affinity for the hydrophobic pocket of AhR comprising 7- and 8-carbons, 1-pyran and a B-ring, and this was consistent with a finding for a cytochrome P450 (CYP) 1A1 [72]. CYP 1A1 is one of the enzymes that are expressed through AhR and metabolize hydrophobic xenobiotics [17, 58, 59]. The similarity between the hydrophobic pockets of AhR and CYP is purposive.

2.3.4. Changes in the IC_{50} values with TCDD level

Table 2.1. Effects of flavonoids on the transformation of AhR[a]

Chemicals	Antagonistic IC_{50} (μM) against		Agonistic ED_{50} (μM or maximum % when added at 100 μM)[b]
	1 nM TCDD	5 nM MC	
Flavones			
Flavone (none)	0.14	0.62	23
Chrysin (5,7-OH)	4.2	3.8	weak (32±6)
Baicalein (5,6,7-OH)	4.3	5.3	no effect
Apigenin (5,7,4′-OH)	3.2	7.1	no effect
Luteolin (5,7,3′,4′-OH)	6.5	8.4	no effect
Tangeretin (5,6,7,8,4′-OCH_3)	9.0	8.2	no effect
Luteolin-3′,7-di-*O*-glucoside	28	-	no effect
Flavonols			
Flavonol (3-OH)	0.42	1.2	weak (33±3)
Galangin (3,5,7-OH)	0.22	1.3	no effect
Kaempferol (3,5,7,4′-OH)	2.1	9.0	no effect
Fisetin (3,7,3′,4′-OH)	5.5	-	no effect
Morin (3,5,7,2′,4′-OH)	7.4	>50	no effect
Quercetin (3,5,7,3′,4′-OH)	1.5	10	weak (15±2)
Myricetin (3,5,7,3′,4′,5′-OH)	7.6	-	no effect
Tamarixetin (3,5,7,3′-OH, 4′-OCH_3)	2.4	-	89
Isorhamnetin (3,5,7,4′-OH, 3′-OCH_3)	10	-	no effect
Quercetin-3-*O*-rhamnoside (quercitrin)	3.7	35	no effect
Quercetin-3-*O*-rutinoside (rutin)	13	>50	no effect
Flavanones (2-3 is saturated)			
Flavanone (none)	0.65	-	weak (12±2)
Naringenin (5,7,4′-OH)	6.7	-	no effect
Eriodictyol (5,7,3′,4′-OH)	11	-	no effect
Hesperetin (5,7,3′-OH, 4′-OCH_3)	14	-	no effect
Naringenin-7-*O*-rutinoside (naringin)	25	-	no effect
Isoflavones (B-ring binds to 3 position)			
Daidzein (7,4′-OH)	>50	>50	weak (34±1)
Genistein (5,7,4′-OH)	>50	>50	no effect
Daidzein-8-*C*-glucoside (puerarin)	>50	-	no effect
Catechins			
(+)-Catechin (R_1=OH, R_2=H, R_3=OH)	>200	-	no effect
(−)-Gallocatechin (R_4=OH, R_5=OH)	>200	-	no effect
(−)-Catechin gallate (R_4=H, R_5=OG)	150	-	no effect
(−)-Gallocatechin gallate (R_4=OH, R_5=OG)	39	-	no effect
(−)-Epicatechin (R_1=H, R_2=OH, R_3=H)	>200	-	no effect
(−)-Epigallocatechin (R_1=OH, R_2=OH, R_3=H)	>200	-	no effect
(−)-Epicatechin gallate (R_1=H, R_2=OG, R_3=H)	81	-	no effect
(−)-Epigallocatechin gallate (R_1=OH, R_2=OG, R_3=H)	35	-	no effect
Known antagonistic and agonistic drugs			
7,8-Benzoflavone (α-naphthoflavone)	0.39	-	31
5,6-Benzoflavone (β-naphthoflavone)	no effect	-	2.7
4,7-Phenanthroline	6.8	-	no effect
5,11-Dimethyl-6*H*-pyrido[3,4-*b*]carbazole (ellipticine)	0.013	-	no effect

[a]The amounts of AhR/DRE complex were determined as a percentage of control incubation without flavonoids, and plotted vs. flavonoid concentrations to calculate the IC_{50} values. [b]'No effect' is up to 100 μM.

The dioxin level in the body is usually much less that the 1 nM in Table 2.1 [15, 16]. Therefore, the changes in the IC_{50} values with TCDD concentration in several flavonoids were determined (Table 2.2). This, together with the results at 1 nM TCDD in Table 2.1, indicated that the antagonistic action of flavones and flavonols depended on the concentration of agonistic TCDD. Thus, flavonoid was available at lower levels against a smaller amount of TCDD.

Table 2.2. Changes in the IC_{50} values of flavonoid with TCDD concentration

Flavonoid	IC_{50} value against TCDD	
	0.2 nM	5 nM
Apigenin	2.4	6.3
Luteolin	0.83	16
Kaempferol	0.54	20
Quercetin	0.50	18
Rutin	11	>100

2.4. Discussion

In this chapter, the author demonstrates that flavonoids, especially flavones and flavonols in aglycones, inhibit the AhR transformation antagonistically, indicating that they protect against dioxin toxicity. Therefore, an important question is whether flavonoids are present in the body at antagonistic levels and reach agonistic levels. The physiological concentrations of dietary flavonoids have been established by several excellent studies.

In absorption experiments in volunteers, onion quercetin, pure rutin and pure aglycone quercetin have been reported to be absorbed into the body at 52, 17 and 24% of the intake amount, respectively [73, 74]. The levels in healthy individuals are 0.72-0.76 μM for rutin and 0.5-1.6 μM for other quercetin glycosides in the blood plasma [75]. Hollman and coworkers have examined the changes in physiological levels with a single dose of flavonoid after washing out the internal flavonoid [73, 74]. Fried onion equivalent to 68 mg (225 μmol) quercetin gives 0.74 μM in plasma as a peak at 0.7-7 h after the ingestion, and pure rutin 0.3 μM. The disposition has an elimination half-life of about 25 h. It was concluded that the levels reflect the amount

ingested and that repeated intake of quercetin throughout the day would lead to a build-up in the plasma. Indeed, in another experiment by Conquer *et al.* [76], a capsule containing free quercetin raised the concentration to 1.5 μM after 28 days at a dose of 1 g/day. Quercetin and its glycosides bind to albumin and are carried in the blood stream to the liver, where they are partly metabolized to isorhamnetin and glucurono-sulfated conjugates before being excreted in the urine [77, 78]. The other flavonoids have a similar fate. Kaempferol is absorbed better or metabolized to a lesser extent than quercetin [74]. A daily supplement of 500 g of broccoli (providing 12.5 mg of kaempferol and 9 mg of quercetin) for 12 days resulted in small amounts in the urine as conjugates of the non-metabolized aglycone (52-78 ng kaempferol/ml urine) [79]. An oral dose of 500 mg of pure naringin gives naringin, naringenin, and naringenin glucuronides in the urine [80]. A capsule of 525 mg of (−)-epigallocatechin gallate (roughly equivalent to six cups of green tea) increases the plasma levels to 4.41 μM after 90 min as the free form but not the conjugates [81].

Thus, aglycones and glycosides from the diet are excreted into urine as free aglycones and conjugates thereof. Since the conjugations occur in the intestines and liver [77, 78], the dietary flavonoids exist as free aglycones at least temporarily in these tissues, which are major routes for dietary dioxins [15, 16]. The physiological levels indicate that the dietary flavones and flavonols can build up to near antagonistic levels and on repeated ingestion reach close to the IC_{50} value, i.e. 1.5 μM for quercetin (Table 2.1), but do not approach agonistic levels such as 100 μM for quercetin. In addition, the dioxin level is usually pM in the body [15, 16], much less that the 1 nM in Table 2.1. Towards the lower concentration of TCDD, flavones and flavonols acted as antagonists at lower concentrations (Table 2.2). Probably, the intake from a plant-based diet is enough to inhibit AhR transformation by TCDD. The present results obtained from experiments in a cell-free system suggest strongly that the dietary flavones and flavonols protect against the toxicity of the environmental contaminant dioxin, because the transformation of AhR has been recognized to be a primary and key step in the development of dioxin toxicity [17, 58, 59].

CHAPTER 3

A New Southwestern Chemistry-based ELISA for Detection of Aryl Hydrocarbon Receptor Transformation: Application to the Screening of Its Receptor Agonists and Antagonists

3.1. Introduction

The aryl hydrocarbon receptor (AhR) is a ligand-dependent transcription factor that is transformed by the binding of ligands including a variety of halogenated aromatic hydrocarbons (HAHs), as well as some polycyclic aromatic hydrocarbons (PAHs) such as benzo(*a*)pyrene, 3-methylcholanthrene (MC), and benzoflavones [1, 45-47, 50]. After the binding of ligands, the cytosolic AhR detaches itself from its associated protein complex, undergoes transformation into a high-affinity DNA binding form, is translocated into the nucleus, and then forms a heterodimer with another helix-loop-helix protein, AhR nuclear translocator (Arnt) [25-27]. The complex of ligand:AhR:Arnt interacts with a specific DNA sequence, the dioxin responsive element (DRE), resulting in transcriptional activation of adjacent genes [25, 28, 82]. Thus, the detection of transformed AhR is a target for the estimation of biological potency of HAHs and PAHs including dioxins.

Because of the adverse effects by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and its related compounds, it has generated considerable concerns worldwide. Therefore, many analytical techniques have been developed for the detection and quantification of HAHs in environmental, biological, and food samples. A method using high-resolution gas chromatography/mass spectrometry (HRGC/MS) has been developed and became the "golden standard" for HAHs analysis [83-86]. Although HRGC/MS provide an accurate measurement of each of the known isomers and congeners in sample extracts, it has some drawbacks of being costly and time-consuming because it requires a complicated separating process and expensive

equipments. This indicates that HRGC/MS is not suitable for rapid screening and analysis of a large number of samples. Moreover, HRGC/MS analysis provides information about the presence and concentration of the known compounds, especially HAHs, but this method does not allow estimation on the biological potency of complex mixtures of HAHs, PAHs, and other known AhR ligands, nor applicable to the identification of novel ligands.

To estimate the biological/toxic potency of such compounds, numerous *in vitro* bioassays have been developed: i.e., cell culture-based risk assessment bioassay and noncell-based hazard assessment bioassay [reviewed in 44]. Most of them are based on the AhR-dependent mechanism of actions because quantitative structure-activity relationship studies have revealed a good correlation between the affinity of HAHs to bind to the AhR and their potency to induce toxicity [2, 15]. These bioassays have been used as the rapid, low-cost, and sensitive screening methods for the detection and quantification of HAHs and PAHs in sample extracts [e.g., 45-47, 50, 52, 54, 87, 88] and for the identification and characterization of novel ligands [e.g., 33, 35, 48, 49, 51, 53]. Cell culture-based bioassays with recombinant cell lines that stably transfected the firefly luciferase gene, such as the chemically activated luciferase expression (CALUX) [45-47, 48, 49] and the green fluorescent protein-based cell bioassay [50, 51], are extremely sensitive and suitable for a high-throughput screening for the assessment of AhR-dependent potential of a variety of pure compounds and extracts from environmental and biologic matrices. However, these assays require equipments for cell cultures and luminometer/fluorometer for a multiple-well microtiter plate. Furthermore, there is no applicability to tissues or primary cultured cells isolated from experimental animals. Regarding noncell-based bioassays, the gel retardation of AhR binding (GRAB) assay is certainly one of the excellent methods for measuring the ability of a compound or extracts to stimulate AhR transformation [48, 49, 52, 53], but this method requires handling and disposal of radioactive compounds to prepare radio-labeled DRE probe. One approach has been done that AhR transformation can be estimated by a dot blot immunoassay with an eluate from DRE probe-conjugated affinity column [54]. Although this assay does not require live animals, cell culture, and radioactive compounds, its sensitivity is relatively low and not suitable for analysis of a

large number of samples.

Here, the author demonstrates the development of a new ELISA system based on southwestern chemical technique (SW-ELISA), which can quantify the transformed AhR protein using DRE oligonucleotide probe. This noncell-based new bioassay has the high sensitivity with wide-working range but does not require any radioactive compounds and any sophisticated instrumentations to perform. In addition, the author also demonstrates the application of SW-ELISA with a 96-well microtiter plate format for the detection and relative quantification of HAHs and PAHs, and for estimation of the antagonistic effect of natural flavonoids on TCDD-induced AhR transformation [55].

3.2. Materials and methods

3.2.1. Reagents, antibodies, and oligonucleotide probes

Dioxins and dibenzofurans were purchased from AccuStandard or Cambridge Isotope Laboratories (Andover, MA). Benzo(*a*)pyrene was obtained from Sigma Chemical Co. Flavonoids and MC were obtained from the same companies as described in Chapter 2. Fluorescein isothiocyanate (FITC)-labeled goat immunoglobulin protein was from Wako Pure Chemical. Nine specific antibodies, which raised against the C-terminal region of human AhR (anti-AhR C-18 and anti-AhR H-211), the N-terminal region of human AhR (anti-AhR N-19), the C-terminal region of mouse AhR (anti-AhR M-20), the C-terminal region of human Arnt (anti-Arnt C-19), and the C-terminal region of mouse Arnt (anti-Arnt M-20), were from Santa Cruz Biotechnology (Santa Cruz, CA), the N-terminal region of mouse AhR (anti-AhR MA1-513 and MA1-514) were from Affinity BioReagents (Golden, CO), and the N-terminal region of mouse AhR (anti-AhR NB100-128) was from Novus Biologicals (Littleton, CO). Two anti-FITC antibodies, mouse monoclonal antibody (mAb, clone DAK-FITC4), and rabbit polyclonal antibody (pAb) were from DakoCytomation (Kyoto, Japan). The detection of the transformed AhR was performed with a 26-bp double-stranded

oligonucleotide DRE probe as described in Chapter 2. DRE probe with 5′-end labeling of FITC was synthesized at Hokkaido System Science, and used for SW-ELISA. Unlabeled DRE probe was also synthesized and radiolabeled as described in Chapter 2.

3.2.2. Preparation of rat hepatic cytosolic fraction

Rat hepatic cytosol was used as a source of AhR to determine its transformation, and prepared as described in Chapter 2 [35].

3.2.3. Transformation of AhR

The cytosol (15 mg protein/ml) was incubated with HAHs or PAHs in dimethylsulfoxide (DMSO) at various concentrations (dose range of each compound was shown in Table) in HEDG buffer at 20°C for 2 h in the dark. Control sample was incubated with DMSO (10 μl/ml) alone as a vehicle. In the antagonism test, flavonoids were added to the cytosolic fraction 10 min before addition of 1 nM TCDD at the following concentrations: galangin was used at 0.5, 1, 2, and 5 μM; apigenin, kaempferol, myricetin, quercetin, and tangeretin were at 2, 5, 10, and 20 μM; hesperetin and naringenin were at 10, 20, 50, 100, and 200 μM; rutin was at 50, 100, 200, 500, and 1000 μM. The reaction mixture for SW-ELISA was prepared by adding 40 μl of treated cytosol to 10 μl of HEDG buffer containing 750 mM KCl (final concentration at 150 mM).

3.2.4. SW-ELISA

A 96-well microtiter plate (Maxisorp; Nalge Nunc International, Tokyo, Japan) was coated with 100 μl of 0.46 μg/ml anti-FITC antibody in 50 mM sodium bicarbonate (pH 9.6) overnight at 4°C. After washing three times with phosphate buffered saline containing 0.05 % Tween 20 (PBST), the plate was blocked with a casein-based blocking buffer (Nakalai Tesque) for 2 h at 20°C, and washed three times with PBST. One hundred microliters of 250 fmol/ml FITC-labeled DRE probe were added to the plate and followed by 2 h incubation at 20°C and then stored at −20°C until samples were applied. The reaction mixture containing transformed AhR (50 μl) was plated into each DRE probe-bound well of the thawed microtiter plates, incubated for 2 h at room

temperature, and the plates were washed three times with PBST. One hundred microliters of the specific antibody against Arnt or AhR was added to each well, incubated for 1 h at 20°C, and then the plates were washed three times with PBST. The detection of the bound specific antibody was carried out using labeled-streptavidin biotin (LSAB) method. Briefly, the plate was incubated with biotinylated antigoat IgG antibody (Jackson Immuno Research Lab., West Grove, PA), and after washing three times with PBST, incubated with peroxidase-conjugated streptavidin (Dako-Cytomation) diluted at 1:2000. The reaction of each reagent was performed by the 30 min incubation time at 20°C. Finally, the microtiter plate was washed four times with PBST and then once with PBS. Bound peroxidase activity was visualized with tetramethylbenzidine (DakoCytomation). Color development with tetramethyl-benzidine was stopped by adding 0.5 M sulfuric acid, and the result was measured using Wallac ARVO sx multi labelcounter (Perkin-Elmer Life Sciences, Boston, MA) at a wavelength of 450 nm. The summary of principle of this technique is presented in Fig. 3.1.

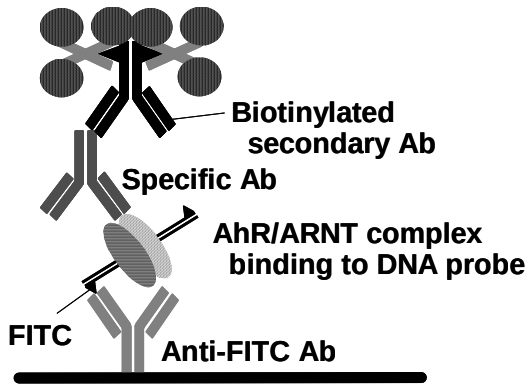


Fig. 3.1. Principle of SW-ELISA using anti-FITC antibody.

3.2.5. Gel retardation assay

To determine transformed AhR, 2.5 µl of treated cytosol, 9.5 µl of HEDG buffer containing 250 ng of poly [dI-dC] and 150 mM (final concentration) KCl, and 0.5 µl of [γ-^{32}P]-labeled DRE probe (30 kcpm, 10 fmol) was subjected to the gel retardation assay as described in Chapter 2 [35].

3.3. Results

3.3.1. Availability of anti-FITC antibodies in ELISA

To determine whether two different antibodies to FITC molecule, anti-FITC pAb and mAb, are applicable to a capturing antibody in immunoassay, a conventional ELISA was examined using anti-FITC antibody-coated plate. In a conventional ELISA, FITC-labeled goat IgG was used as a control protein instead of a complex that consists of FITC-labeled DRE probe, transformed AhR, and the bound specific antibody obtained from goat. When the protein concentration of FITC-labeled goat IgG protein was measured, a good dose-response curve was obtained in ELISA using the microtiter plates coated with 0.46 μg/ml anti-FITC pAb, and a significant increase was observed at

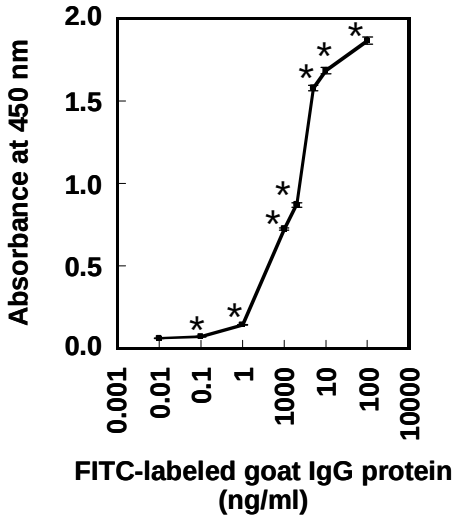


Fig. 3.2. Availability of anti-FITC pAb as a capturing antibody in ELISA. To assess anti-FITC pAb as a capturing antibody, FITC-labeled goat IgG protein was measured by a conventional sandwich-ELISA. A 96-well microtiter plate was coated with anti-FITC pAb (0.46 μg/ml, titer 1:5000) overnight at 4°C. After washing the plate three times with PBST, FITC-labeled goat IgG protein was added to each well at the concentrations of 0 (control), 0.01, 0.1, 1, 10, 20, 50, 100, and 1000 ng/ml and incubated for 1 h at room temperature. The plate was washed again, and peroxidase-conjugated anti-goat IgG antibody (titer 1:10000) was added and incubated for 1 h at room temperature. The activity of peroxidase was measured using tetramethylbenzidine. Values represent the means ± S.E. of independent triplicate determination. Asterisks indicate significant differences ($p < 0.05$, Student's *t*-test) from the control value (0.059 ± 0.001).

a range of 0.1 to 1000 ng/ml in the assay (Fig. 3.2). However, FITC-labeled goat IgG protein was undetectable in ELISA using anti-FITC mAb at a high concentration (data not shown).

3.3.2. Selection of specific antibodies

Transformation of the AhR is known to require binding of ligand such as HAHs and PAHs to the receptor protein, and subsequent interaction of this ligand:AhR complex with Arnt for binding to DRE sequence [25-27]. Therefore, the specific

antibody against Arnt, in addition to that against AhR, is capable of detecting transformed AhR. We compared nine specific antibodies (anti-AhR C-18, anti-AhR H-211, anti-AhR N-19, anti-AhR M-20, anti-AhR MA1-513, anti-AhR MA1-514, anti-AhR NB100-128, anti-Arnt C-19, and anti-Arnt M-20) that recognize AhR or Arnt proteins to clarify their availability to SW-ELISA in a TCDD-treated cytosolic fraction. Among these antibodies, anti-AhR MA1-514, anti-AhR NB100-128, anti-Arnt C-19 and anti-Arnt M-20 detected AhR transformation induced by 5 nM TCDD (Fig. 3.3.A). Particularly, anti-Arnt C-19 and anti-AhR MA1-514 exhibited higher reactivity to native TCDD:AhR:Arnt complex, and the absorbance was increased over twofold by TCDD treatment compared to that of control, indicating that these antibodies were available for the detection of TCDD-induced AhR transformation. Thus, the author determined a suitable antibody titer for SW-ELISA (Fig. 3.3.B and C), and the dilutions of them were decided 1:5000 and 1:1000, respectively.

Although these antibodies were available for SW-ELISA, this method revealed relatively high background. Background absorbance was increased by the concentration of antibodies dose-dependently (Fig. 3.3.B and C). When the author changed the concentration and volume of the cytosolic fraction, background absorbance did not alter (data not shown). These data indicated that background absorbance is due to antibodies but not to cytosolic protein. To reduce or eliminate this background absorbance, several blocking solutions were used; however, there was no solution that gave better results than the solution used in the current system (data not shown). To normalize the results between experiments, background absorbance of control treatment (DMSO alone) was subtracted from that of the HAHs- or PAHs-treated cytosol fraction in all subsequent experiments.

3.3.3. Quantification of TCDD-induced AhR transformation by SW-ELISA

Because anti-Arnt C-19 and anti-AhR MA1-514 were superior to other antibodies, the author used these antibodies for SW-ELISA and determined the dose-dependence and relative sensitivity of TCDD-induced AhR transformation. Although both antibodies could detect TCDD-induced transformation

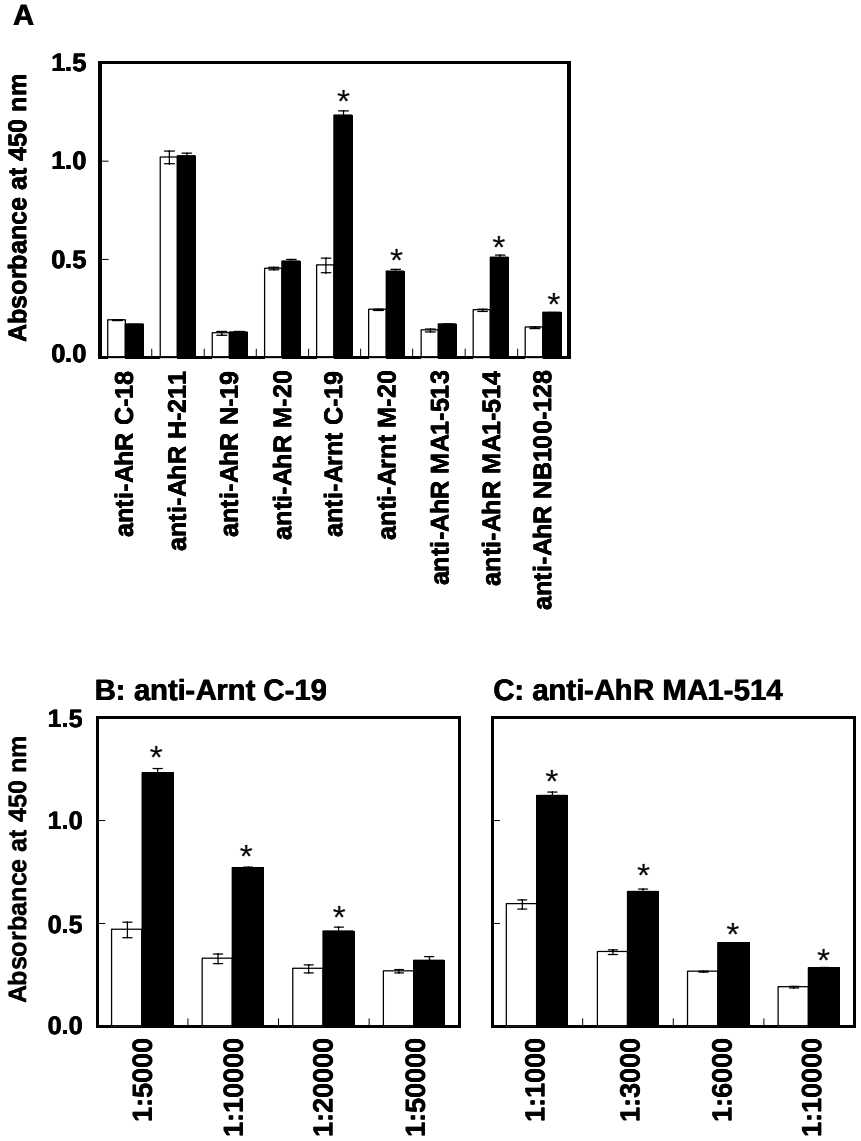


Fig. 3.3. Selection of specific antibodies for detecting the transformed AhR. Transformation of the AhR was induced by treatment of the rat hepatic cytosolic fraction with 5 nM TCDD (closed bar) or DMSO as vehicle control (open bar) as described in Materials and methods. (A) The TCDD:AhR:Arnt complex was detected by SW-ELISA with 9 antibodies; anti-AhR C-18 (titer 1:3000), anti-AhR H-211 (titer 1:3000), anti-AhR N-19 (titer 1:3000), anti-AhR M-20 (titer 1:3000), anti-Arnt C-19 (titer 1:5000), anti-Arnt M-20 (titer 1:3000), anti-AhR MA1-513 (titer 1:3000), anti-AhR MA1-514 (titer 1:2000), and anti-AhR NB100-128 (titer 1:4000). Dose responses of anti-Arnt C-19 (B) and anti-AhR MA1-514 (C) were measured to determine the suitable antibody titer for SW-ELISA. Values represent the means ± S.E. of independent triplicate determination. Asterisks indicate significant differences ($p < 0.05$, Student's *t*-test) from the value of corresponding vehicle control.

dose-dependently, anti-Arnt C-19 was more sensitive than anti-AhR MA1-514 (Fig. 3.4.A). The sensitivity was estimated by a minimum detection limit (MDL), which was defined by a significant increase in the absorbance in TCDD treatment from that in control treatment. SW-ELISA with anti-Arnt C-19 revealed an MDL of 2 pM (0.026 pg/assay), a median effective concentration (EC_{50}) value of 0.125 nM

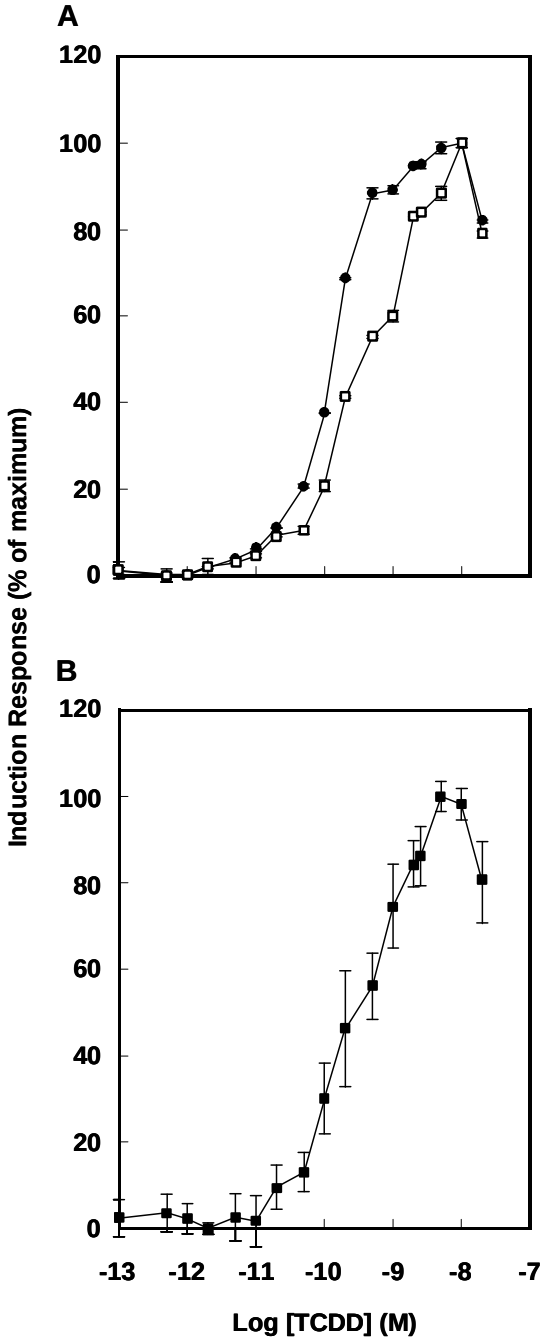


Fig. 3.4. Dose-dependent induction of AhR transformation by TCDD in SW-ELISA (A) and gel retardation assay (B). AhR transformation was induced by treatment of the rat hepatic cytosolic fraction with TCDD at indicated concentrations, and the transformed AhR was measured by both SW-ELISA using anti-Arnt C-19 (●) or anti-AhR MA1-514 (□) and gel retardation assay as described in Materials and methods. Data are expressed as percentage (%) of maximum induction response, and values represent the means ± S.E. of independent triplicate determination. In SW-ELISA, basal and maximal values (absorbance at 450 nm) were 0.463 ± 0.007 and 1.257 ± 0.021, respectively, for anti-Arnt C-19; and 0.598 ± 0.005 and 1.184 ± 0.013, respectively, for anti-AhR MA1-514. In gel retardation assay, basal and maximal values (arbitrary unit) were 10 ± 5 and 157 ± 5, respectively. Induction responses in SW-ELISA using anti-Arnt C-19 and anti-AhR MA1-514, and gel retardation assay were significantly greater than corresponding controls at 2, 5, and 100 pM TCDD, respectively.

(1.6 pg/assay), and a maximum response at 10 nM (129 pg/assay), while SW-ELISA with anti-AhR MA1-514 showed the MDL of 5 pM (0.064 pg/assay), the EC_{50} value of 0.15 nM (1.9 pg/assay), and maximum induction at 10 nM. In the case of the gel retardation assay using the rat hepatic cytosol fraction, it showed an MDL of 100 pM (0.08 pg/assay), the EC_{50} value of 0.27 nM (0.22 pg/assay), and maximum induction at 5 nM (4.0 pg/assay; Fig. 3.4.B). Although

the maximum response of SW-ELISA against background was lower than that of the gel retardation assay (SW-ELSA; about threefold vs. the gel retardation assay; about 16-fold), SW-ELSA showed lower standard deviation (S.D.) in each data point than the gel retardation assay variation. These results indicated that SW-ELISA is more sensitive and quantitative than the gel retardation assay at least under our experimental conditions.

Regarding the reproducibility of SW-ELISA with anti-Arnt C-19, the author measured AhR transformation induced by TCDD in 12 wells of each plate in three different plates run on three successive days as described previously [50]. The induction values (increased absorbance at 450 nm) between the runs, which expressed as means ± S.D. for these analyses, were 1.110 ± 0.081, 1.181 ± 0.068, and 1.128 ± 0.052. This similarity of the results indicates that SW-ELISA has a high degree of the reproducibility.

3.3.4. Analysis of HAHs and PAHs by SW-ELISA

To assess the agonistic effects of other AhR ligands, 12 dioxin-congeners including TCDD, six dibenzofuran-congeners, and three PAHs, were tested by SW-ELISA with anti-Arnt C-19 (Table 3.1). Dose-response experiments for the induction of AhR transformation by treatment with each compound were carried out, and the results from independent triplicate determination for the selected compounds were shown in Figure 3.5. The MDL, EC_{50} value and the maximum response concentration of each compound were determined from the dose-response curve (Table 3.1). From these results, SW-ELISA showed relatively high reactivity against congeners, especially against TCDD, with high toxic equivalency factor (TEF) [89] such as 1,2,3,7,8-pentachlorodibenzodioxin and 2,3,4,7,8-pentachlorodibenzofuran, although nontoxic congeners such as 2,3,7-trichlorodibenzodioxin and 1,2,7,8-tetrachloro-dibenzodioxin also showed reactivity. Because the maximum induction response of each compound was lower than that of TCDD (Fig. 3.5), the induction response dose at 50% of that of TCDD (EC_{50} [TCDD]) could be determined concerning only six compounds out of 18. However, when the correlation coefficient between a reciprocal number of each EC_{50} value and WHO-TEF was calculated, a significant value (r=0.754)

was obtained.

Because certain PAHs, such as benzo(*a*)pyrene, MC, and β-naphthoflavone, are known as AhR agonist, many *in vitro* bioassays attempt to measure their induction potency of AhR transformation [1, 45-47, 49]. The results also showed that these compounds induced AhR transformation in a dose-dependent manner (Fig. 3.5.C), but their induction potencies were significantly less than TCDD (Table 3.1). Taken together with these data in this study, the results in SW-ELISA are consistent with previously published observations in bioassays based on AhR-dependent mechanism, indicating that SW-ELISA was useful tool for the detection of AhR ligands.

3.3.5. Application of SW-ELISA to determine the antagonistic effects of flavonoids on TCDD-induced AhR transformation

In Chapter 2, it was demonstrated that certain flavonoids, such as galangin and quercetin, show the antagonistic effects on TCDD-induced AhR transformation at the dietary levels by gel retardation assay using rat hepatic cytosolic fraction (4 mg protein/ml) [35]. Then, the author investigated whether SW-ELISA is applicable to determine the antagonistic effects of nine flavonids on AhR transformation induced by 1 nM TCDD at a suitable protein concentration (15 mg protein/ml) for SW-ELISA. Dose-response experiments for the inhibition of TCDD-induced AhR transformation were carried out in each flavonoid, and typical results for selected flavonoids were shown in Figure 3.6.A. These flavonoids revealed the inhibitory effects on TCDD-induced AhR transformation in a dose-dependent manner expectedly in either SW-ELISA or gel retardation assay. The results from dose-response experiments in SW-ELISA and gel retardation assay were evaluated by the IC_{50} values, which were determined by plotting the percentage of AhR transformation against each logarithmic concentration of flavonoids as described previously [35]. Of nine flavonoids (apigenin, galangin, hesperetin, kaempferol, naringenin, quercetin, rutin, and tangeretin) tested here, galangin showed the lowest IC_{50} values in both SW-ELISA (2.5 μM) and gel retardation assay (1.3 μM) (Fig. 3.6.B). Although the IC_{50} values from SW-ELISA showed a higher tendency than those from gel retardation assay, the results of SW-ELISA closely correlated with those of gel retardation assay (Fig. 3.6.C) with a

significant correlation coefficient (r=0. 935). Thus, SW-ELISA was applicable to the

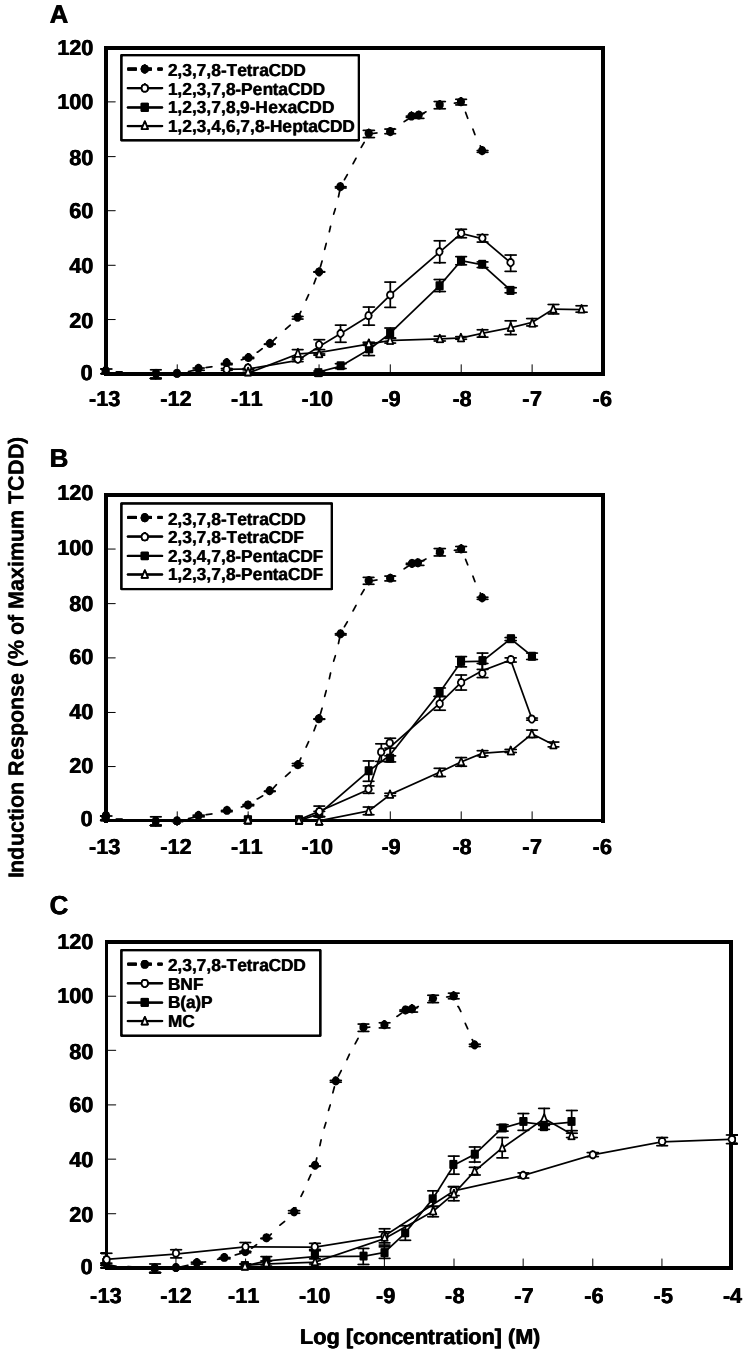


Fig. 3.5. Dose-dependent induction of AhR transformation by HAHs and PAHs in SW-ELISA. AhR transformation was induced by treatment of the rat hepatic cytosolic fraction with dioxins (A), dibenzofurans (B), and PAHs (C) at indicated concentrations, and the transformed AhR was measured by SW-ELISA as described in Materials and methods. To compare the induction response of each compound to that of TCDD, the dose-response curve of TCDD was shown in each panel. Data are expressed as percentage (%) of maximum induction response, and values represent the means ± S.E. of independent triplicate determination. TetraCDD, tetrachlorodibenzodioxin; PentaCDD, pentachlorodibenzodioxin; HexaCDD, hexachlorodibenzodioxin; HeptaCDD, heptachlorodibenzodioxin; TetraCDF, tetrachlorodibenzofuran; PentaCDF, pentachlorodibenzofuran; BNF, β-naphthoflavone; B(a)P, benzo(*a*)pyrene; and MC, 3-methylcholanthrene.

determination of antagonistic effects of chemicals such as flavonoids on the AhR transformation.

Table 3.1. Responses of SW-ELISA to selected HAHs and PAHs

Compounds	Dose range	Each compounds			EC_{50}
		MDL (nM)	EC_{50} (nM)	Max-Res (nM)	[TCDD] (nM)
Dioxins					
2,3-DiCDD[a]	10 pM-2 μM	2	100	1000	ND[b]
2,3,7-TriCDD	10 pM-200 nM	0.5	5.0	100	ND
1,2,3,4-TetraCDD	10 nM- 5 μM	500	2200	5000	ND
1,2,7,8-TetraCDD	100 pM-50 nM	2	5.0	20	ND
1,3,7,8-TetraCDD	1 nM-2 μM	50	150	1000	ND
2,3,7,8-TetraCDD	0.1 pM-20 nM	0.002	0.125	10	0.125
1,2,3,7,8-PentaCDD	1 pM-50 nM	0.005	0.7	10	7.5
1,2,3,4,7,8-HexaCDD	10 pM-20 nM	0.5	1.2	100	ND
1,2,3,6,7,8-HexaCDD	10 pM-40 nM	0.05	1.5	20	14.8
1,2,3,7,8,9-HexaCDD	100 pM-50 nM	0.2	1.8	10	ND
1,2,3,4,6,7,8-HeptaCDD	100 pM-500 nM	0.5	20	200	ND
OctaCDD	100 nM-50 μM	1000	18000	50000	ND
Dibenzofurans					
2,3,7,8-TetraCDF	50 pM-100 nM	0.5	1.3	50	8.7
2,3,4,7,8-PentaCDF	10 pM-100 nM	0.01	1.6	50	5.9
1,2,3,7,8-PentaCDF	10 pM-200 nM	0.5	4.0	100	ND
1,2,3,6,7,8-HexaCDF	100 pM-10 nM	0.2	1.1	5	1.5
1,2,3,4,6,7,8-HeptaCDF	100 pM-200 nM	5	5.0	100	ND
OctaCDF	100 nM-50 μM	500	5000	25000	ND
Polycyclic aromatic hydrocarbons					
Benzo(*a*)pyrene	10 pM-500 nM	1	6.0	100	100
3-Methylcholanthrene	10 pM-500 nM	0.5	10	200	45
β-Naphthoflavone	0.1 pM-100 μM	0.001	1.0	100000	ND

Dose-response experiments were carried out triplicate in each compound. The minimum detection limit (MDL), median effective concentration (EC_{50}) value, and the maximum response concentration (Max-Res) of each compound were determined from the dose-response curve, and the induction response dose at 50% of that of TCDD (EC_{50} [TCDD]) was also calculated. [a]DiCDD, dichlorodibenzodioxin; TriCDD, trichlorodibenzodioxin; TetraCDD, tetrachlorodibenzodioxin; PentaCDD, pentachlorodibenzodioxin; HexaCDD, hexachlorodibenzodioxin; HeptaCDD, heptachlorodibenzodioxin; OctaCDD, octachloro-dibenzodioxin; TetraCDF, tetrachlorodibenzofuran; PentaCDF, pentachlorodibenzofuran; HexaCDF, hexachlorodibenzofuran; HeptaCDF, heptachlorodibenzofuran; OctaCDF,octachlorodibenzofuran. [b]ND, not determinable.

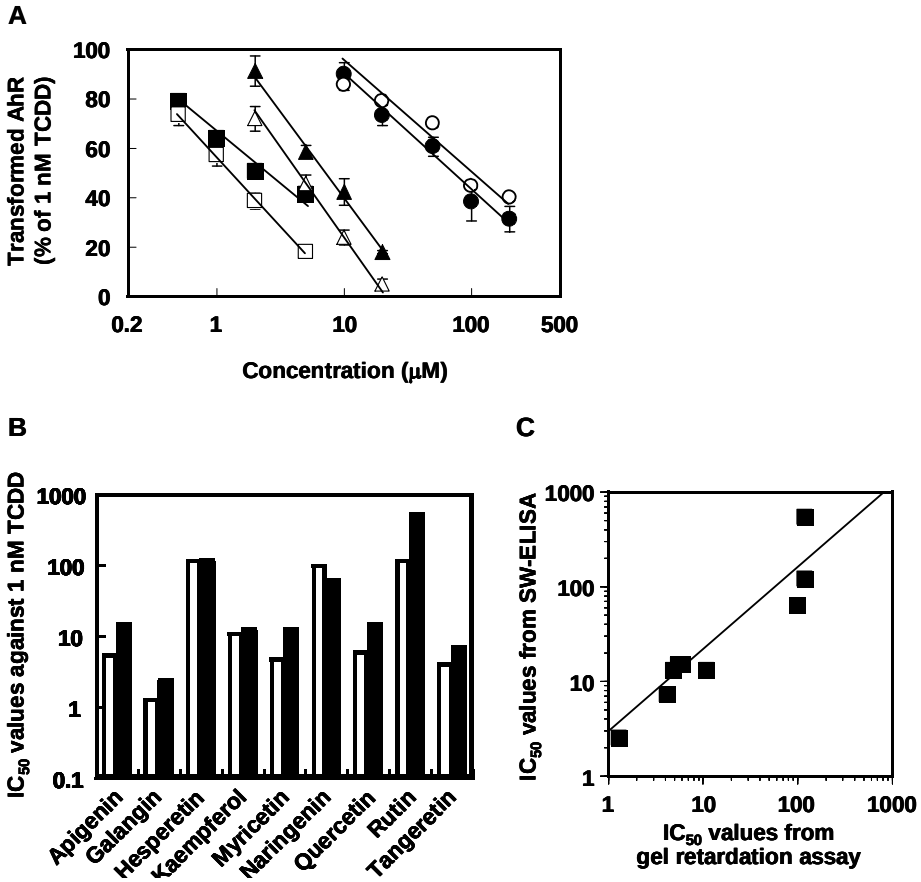


Fig. 3.6. The antagonistic effects of flavonoids on TCDD-induced AhR transformation. Flavonoids at various concentrations were added to the rat hepatic cytosolic fraction 10 min before addition of 1 nM TCDD, and the transformed AhR was measured by both SW-ELISA and gel retardation assay as described in Materials and methods. (A) Dose-dependent inhibition of galangin (□ and ■), tangeretin (Δ and ▲), and naringenin (○ and ●) on TCDD-induced AhR transformation was shown. Closed symbols, SW-ELISA; and open symbols, gel retardation assay. Data are expressed as percentage (%) of transformed AhR induced by 1 nM TCDD, and values represent the means ± S.E. of independent triplicate determination. The IC_{50} value of each flavonoid was calculated from the corresponding dose-dependent curve. (B) Comparison of the IC_{50} value from gel retardation assay (open bar) to that from SW-ELISA (closed bar); and (C) correlation between both methods.

3.4. Discussion

In this chapter, the author has developed ELISA based on southwestern chemistry for the evaluation of the transformed AhR with an increase in the DNA binding activity. ELISA has been extensively used for detecting various molecules, proteins and peptides in particular, in research and clinical settings [90]. Among several ELISA methods, a sandwich-ELISA using two antibodies is a suitable method for quantitative analysis [91]. Advanced ELISAs, combined with sugar chain recognition by lectin [92],

reverse transcript-polymerase chain reaction (RT-PCR) [93], and determination of phosphorylated state by phosphospecific antibody [94], were also reported as routinely applicable techniques. Namely, the use of ELISA and ELISA-modified techniques seem to be profitable in a large-scale screening. Southwestern chemistry underlying DNA-protein interaction is also an indispensable technique for biochemical and histochemical analyses of transcriptional activation and has been widely employed [95, 96]. The increased binding activity of the transformed AhR to DRE *in vivo* by southwestern histochemistry has been observed in hepatic nuclei from rats dosed with MC [97]. This provided a clue for the development of SW-ELISA with the 96-well microtiter plate format for the detection and relative quantification of HAHs and PAHs.

In designing SW-ELISA, the author focused on its detection threshold. SW-ELISA proposed here was achieved by combining amplification based on a high-affinity streptavidin-biotin (SAB) system. Because biotinylated DNA probes were widely used for binding of its probe to the streptavidin-coated solid phase, we first tried to apply this system to coat the microtiter plate. However, it was not useful because of a lack of sufficient sensitivity (data not shown). Due to enhancement of its detection sensitivity by the SAB system, an FITC-labeled DRE probe and anti-FITC antibody were employed as another hapten detection system instead of the SAB system. The results in this chapter demonstrated that this combination was suited to obtain higher sensitivity and applicability. With regard to specific antibody for detecting the ligand:AhR:Arnt complex, four out of nine commercially available antibodies could detect AhR transformation. The differences of the availability distinct specificity between these antibodies may be attributed to the stereological conformation of epitopes present in the complex. Consequently, the author selected anti-Arnt C-19, because it showed the highest sensitivity with wide dynamic range against TCDD-induced AhR transformation.

Although numerous bioassays based on AhR-dependent mechanism have been developed, the majority of them are cell culture-based bioassays [45-47, 50, 51, 87]. Because of the sensitivity, rapidity, and cost performance, these assays have been used to detect and quantify known AhR agonists to estimate the induction potency of

complex mixtures and to identify novel agonists. The results in this chapter clearly demonstrated that the sensitivity and dynamic ranges of detection were comparable to cell culture-based bioassays. The MDL and maximum induction of TCDD in SW-ELISA with anti-Arnt C-19 were 2 pM (0.026 pg/assay) and 10 nM, respectively, whereas those in the green fluorescent protein-based cell bioassay [50] were 1 pM (0.03 pg/assay) and 0.3 nM, respectively. However, there is discrepancy between SW-ELISA and cell culture-based bioassays on the responsibility against other HAHs and PAHs. SW-ELISA showed that the maximum induction levels varied on each test compound, whereas cell culture-based bioassays generally showed almost the same level among compounds. When gel retardation assay was carried out using the selected HAHs at their corresponding maximum induction concentration, the induction level of each compound from gel retardation assay was almost identical with that from SW-ELISA (data not shown). This indicates that the difference of the responsibility may be due to a characteristic of cell-free assays using the rat liver cytosolic fraction, and that these assays have limited responsibility.

Because the basic principles of SW-ELISA were similar to those of gel retardation assay, the author also compared the MDL and maximum induction in SW-ELISA to those in the gel retardation assay. The sensitivity of gel retardation assay was lower than that of SW-ELISA, and the standard deviation in each data point was relatively large at least under the present experimental conditions. Previously, Seidel *et al.*, [47] demonstrated that gel retardation assay using the cytosolic fraction prepared from the liver of guinea pigs (GRAB assay) showed an MDL of 1 pM without large standard deviations. The difference between the present and the previous results was the source of cytosolic fraction. Although male guinea pigs are the most sensitive strain to TCDD, the sensitivity of SW-ELISA remained unchanged when hepatic cytosol from guinea pigs was used (data not shown). Thus, the author used the cytosolic fraction prepared from the liver of male SD rats to develop the SW-ELISA. However, the differential activation of the AhR to interact with DRE was observed between these animal strains [88], probably because the significant variations were observed in the ligand domain and the C-terminal region between these species [98]. Although further studies are

needed to optimize SW-ELISA to obtain higher sensitivity, SW-ELISA in this chapter is the comparable method to previous bioassays, such as the green fluorescent protein-based cell bioassay and GRAB assay without cell culture or radioactive chemicals.

SW-ELISA detected AhR transformation that was induced by not only by TCDD but also by other HAHs and PAHs. These results were consistent with previous studies concering the potency of each compound to induce the AhR-dependent actions [45-47, 50, 54, 87]. However, some nontoxic congeners, such as 2,3,7-trichlorodibenzodioxin and 1,2,7,8-tetrachlorodibenzodioxin, also showed the reactivity in SW-ELISA, though they do not have TEF, suggesting that they have week binding potency to the AhR protein. When 1,2,7,8-tetrachlorodibenzodioxin was co-treated with TCDD, the author observed antagonistic effects in SW-ELISA (data not shown). It has been reported that certain non-toxic polyhalogenated biphenyls exhibit the antagonistic activity to AhR [99, 100]. These data suggest that these nontoxic HAHs also have a potency interact with the AhR. Therefore, this method is useful for determining the transformation potency of chemicals or their mixture but not quantifying the toxic congeners *per se*.

In Chapter 2, the author demonstrated that certain flavonoids act as antagonists of the AhR in the gel retardation assay [35]. It has also been found in numerous novel ligands that exhibit the agonistic and/or antagonistic effects to the AhR transformation [e.g., 33, 48, 49, 51, 53]. In this study, SW-ELISA succeeded in determining the antagonistic effects and their efficiencies. Moreover, the IC_{50} values of flavonoids from SW-ELISA were consistent with those from the gel retardation assay, and both parameters highly correlated with each other. Thus, SW-ELISA is available for a screening of novel agonists and antagonists.

SW-ELISA is a convenient and useful assay for the detection of the transformed AhR, but this method has some limitations. In contrast to the green fluorescent protein-based cell bioassay [51], which succeeded in measuring 12,090 chemicals by one person within less than 1 week, longer times are required for measuring such a large number of the samples within a limited time. In addition, *in vitro* methods, including SW-ELISA, could not directly estimate the substantial toxic potential of the sample. SW-ELISA will assess only the ability of a chemical mixture or extracts, which

were prepared from environmental and biologic matrices, to induce AhR transformation, although this method allows determining the transformation status of AhR in the tissue sample after preparation of nuclear extracts.

In conclusion, SW-ELISA is a sensitive and useful noncell-culture method to determine the potency of AhR transformation. This assay will be applicable to a primary screening tool that provides information about the presence of compounds that induced AhR transformation in the extracts from environmental and biologic matrices. When positive results are observed in SW-ELISA, analytical approaches, such as HRGC/MS, can be used to identify the specific compounds. Further studies will lead to a search for novel ligands of the AhR and estimation of the potency of agonists or antagonists in this assay.

CHAPTER 4

Pigments in Green Tea Leaves (*Camellia sinensis*) Suppress Transformation of the Aryl Hydrocarbon Receptor Induced by Dioxin

4.1. Introduction

Since the aryl hydrocarbon receptor (AhR) is one of the orphan receptors, numerous studies have been carried out to search for the agonists and antagonists of AhR, besides dioxins and polycyclic aromatic hydrocarbons (PAHs). As agonists of AhR, tryptophan and its metabolites [48], beta-apo-8′-carotenal, canthaxanthin, and astaxanthin [101] are reported to induce the AhR transformation and its downstream events, CYP1A1 and CYP1A2 expression. Indigo and indirubin are also possible agonists of AhR, and they interact with human AhR *in vitro* [34]. As antagonists of AhR, 3′,4′-dimethoxyflavone [33] and LY294002 [2-(4-morpholinyl)-8-phenyl-*H*-1-benzo-pyran-4-one] [102] have been reported to suppress expression of *CYP1A1* mRNA and its enzyme activity through the AhR-dependent pathway. Natural flavonoids also suppress the transformation of AhR [35] and CYP1A1 activity [38, 103]. In addition, resveratrol [39] and curcumin [40] are reported to act as antagonists of AhR. Thus, it is important to search for novel AhR ligands. From the viewpoint of the prevention of toxicities by dioxins, it is critical to search for natural compounds possessing the antagonistic effects toward AhR. Because dioxins unexpectedly enter the body mainly through diet [104, 105], antagonists of AhR should be components of food to prevent or reduce the toxicities.

In Chapter 2, the author demonstrates that flavones and flavonols have an ability to suppress AhR transformation at the dietary level, while catechins show a moderate effect. The IC_{50} value of galangin, apigenin, kaempferol, and quercetin against 1 nM TCDD-induced AhR is less than 3 μM, while that of (−)-epigallocatechin gallate is 10 times larger [35]. Another report showed that green tea extract inhibited *CYP1A1* gene

expression [36], but the green tea extract used was an artificial mixture enriched with catechins (more than 81%) and different from commercially available ones, which contain only 15-25% catechins as dry weight matter [106]. A recent report also showed that antagonistic compounds isolated from green tea extract were catechins [37], although the isolation method was focused on flavonoids. Thus, there is a possibility that plants including green tea leaves contain novel ligand(s) of AhR. In this chapter, the author has isolated and identified antagonists of AhR from green tea leaves and determined their activities *in vitro* [43].

4.2. Materials and methods

4.2.1. Materials

Dried green tea leaves (*Camellia sinensis*) were manufactured in the Shizuoka Prefecture in Japan. 2,3,7,8-Tetarachlorodibenzo-*p*-dioxin (TCDD), catechins, and the other flavonoids were purchased from the same companies as described in Chapter 2. β-Cryptoxanthin and zeaxanthin were obtained from Extrasynthèse. Lutein, chlorophyll a, chlorophyll b, lycopene, and astaxanthin were from Sigma Chemical Co., and β-carotene was purchased form Nacalai Tesque. For electrophoretic mobility shift assay (EMSA), oligonucleotide probe for dioxin responsive element [3] (DRE; 5′-GAT CCG GAG TTG CGT GAG AAG AGC CA-3′ (coding) and 5′-GAT CTG GCT CTT CTC ACG CAA CAC CG-3′ (non-coding)) was synthesized. All other reagents used were of the highest grade available from a commercial source.

4.2.2. Extraction and fractionation of green tea leaves

To identify the green tea components having suppressive effects on AhR transformation, dried green tea leaves (500 g) were extracted three times with 1 l of 75% ethanol for 24 h, and the extract was evaporated and used as the ethanol extract. The ethanol extract was suspended in 1 l of distilled water and partitioned stepwise with 2 l of *n*-hexane, chloroform, ethyl acetate, and *n*-butanol, three times each. Each fraction obtained was quantitatively recovered, evaporated, and assayed for antagonistic effects on AhR transformation. The *n*-hexane-partitioned fraction was

further fractionated by column chromatography and high-performance liquid chromatography (HPLC) in the following way: An aliquot of 200 μl of the *n*-hexane-partitioned fraction (100 mg/ml methanol solution) was applied to a 300- × 20-mm i. d. MCI gel column in methanol as the immobile phase. The column was eluted with 500 ml of methanol and then with 200 ml of acetone. The eluate with methanol was fractionated into four subfractions (referred to as the methanol fraction-1-4) by measuring the absorbance at both 210 and 350 nm, while the elute with acetone was collected in a lump fraction. This column chromatography was repeated 5 times under the same conditions, and each subfraction was collected and combined. Each subfraction was dried *in vacuo*, dissolved in methanol at a concentration of 100 mg/ml, and subjected to the preparative HPLC equipped with a photodiode array detector (Waters 2695 and 2996 PDA system). The HPLC conditions were as follows: Column, 250- × 20-mm i. d., 5-μm Js80H ODS column (YMC Co., Ltd., Kyoto, Japan) maintained at 40 °C; elution was performed with 100% methanol at a flow rate of 8 ml/min; injection volume of each fraction was 200 μl; and the detection was UV at 210 nm. The HPLC was carried out repeatedly under the same conditions, and each peak was collected and combined.

4.2.3. Instrumental analysis

The active compounds isolated from green tea leaves were purified by rechromatography with HPLC using a Waters 600E Multisolvent Delivery System and a 486UV/Vis detector (Nihon Waters K. K., Japan), and mass spectra (MS) were obtained using a JEOL JMS-SX 102 mass spectrometer (JEOL Ltd., Japan). ^{1}H- and ^{13}C-NMR spectra were recorded on a JEOL JNM-A 400 at 400.00 and 100.4 MHz, respectively (JEOL Ltd.), and chemical shifts were given in δ (ppm) with tetramethylsilane used as an internal standard.

4.2.4. Determination of polyphenols content in green tea extract

To determine the contents of polyphenols, chlorophylls, and lutein in green tea extract, a hot-water extract was separately prepared in addition to the ethanol extract. These extracts were dissolved in methanol at 1 mg/ml, and aliquots of 10 μl were

injected into the HPLC. Analytical conditions were as follows: for detection of polyphenols, a 250- × 4.6-mm i. d. Wako pack C18HG column maintained at 40 °C; mobile phase, 22% methanol solution in 0.1% phosphate buffer; flow rate, 1 ml/min; and wavelength at 230 and 280 nm; and for chlorophylls and lutein, a 250- × 4.6-mm i. d. Js80H column (YMC Co., Ltd.) maintained at 30 °C or room temperature; mobile phase, 100% methanol; flow rate, 1 ml/min; and monitoring with a Waters 486 UV/Vis at 210 nm.

4.2.5. Preparation of the cytosol fraction from rats

Livers from male Sprague-Dawley rats (6 weeks old, 140-170 g, obtained from Japan SLC) were subjected to preparation of the cytosol fraction as described in Chapter 2 [35]. After measuring the protein contents [68], the cytosolic fraction was used for estimation of the antagonistic effects of green tea extract or its components on AhR transformation.

4.2.6. Estimation of the antagonistic effects of green tea components on AhR transformation

To estimate the antagonistic effects of the green tea components on AhR transformation, the cytosolic fraction (4.0 mg protein/ml) in HEDG buffer (25 mM HEPES of pH 7.4, 1.5 mM EDTA, 1.0 mM DTT, 10% glycerol) was incubated with various concentrations of green tea extract or its components dissolved in DMSO at 20 °C for 10 min and then with 0.1 or 1 nM TCDD or with DMSO (10 μl/ml) alone as a vehicle control for a further 2 h. The resultant mixture was subjected to EMSA in the manner described in Chapter 2 [35].

4.3. Results

4.3.1. Evaluation of the suppressive effects of green tea extract on AhR transformation

To confirm whether crude green tea extract can suppress AhR transformation, green tea leaves were extracted with 75% ethanol to obtain almost all of the low-molecular weight components. As shown in Fig. 4.1.A, the ethanol extract suppressed AhR transformation induced by 1 nM TCDD in a dose-dependent manner, while the ethanol

extract itself did not induce the transformation (Fig. 4.1.A, Lane 8). This indicates that green tea extract possesses suppressive effects toward TCDD-induced AhR transformation and that total antagonistic activities in the extract overcome total agonistic ones. To determine the 50% inhibitory concentration (IC_{50}) value, the density of each specific band was analyzed, and a log of the concentration of the ethanol extract against the ratio of transformed AhR was plotted (Fig. 4.1.B). The IC_{50} value of green tea extract against the 1 nM TCDD-induced AhR transformation was 110 μg/ml.

The ethanol extract was then stepwisely partitioned with *n*-hexane, chloroform, ethyl acetate, and *n*-butanol, and the suppressive effect of the obtained fractions on AhR transformation was examined (Fig. 4.2). The *n*-hexane and the ethyl acetate fractions showed strong effects with IC_{50} values of 41 and 42 μg/ml, respectively, while the other fractions showed weak effects with the IC_{50} values of > 100 μg/ml. Hence, the author searched for the antagonistic compounds in the *n*-hexane and the ethyl acetate fractions.

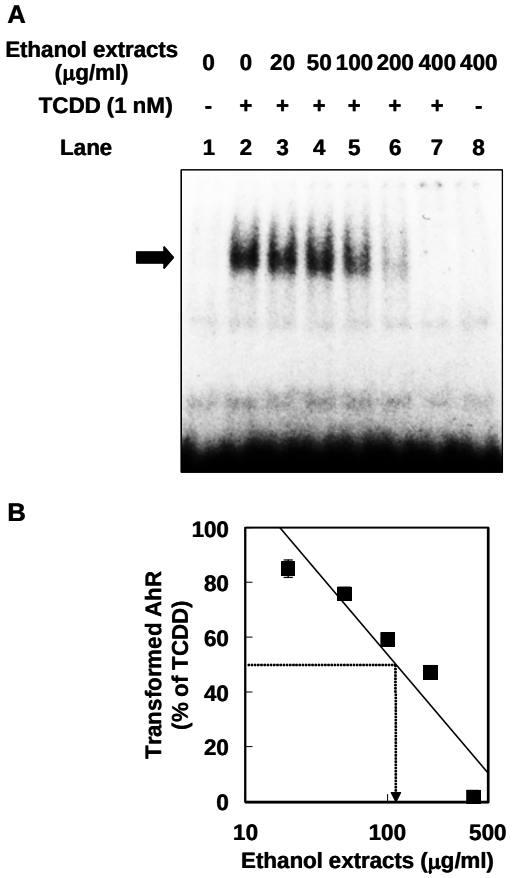


Fig. 4.1. Suppressive effects of the ethanol extract from green tea leaves on AhR transformation. (A) Representative EMSA result, and the arrow indicates AhR/DRE complex. (B) Quantified density of AhR/DRE complex. Data are represented as the mean ± SE from the independent triplicate experiments. The IC_{50} value of the ethanol extract against 1 nM TCDD was determined by plotting a log of the concentration of the ethanol extract against the ratio of transformed AhR.

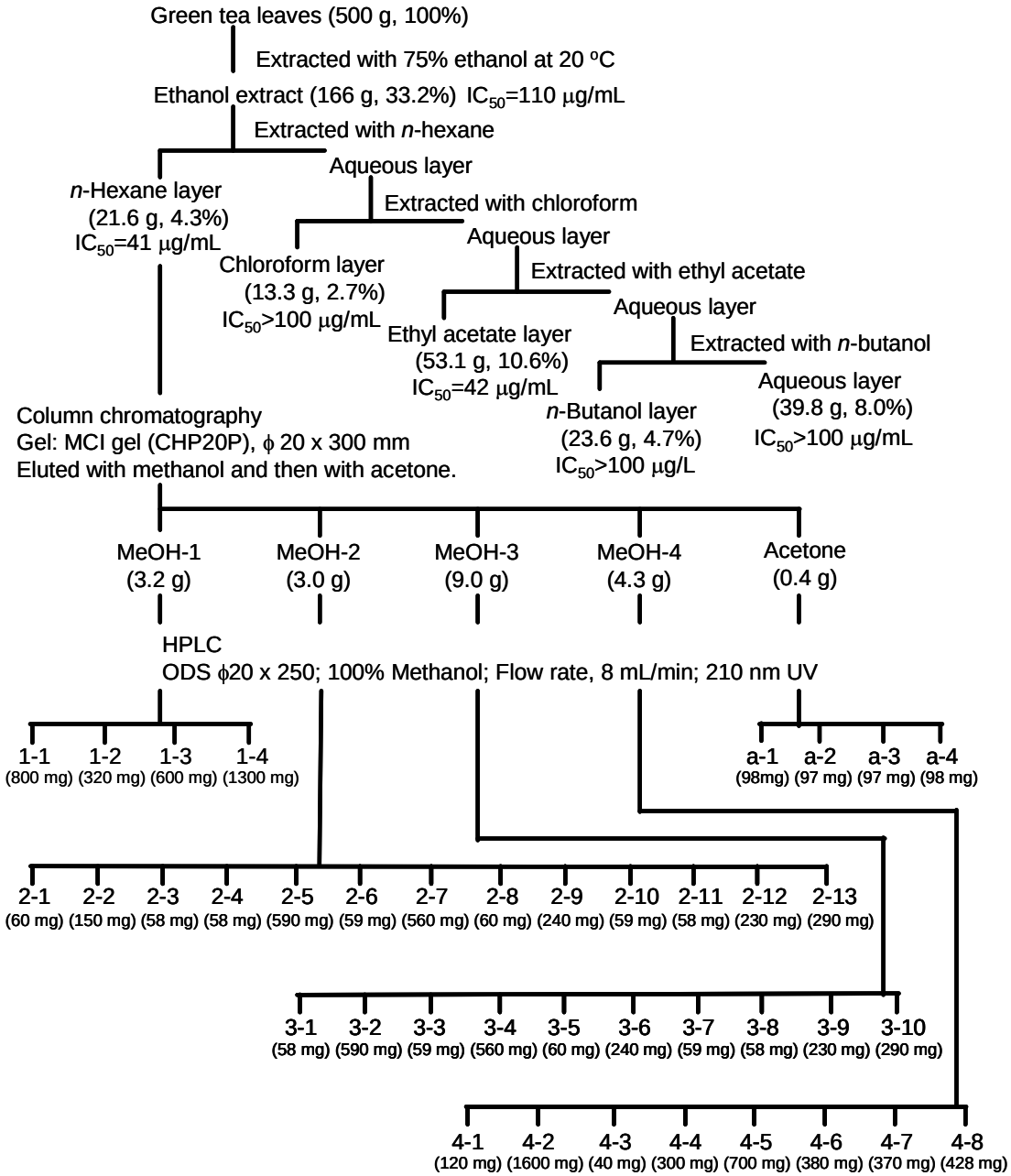


Fig. 4.2. Extraction and fractionation procedures of green tea leaves. Weight and percent in parentheses are the yield of each fraction. The IC_{50} values were determined against 1nM TCDD-induced AhR transformation.

4.3.2. Isolation of antagonists of AhR from n-hexane fraction

The *n*-hexane fraction is rich in various pigments such as chlorophylls, pheophytins, carotenoids, and so on. It has not yet been investigated whether these pigments in tea leaves can suppress AhR transformation. Consequently, the *n*-hexane fraction was subjected to further fractionation into subfractions using column chromatography (Fig. 4.2). Among the subfractions, an acetone subfraction showed the most suppressive

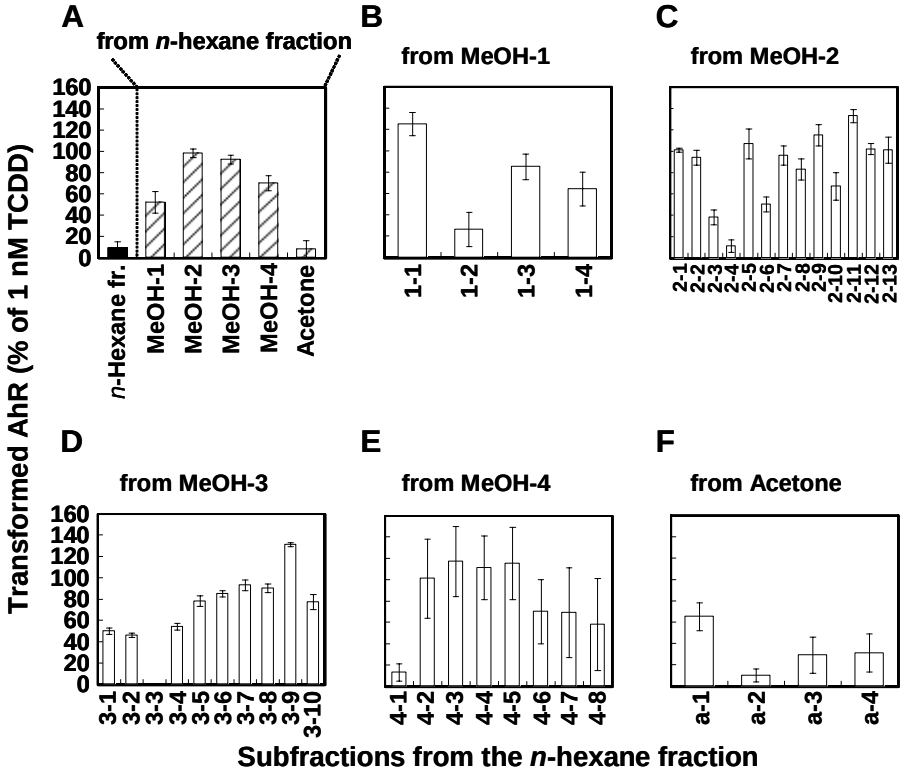


Fig. 4.3. Suppressive effects of the subfractions from the *n*-hexane fraction on the AhR transformation. Suppressive effects of subfractions at 10 μg/mL (A) or 5 μg/mL (B-F) were determined against 1 nM TCDD-induced AhR transformation. Data are shown as the percent of transformed AhR (% of TCDD) and represented as the mean ± SE from the independent triplicate experiments.

effects on AhR transformation and the methanol fraction-1 also showed moderate effects (Fig. 4.3.A). Each subfraction was subjected to preparative HPLC and separated into 4 parts from the methanol fraction-1, 13 from the methanol fraction-2, 10 from the methanol fraction-3, 8 from the methanol fraction-4, and 4 from the acetone fraction (Fig. 4.2). Each obtained part was dried *in vacuo*, dissolved in DMSO containing 20% acetone, and examined for the suppressive effect on AhR transformation. As a result, the HPLC-fractions 1-2, 2-4, 3-3, 4-1, a-2, a-3, and a-4 showed strong effects (Fig. 4.3, panels B-F). These HPLC fractions were purified by rechromatography with HPLC and subjected to identification by instrumental analyses.

4.3.3. Identification of the antagonists of AhR in green tea extract

A major compound in the HPLC fractions 3-3 and 4-1 was identified as lutein by ^{1}H-NMR, ^{13}C-NMR, and FAB-MS (*m*-nitrobenzyl alcohol matrix): *m*/*z* = 568[M]$^{+}$, and obtained NMR data was consistent with literature ones [107-109]. The compounds in

the HPLC fractions a-2 and a-4 were identified as chlorophyll b and chlorophyll a from the molecular ion peak in their FAB-MS, respectively [110, 111]. Also, their R_f values on thin-layer chromatography were the same as those of authentic samples. Regarding the HPLC fractions 1-2 and a-3, they mainly contained caffeine and pheophytins, respectively (data not shown), but the authentic compounds did not show any effects. Probably, minor compounds in these fractions showed the strong suppressive effects on AhR transformation. In the case of the HPLC fraction 2-4, the active compound(s) could not be identified because this fraction contained at least several compounds.

4.3.4. Antagonistic effects of lutein and chlorophyll a and b on AhR transformation

Because the author identified lutein and chlorophyll a and b in green tea leaves, their antagonistic effects on AhR transformation were further investigated using the corresponding authentic compounds. Lutein showed suppressive effects on AhR transformation in a dose-dependent manner against 1 and 0.1 nM TCDD (Fig. 4, panels A and B, respectively). Chlorophyll a and b also suppressed the transformation dose-dependently (Fig. 4.4, panels C and D, respectively). The IC_{50} values of lutein and chlorophyll a and b against 0.1 nM TCDD-induced AhR transformation were 3.2, 5.0, and 5.9 μM, respectively. Moreover, these compounds did not show the agonistic effects toward AhR (data not shown).

Among these compounds, lutein is a novel antagonist for AhR. Thus, the author examined the effects of other commercially available carotenoids on AhR transformation. As shown in Fig. 4.5, β-cryptoxanthin and zeaxanthin also suppressed AhR transformation to about 50% at 50 μM. At the same concentration, lutein completely suppressed AhR transformation (Fig. 4.4.B and Fig. 4.5). These results indicate that lutein has a unique property for AhR in carotenoids.

4.3.5. Contents of polyphenols in green tea extract and their antagonistic effects on AhR transformation

The ethyl acetate-partitioned fraction from green tea extract suppressed the TCDD-induced AhR transformation, as did the *n*-hexane-partitioned fraction. Green

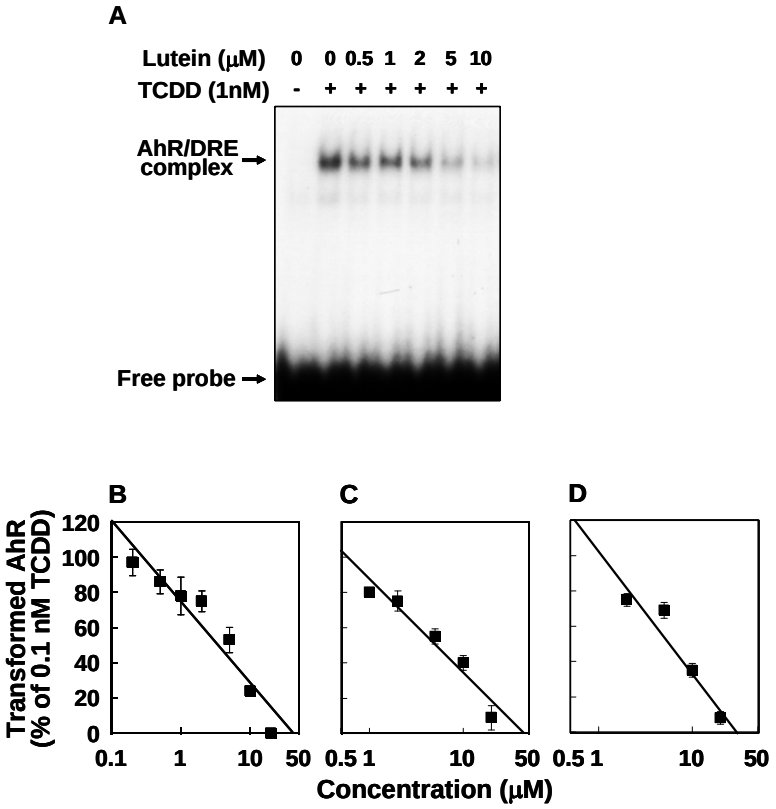


Fig. 4.4. Dose-dependent suppressive effects of lutein or chlorophylls at the indicated concentrations on AhR transformation. The representative EMSA result of lutein against 1 nM TCDD is shown in panel A, and the arrow indicates AhR/DRE complex. The quantified density of the AhR/DRE complex of each compound against 0.1 nM TCDD is shown in panels B-D. Data are represented as the mean ± SE from the independent triplicate experiments. The IC_{50} values of lutein and chlorophyll a and b against 0.1 nM TCDD were determined by plotting a log of the concentration of the compounds against the ratio of transformed AhR.

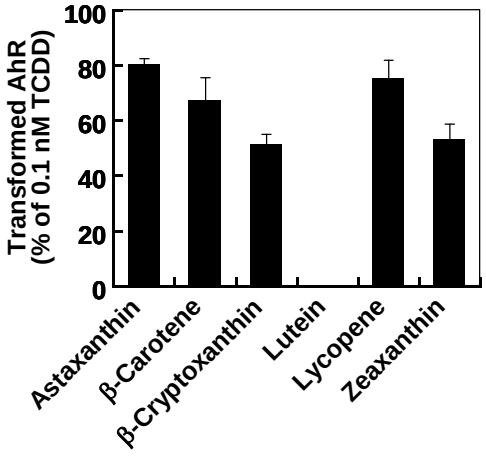


Fig. 4.5. Suppressive effects of carotenoids on the AhR transformation. Suppressive effects of carotenoids at 50 μM were determined against 0.1 nM TCDD-induced AhR transformation. Data are shown as the percent of transformed AhR (% of TCDD) and represented as the mean ± SE from the independent triplicate experiments.

tea extract contains various flavonoids, mainly catechins, and their aglycones are expected to be distributed in the ethyl acetate-partitioned fraction because of their

chemical properties [35]. When the author determined the contents of catechins and caffeine in each fraction from green tea extract, catechins were mainly contained in the ethyl acetate fraction, and caffeine was in the chloroform fraction (Table 4.1). In the current study, green tea leaves were extracted with 75% ethanol and further fractionated to determine their effects on AhR transformation. However, green tea is generally drunk as a hot-water extract, although powdered green tea is also drunk as a suspension (e.g., in a tea ceremony or in commercial beverages). To compare the contents of compounds between the ethanol extract and the hot-water extract, each of them was subjected to an HPLC analysis. The ethanol extract contained all compounds that were contained in the hot-water extract (Table 4.2). For example, the content of

Table 4.1. Contents of catechins in the organic solvent-partitioned fraction from the ethanol extract of green tea leaves[a]

Compounds	Fraction				
	n-Hexane	Chloroform	Ethyl acetate	*n*-Butanol	Aqueous
(+)-Catechin	nd[b]	nd	trace	trace	nd
(−)-Epicatechin	nd	trace	9.0	nd	nd
(−)-Gallocatechin	nd	trace	1.4	trace	nd
(−)-Epigallocatechin	trace	trace	23.2	10.5	nd
(−)-Epicatechin gallate	nd	trace	10.3	nd	nd
(−)-Epigallocatechin gallate	trace	1.1	58.7	3.2	nd
Caffeine	trace	77.9	trace	nd	nd

[a]Contents of various catechins and caffeine were determined by HPLC analysis and represented as w/w percent in each organic solvent-partitioned fraction. [b]nd, not detected.

(−)-epigallocatechin gallate in the ethanol extract was 4 times as much as that in the hot-water extract. (−)-Epicatechin gallate, kaempferol, chlorophyll a, and lutein were detected in the ethanol extract but not in the hot-water extract. These results indicate that catechins, the major polyphenols in green tea leaves, are contained abundantly in the ethanol extract and distributed in the ethyl acetate-partitioned fraction.

In Chapter 2, it was demonstrated that flavonoids, including catechins, suppress the AhR transformation induced by 1 nM TCDD [35]. In the present study, the author examined the suppressive effects of catechins and other polyphenols contained in green tea leaves on the AhR transformation induced by 0.1 nM TCDD, a lower concentration close to the physiological conditions. As shown in Table 4.2, (−)-catechin

gallate and (−)-epigallocatechin gallate showed the strong effects, with the IC_{50} values of 0.5 and 1.7 μM, respectively, while others showed moderate or weak effects. (−)-Epigallocatechin gallate at this concentration can exist in the body [81], whereas the amount of (−)-catechin gallate is small in green tea extract (Tables 4.1 and 4.2). Other flavonoids, including luteolin, quercetin, and kaempferol, also strongly suppressed the transformation, with IC_{50} values of 0.52, 0.84, and 0.63 μM, respectively. The other compounds, kaempferol-3-glucoside, caffeine, gallic acid, *n*-butylgallate, theanine, and theobromine, did not affect AhR transformation. These results suggest that catechins, especially (−)-epigallocatechin gallate, are major effective compounds in the ethyl acetate-partitioned fraction.

Table 4.2. Contents and IC_{50} values against 0.1 nM TCDD-induced AhR transformation of selected compounds in the ethanol and the hot-water extracts from green tea leaves[a]

Compounds	Ethanol extract (%)	Hot-water extract (%)	IC_{50} value (μM)
(+)-Catechin	1.3	1.1	69
(−)-Epicatechin	4.6	1.6	93
(−)-Gallocatechin	1.8	1.2	34
(−)-Epigallocatechin	13.6	3.5	24
(−)-Catechin gallate	nd[b]	nd	0.5
(−)-Epicatechin gallate	5.0	nd	20
(−)-Gallocatechin gallate	nd	nd	8.3
(−)-Epigallocatechin gallate	21.6	5.1	1.7
Luteolin	0.33	0.25	0.52
Quercetin	nd	nd	0.84
Kaempferol	0.06	nd	0.63
Kaempferol-3-glucoside	N.D.[c]	N.D.	N.E.[d]
Chlorophyll a	0.023	nd	5.0
Chlorophyll b	nd	nd	5.9
Lutein	0.015	nd	3.2
Caffeine	9.5	9.4	N.E.
Gallic acid	N.D.	N.D.	N.E.
n-Butylgallate	N.D.	N.D.	110
Theanine	N.D.	N.D.	N.E.
Theobromine	N.D.	N.D.	N.E.

[a]Compounds in the ethanol and the hot-water extract were detected by HPLC analysis. [b]nd, under the detection limit. [c]N.D., not determined. [d]N.E., no effect is up to 100 μM.

4.4. Discussion

Dioxins, the environmental contaminants, express toxicities through the transformation of AhR [5, 10, 12] and enter the body mainly through diet [104, 105]. It is important to search for natural antagonists of AhR in food. Previous reports showed that green tea extract and its major compounds, catechins, suppressed AhR transformation and its downstream event, *CYP1A* gene expression [36, 37]. In this chapter, the author has confirmed that green tea extract suppresses the transformation of AhR in a dose-dependent manner, and found that lutein and chlorophyll a and b are the novel antagonists of AhR from the *n*-hexane-partitioned fraction of green tea extract.

These pigments, in addition to catechins, especially (−)-epigallocatechin gallate, contribute to the suppressive effects of green tea extract on AhR transformation.

Lutein is a common carotenoid in the plant kingdom and has various biological functions such as antioxidant activity, anticarcinogenic activity, and protection against the development of age-related macular degeneration [112-114]. Several carotenoids, excluding lutein, have been reported to be ligands of AhR. Gradelet *et al.* [101] reported that β-apo-8′-carotenal, canthaxanthin, and astaxanthin induce CYP1A1 and 1A2 in the rat. Some synthetic retinoids have also been reported to induce CYP1A1 via AhR transformation [115]. In this chapter, the author has isolated and identified lutein as a novel antagonist with the IC_{50} value against 0.1 nM TCDD of 3.2 μM (Figure 4.4 and Table 4.2). Although the mechanism of the suppression has not yet been elucidated, the suppressive effect is similar to that of catechins having gallate moieties (Table 4.2). Other xanthophylls such as β-cryptoxanthin and zeaxanthin also showed a moderate suppressive effect, whereas β-carotene, lycopene, and astaxanthin showed a weaker effect. These results suggest that the polyene structure is not so important, but the hydroxyl groups in 6C rings are needed to exhibit the effects.

In this chapter, chlorophylls, as well as lutein, were identified as the novel antagonists of AhR. Chlorophylls, as well as carotenoids, widely distributed in the plant kingdom, are not considered to be incorporated *per se* into our body, although uptake of chlorophyll derivatives by human intestinal cells has been demonstrated [116]. Chlorophylls are reported to inhibit absorption and accelerate excretion of

dioxins in rat [117] and in human intestinal Caco-2 cells [118]. Chlorophyllin, a copper/sodium salt of chlorophyll, forms a molecular complex with heterocyclic amines, which have been reported to interact with AhR [119, 120]. Moreover, bilirubin induced CYP1A1 in hepatocytes via AhR-dependent action [121, 122]. These results suggest that the compounds having a porphyrin ring might have the potency to interact with TCDD and/or AhR, and suppress the transformation.

Catechins, flavonols, and flavones are natural antagonists of AhR, as demonstrated in Chapter 2 [35], and these pigments were contained mainly in the ethyl acetate fraction (Table 4.1). The results in this chapter demonstrated that the IC_{50} value of (−)-epigallocatechin gallate against the 0.1 nM TCDD-induced AhR transformation was 1.7 μM (Table 4.2). (−)-Epigallocatechin gallate at this concentration can exist in the body, since Nakagawa *et al.* [81] reported that the plasma concentration of (−)-epigallocatechin gallate rose to 4.4 μM after an intake of 525 mg. Thus, (−)-epigallocatechin gallate would suppress the AhR transformation at the physiological level. Previous reports have also demonstrated that not only green tea extract but also catechins suppress AhR transformation and *CYP1A1* gene expression [35, 37, 105, 123], but their suppressive effects were weaker than those in this chapter. This difference is due to TCDD concentration because higher concentrations were used. In Chapter 2, it was showed that the lower concentrations of these compounds were needed to suppress the lower concentration of TCDD [35]. The level of TCDD in the environment is much lower than those in the experiments, including the current study. Therefore, flavonoids, including catechins, are attributive compounds for the prevention of dioxin toxicity.

Among the antagonistic pigments, (−)-catechin gallate, luteolin, quercetin, and kaempferol showed potentially strong effects from the IC_{50} values against 0.1 nM TCDD, though these strong antagonistic compounds were scarcely present in green tea leaves (Table 4.2). Similarly, lutein is contained in the ethanol extract from green tea leaves but not detected in the hot-water extract. These compounds are abundantly present not only in green tea leaves but in common plants. After intake of vegetables and fruits, plasma levels of flavonols and lutein were around 0-0.14 μM and 0.2-0.5 μM, respectively [124-126]. Therefore, intake of vegetables and fruits leads to an increase in

the physiological or intestinal levels of these antagonistic pigments, and contributes to protecting us from dioxin toxicity.

The present findings are based on only *in vitro* assays using rat liver cytosol, and the actual effects on dioxin toxicity need future animal studies. Maliakal *et al.* [127, 128] demonstrated that tea consumption modulates both phase I and phase II enzyme activities in rats, suggesting that tea components have the potency to interact with AhR *in vivo*. Because the transformation of AhR is the initial step of dioxin toxicity, intake of green tea can possibly protect against dioxin toxicity. This possibility will be discussed in Chapter 6.

CHAPTER 5

Black Tea Theaflavins Suppress Dioxin-induced Transformation of the Aryl Hydrocarbon Receptor

5.1. Introduction

Since transformation of the aryl hydrocarbon receptor (AhR) is the initial step in the expression of dioxin toxicity, inhibition of the transformation would protect humans from its toxic effects. It is, therefore, necessary to search for food factors that possess antagonistic effects against the AhR, because dioxins enter the body mainly through diet [104, 105]. In Chapter 2, the author showed that flavones and flavonols can suppress the transformation at dietary levels [35]. Other natural flavonoids, catechins [36, 37, 129] and galangin [38] are also reported to suppress AhR transformation and the downstream expression of *CYP1A1*. In Chapter 4, the author has shown that pigments including catechins in green tea suppress AhR transformation [43, 130]. In addition, resveratrol [39], curcumin [40], and certain vegetable constituents [41, 42] act as antagonists of the AhR. These results indicate that polyphenols present in food are possible antagonists of the AhR.

Black tea, which is consumed worldwide, has beneficial effects on human health such as antioxidative, anti-carcinogenic, and anti-mutagenic activities [56, 131]. These effects are assumed to be exhibited by polyphenols present in black tea such as theaflavins (Tfs) and thearubigins. Black tea polyphenols consist of 50% thearubigins, 10% Tfs, and 30% catechins along with other compounds [57]. Tfs and thearubigins are products of oxidation and condensation reactions of catechins during fermentation, and are different from the polyphenols found in green tea. Few studies on the properties of thearubigins have been conducted, but the bioactivity of Tfs, red pigments that contribute to the color of black tea, have recently become the focus of attention due to its antioxidative activity associated with its polyphenolic structure

[132]. The intake of 700 mg of Tfs results in a plasma concentration of 1.0 μg/l and a concentration of 4.2 μg/l in urine after 2 h [133], indicating that Tfs are, at least in part, absorbed into the body, whereafter they affect physiological functions. Recently, black tea Tfs and theafulvins are reported to suppress CYP1A1 expression and its activity in the rat intestine [134], suggesting that these polyphenols suppress AhR transformation induced by halogenated and/or polycyclic aromatic hydrocarbons in the liver. In the present chapter, the author determine whether black tea suppresses transformation of the AhR *in vitro*, and also examine the inhibitory effect of Tfs, specific polyphenols contained in black tea, on AhR transformation.

5.2. Materials and methods

5.2.1. Materials

Green (*Sen-cha*, *Gyokuro*, and *Ban-cha*), oolong (*Tekkannon*, *Shikisyu*, and *Suisen*), and black (*Uva*, *Nuwara eliya*, and *Dimbula*) tealeaves, were manufactured in the Shizuoka Prefecture in Japan, China, and Sri Lanka, respectively. Each tea leaves (20 g) were extracted with 200 ml of hot-water for 30 min, and after evaporation and lyophilization, the obtained powders were used as each tea extract. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) was purchased from the same company as described in Chapter 2 [35]. All other reagents used were of the highest grade available from commercial sources.

5.2.2. Determination of catechins and caffeine contents in tea extract

To determine the contents of catechin and caffeine contents in each tea extract, tea extract was dissolved in methanol at 1 mg/ml, and aliquots of 10 μl were injected into the HPLC (Waters Alliance 2690 Separations Module, 2487 Dual Wavelength Absorbance Detector and data acquisition and analysis were preformed with the Empower Software Systems, Nihon Waters K. K.). Analytical conditions were as follows: Column, Wako pack C18HG (ϕ 4.6 × 250 mm, Wako Pure Chemical Co.) maintained at 40°C; mobile phase, 22 % methanolic solution in 0.1 % phosphoric acid (22:78:0.1, v/v); flow rate, 1 ml/min; and monitoring at 230 and 280 nm.

5.2.3. Extraction and isolation of Tfs from black tealeaves

To isolate active compounds from black tealeaves, dried leaves (500 g) were extracted 3 times with 1 l of 75% ethanol for 24 h, and the extract was evaporated and lyophilized and used as the ethanol extract. The extract was suspended in 1 l of distilled water and stepwisely partitioned with 2 l of *n*-hexane, chloroform, ethyl acetate, and *n*-butanol 3 times each. Each fraction obtained was collected and lyophilized. The *n*-hexane-partitioned fraction was further fractionated by an MCI gel column chromatography (ϕ 20 × 300 mm) as described in Chapter 4 [43]. The eluate with methanol was fractionated into two subfractions (referred to as the methanol fraction-1 and -2) by measuring the absorbance at both 210 and 350 nm, and then with acetone was collected in a lump fraction.

The ethyl acetate-partitioned fraction was loaded onto a Capcell pack AG120-ODS column (ϕ 50 × 250 mm, Shiseido Co., Ltd., Japan) in methanol/1% acetic acid (35:65, v/v) with a flow rate of 40 ml/min, and a crude Tfs fraction was obtained by measuring the absorbance at 350 nm. The crude Tfs fraction was then separated into Tf, theaflavin-3-gallate (Tf3g), theaflavin-3′-gallate (Tf3′g), and theaflavin-3,3′-digallate (Tfdg) by HPLC (Waters 600E multisolvent system) under the following conditions: Column, J'sphere ODS-H80 (ϕ 20 × 250 mm, YMC Co., Ltd.); mobile phase, methanol/0.1% acetic acid (30:70, v/v); flow rate, 12 ml/min; and monitoring at 350 nm using a Waters 486UV/VIS (Nihon Waters K. K.).

5.2.4. Estimation of antagonistic effects of black tea components on AhR transformation

To estimate the antagonistic effects of the black tea components on AhR transformation, the cytosolic fraction (4.0 mg protein/ml) prepared from the rat livers in HEDG buffer (25 mM HEPES of pH 7.4, 1.5 mM EDTA, 1.0 mM DTT, 10% glycerol) was incubated with various concentrations of black tea extract or its components dissolved in DMSO at 20 °C for 10 min and then with 0.1 or 1 nM TCDD or with DMSO (10 μl/ml) alone as a vehicle control for a further 2 h. The resultant mixture was subjected to an electrophoretic mobility shift assay (EMSA) in the manner described in Chapter 2 [35].

5.3. Results

5.3.1. Black tea extract suppresses AhR transformation

In Chapter 4, the author demonstrated that green tea extract suppresses AhR transformation induced by 1 nM TCDD in a dose-dependent manner under the cell-free conditions [43]. The author first confirmed whether black tea extract can suppress AhR transformation. Each 3 kinds of green, oolong, and black tea extracts were examined their suppressive effect at 10 μg/ml, and found that all black tea extracts and *Tekkannon* extract significantly suppressed AhR transformation induced by 0.1 nM TCDD (Fig. 5.1). Since one of the active compounds in green tea leaves is (−)-epigallocatechin gallate [43], the contents of catechins and caffeine in each extract were determined by HPLC analyses (Fig. 5.2). As a result, *Sen-cha* extract contained a largest amount of catechins while *Dimbula* extract did not so much. The contents of catechins in *Uva* extract, which suppressed AhR transformation to 50% of that induced

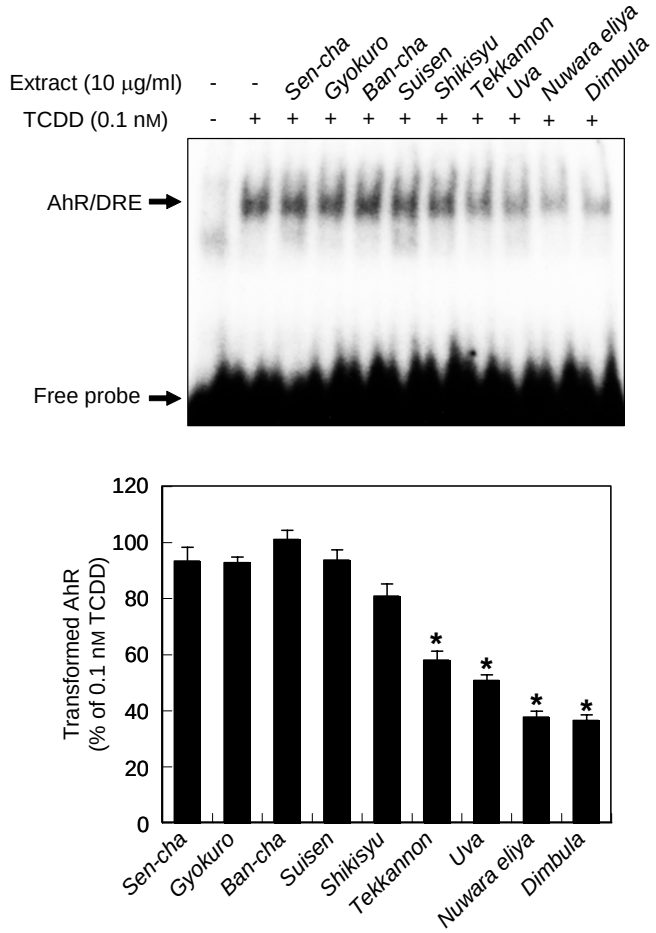


Fig. 5.1. Suppressive effects of various tea extracts on AhR transformation. Hot-water extracts were prepared from 9 tealeaves and they were treated at 10 μg/ml with the rat liver cytosol 10 min prior to addition of 0.1 nM TCDD. AhR transformation was determined by EMSA as described in Materials and methods. Upper panel shows a representative EMSA result, and lower panel shows quantified density of AhR/DRE complex. Data are represented as the means ± SE (n = 3), and asterisks indicate significant differences ($p < 0.05$, Student's *t*-test) from 0.1 nM TCDD-induced AhR transformation.

by 0.1 nM TCDD, was as much as that in *Gyokuro* extract, which did not suppress the transformation (Figs. 5.1 and 5.2). The content of caffeine did not significantly change in each extract, and in addition, caffeine has no suppressive effect on AhR transformation [43]. These data indicated that the suppressive effects of black tea on AhR transformation was not due to the contents of catechins and the specific compounds in black tea extract were much effective.

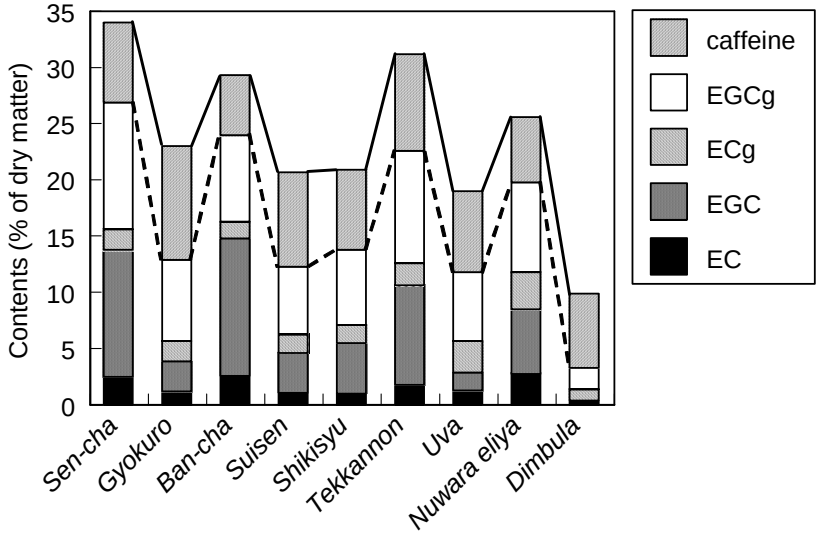


Fig. 5.2. Contents of catechins and caffeine in tea extracts. Each tea extract was dissolved in methanol, and the contents of catechins and caffeine were determined by HPLC as described in Material and methods.

5.3.2. Tfs suppress TCDD-induced AhR transformation

In Chapter 4, the author also demonstrated that the active components in green tea extract were low-molecular compounds and that they were contained in 75% ethanol extract much more than in hot-water extract [43]. Therefore, to determine which components in black tea are effective to suppress AhR transformation, *Uva* tea leaves were subjected to extraction with 75% ethanol and stepwisely partitioned with *n*-hexane, chloroform, ethyl acetate, and *n*-butanol (Fig. 5.3). The ethanol extract and its partitioned fractions were subjected to EMSA to evaluate the suppressive effect in a cell-free system using the rat liver cytosol. The ethanol extract (20 μg/ml) from black tea leaves suppressed 1 nM TCDD-induced transformation by 30% (Fig. 5.4, Lane 3). After fractionation of the ethanol extract, the suppressive components were broadly distributed in the *n*-hexane, chloroform, ethyl acetate, and *n*-butanol fractions with

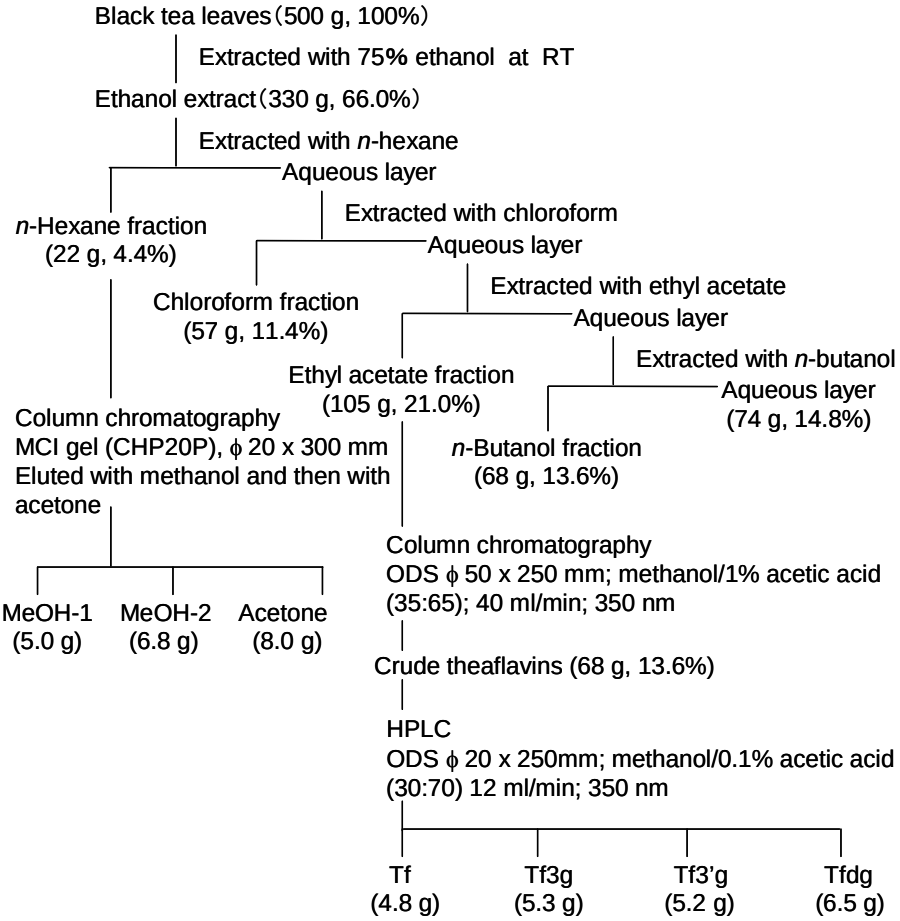


Fig. 5.3. Extraction and fractionation of black tea leaves. Weight and percent in parentheses are the yield of the each fraction.

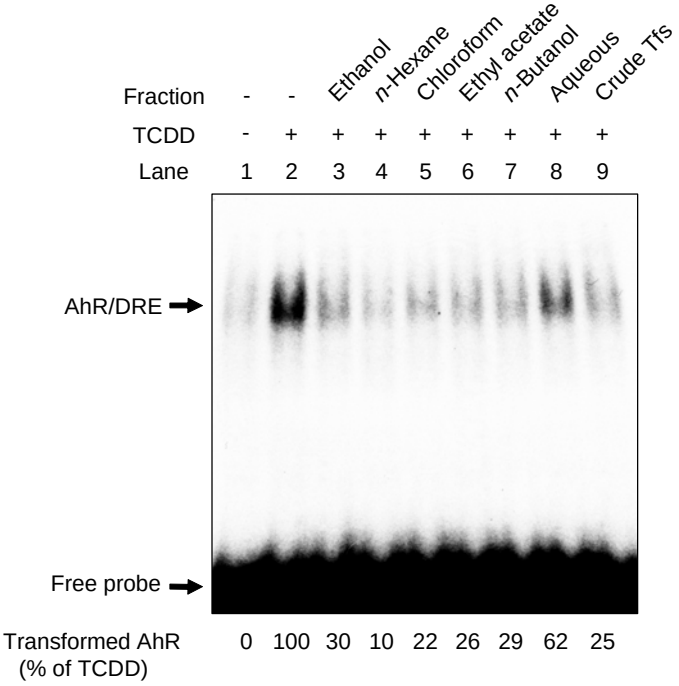


Fig. 5.4. Suppressive effects of black tea extract on AhR transformation. Ethanol extract of black tea leaves was stepwisely partitioned with organic solvents as described in Fig. 5.3. A representative EMSA result of the suppressive effects of the obtained fractions (20 μg/ml) on AhR transformation induced by 1 nM TCDD. Numbers below the panel are the % of transformed AhR induced by TCDD.

suppression levels of 90, 78, 74, and 71%, respectively (Fig. 5.4, Lanes 4-7). The aqueous-residue exhibited a moderate suppressive effect (Fig. 5.4, Lane 8). The *n*-hexane-partitioned fraction, which mainly consisted of pigments such as chlorophylls and carotenoids, was most effective in suppressing AhR transformation. When *n*-hexane-partitioned fraction was further fractionated into 3 subfractions, methanol fraction-1 (MeOH-1), methanol fraction-2 (MeOH-2), and acetone fraction (Acetone), with MCI gel column chromatography (Fig. 5.3), Acetone at 5 μg/ml showed a strong suppressive effect on AhR transformation induced by 1 nM TCDD (Fig. 5.5, Lane 6). In Chapter 4, the author has purified and identified the active compounds in the same acetone fraction from green tea extract are chlorophyll a and b [43]. The author, therefore, focused on the ethyl acetate-partitioned fraction, which possessed the highest yield (21.0%), and this fraction abundantly contained Tfs with a yield of 13.6% (Fig. 5.3). The EMSA results showed that the crude Tfs fraction (mixture of 4 components) had a strong suppressive effect on the TCDD-induced transformation of AhR (Fig. 5.4, Lane 9). The crude Tfs fraction at 20 μg/ml suppressed TCDD-induced AhR transformation by 25%, which was the almost same suppressive effect as that of the ethyl acetate-partitioned fraction at the same concentration. These results suggest that the suppressive effect of the ethyl acetate-partitioned fraction were mainly due to Tfs and that contributions by other compounds were negligible.

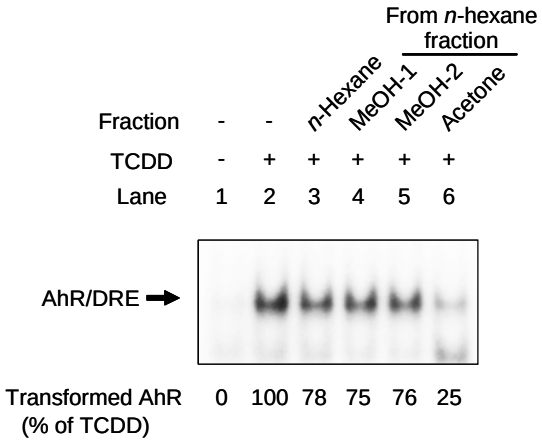


Fig. 5.5. Suppressive effects of the subfractions from *n*-hexane-partitioned fraction on AhR transformation. The *n*-hexane-partitioned fraction was further fractionated by a column chromatography as described in Fig. 5.3. A representative EMSA result of the suppressive effects of the obtained subfractions at 5 μg/ml on AhR transformation induced by 1 nM TCDD. Numbers below the panel are the % of transformed AhR induced by TCDD.

Tf, Tf3g, Tf3'g, and Tfdg were isolated from the crude Tfs fraction by HPLC (Fig. 5.3), and identified by comparison of their NMR spectral data with that found in the literature [135]. As shown in Fig. 5.6, all isolated Tfs exerted a suppressive effect equal

to that of flavone and flavonol [35] in a dose-dependent manner. The IC_{50} values of Tf, Tf3g, Tf3'g, and Tfdg were 4.5, 2.3, 2.2, and 0.7 μM, respectively. Of these, Tfdg had the greatest effect, and its yield of 6.5 g was also the greatest among four Tfs (Fig. 5.3). Moreover, the Tfs did not show any agonistic effect toward AhR (data not shown). These results indicate that Tfs are novel antagonists of AhR in black tea.

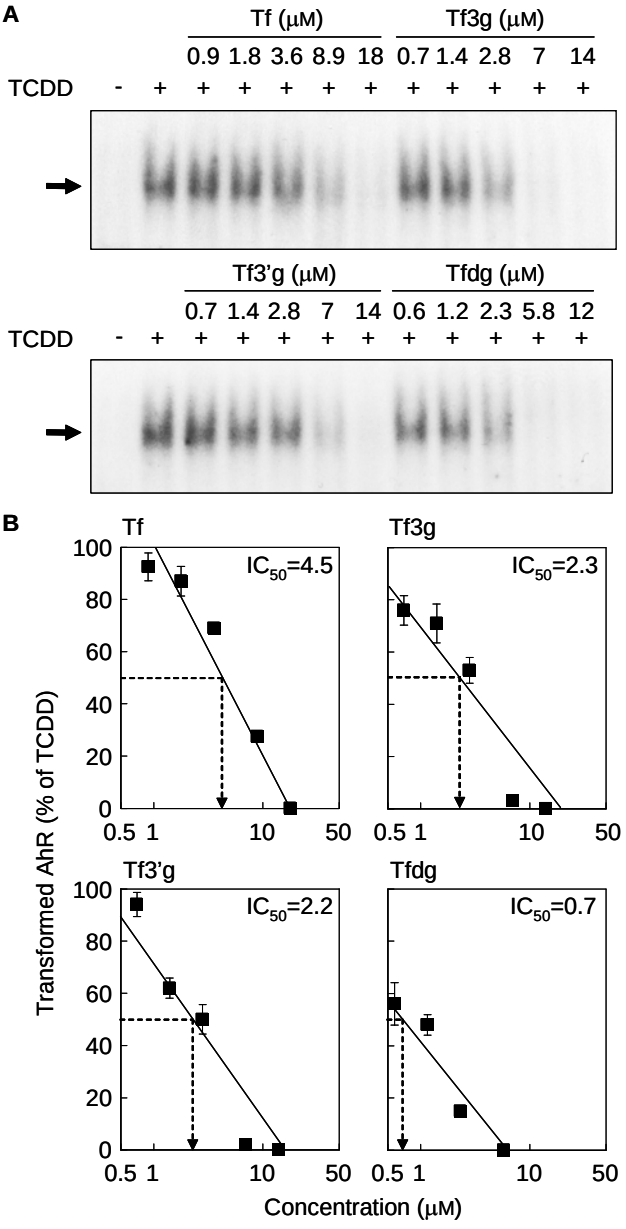


Fig. 5.6. Tfs suppress AhR transformation in a dose-dependent manner. (A) Representative EMSA results of the suppressive effects of Tf, Tf3g, Tf3'g, and Tfdg against 1 nM TCDD-induced AhR transformation. The arrows indicate the AhR/DRE complex. (B) Quantified den-sity of the AhR/DRE complex of each compound. The IC_{50} value (μM) against 1 nM TCDD-induced AhR transformation was determined by plotting the log of the concentrations of each compound against the ratio of TCDD-induced AhR transformation. Data are represented as the means ± SE (n = 4).

5.3.3. Post-treatment with black tea extract and Tfdg suppress AhR transformation under the cell-free systems

Black tea extract and Tfdg suppressed AhR transformation when they applied to the liver cytosol 10 min prior to the addition of TCDD (Figs. 5.4 and 5.6). The author

investigated whether they suppress AhR transformation even when they were treated simultaneously or after the addition of TCDD. When the ethanol extract (Fig. 5.7.A) and Tfdg (Fig. 5.7.B) were applied 10 min prior to the addition of TCDD, both suppressed AhR transformation by 20-30% of that induced by TCDD. Simultaneous co-treatment with the ethanol extract or Tfdg and TCDD also suppressed the transformation by 30-40%. The suppressive effect was diminished but still revealed when they were applied 10-30 min after the addition of TCDD; the effect was around 30-70% of that induced by TCDD. These results indicate that the ethanol extract and Tfdg may inhibit not only the binding of TCDD to the AhR but also binding of the transformed AhR to a DRE probe.

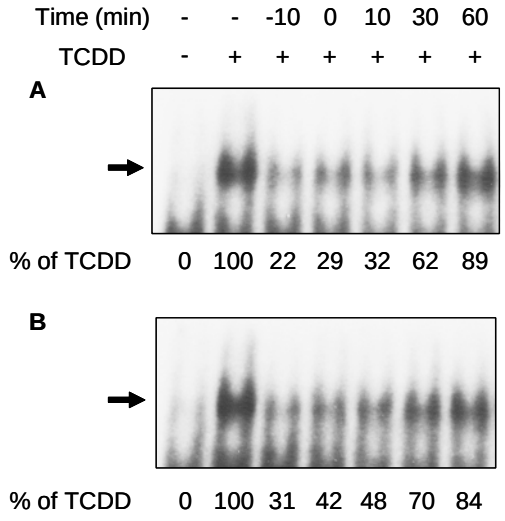


Fig. 5.7. Effect of pre- and post-treatment with black tea extract and Tfdg on AhR transformation. (A) The ethanol extract (20 μg/ml) or (B) Tfdg (2 μM) from black tealeaves was treated with the rat liver cytosol 10 min prior (-10), simultaneously (0), 10, 30, 60 min posterior (10, 30, and 60) to addition of 1 nM TCDD, and AhR transformation was determined by EMSA. Representative data is shown, and numbers below the panel are the % of transformed AhR induced by TCDD.

5.4. Discussion

Dioxins, which are environmental contaminants, induce adverse effects through the AhR transformation [10, 12, 15]. Since dioxins enter the body mainly through diet [83, 84], it is necessary to determine natural antagonists of AhR in foods. In this chapter, it was demonstrated that black tea extract and Tfs suppressed AhR transformation and the effects was kept even they were treated after addition of TCDD to the rat liver cytosol. Previous studies demonstrated that natural flavonoids [35, 36, 129], resveratrol [39], curcumin [40], and certain pigments in green tea, especially (−)-epigallocatechin

gallate [43, 130] suppress transformation of the AhR and/or subsequent downstream events such as expression of *CYP1A1*. These results indicate that the active components in black tea are polyphenols such as Tfs and catechins. However, no correlation was found between the contents of catechins and the suppressive effects on AhR transformation among 9 kinds of tea extracts. Therefore, the active compounds of black tea extract were the specific ones including Tfs, which generated during the fermentation step.

The results in this chapter showed that the IC_{50} values of Tfs against 1 nM TCDD ranged from 0.7 to 4.5 μM, indicating that their suppressive effects are strong, because the IC_{50} values of Tfs are almost the same as those of effective flavonoids aglycones such as galangin, kaempferol, and chrysin [35]. Previous report demonstrated that Tf did not suppress AhR transformation under the cell-free system [41], and that was inconsistent with the results in this chapter. This difference was due to the higher concentration of TCDD was used in the previous one, and so that the effect of Tf was disappeared. Although the bioavailability of Tfs is unknown, a part of Tfs is absorbed into the body and affect physiological functions: A previous report showed that the maximum concentration of Tfs is 1.0 μg/l in the plasma 2.0 h after the ingestion of 700 mg Tfs, which is equivalent to 30 cups of black tea [133]. Since the molecular weights of Tfs range between 564 and 869, and 1.0 μg/l is estimated to be the range from 1.2 to 1.8 nM. However, the concentration of excreted Tfs in the urine (4.2 μg/l) is higher than that in the plasma [133], suggesting that their absorption rates might not be high and/or that they have a short life span in the body. Although the plasma concentration of Tfs is lower than that of other flavonoids [124], Tfs are possible candidate for the antagonists of AhR. The author has demonstrated that the IC_{50} value of antagonist is depending on the TCDD concentration in Chapter 2, i.e., a lower antagonists concentration suppresses AhR transformation induced by a lower concentration of TCDD [35]. Dioxin levels in the body are estimated to be of the pM order [136, 137], and the TCDD level used in the current study was 1000-fold higher than that present in the body. Previous report demonstrated that a long-term ingestion of Tfs (20 mg/kg body weight) suppressed both expression and activity of CYP1A1 in the rat intestine [134]. Taken together results in this chapter and previous reports, continual intake of

black tea would lead an increase in the physiological and intestinal levels of Tfs, and have a possibility of suppressing AhR transformation and CYP1A1 expression.

Regarding the antagonistic mechanism of Tfs on the AhR, they are too large to fit into the AhR binding pocket which has van der Waals dimensions of 14 × 12 × 5 Å [18]. The order of strength of the suppressive effects was TFdg > TF3g = TF3'g > TF (Fig. 5.4), although a pyrogallol-type chemical, gallic acid, did not exert a suppressive effect (data not shown). These results suggest that not the skeletal structure of Tf but the part of the structure possessing the gallate moiety adjusts to the AhR binding pocket and plays a role in the suppression of AhR transformation. From the results shown in Fig. 5.5, Tfdg appears to inhibit binding of the transformed AhR to a DRE probe in addition to the binding of TCDD to the AhR. In Chapter 2, it was demonstrated that flavones and flavonols cannot affect AhR transformation when they are applied after the addition of TCDD [35, 129], suggesting flavonoids suppress the transformation by blocking the binding of TCDD to the AhR. Therefore, Tfs are specific compounds, which can reduce AhR transformation even after exposure to dioxins by inhibiting the binding of transformed AhR to DNA. In conclusion, black tea and its components Tfs suppress AhR transformation induced by TCDD. It is suggested a possibility that black tea is one of the candidates of beverages and foods for reduction of dioxin toxicity through suppressing the AhR transformation pathway.

CHAPTER 6

Drinking Tea Has a Potency to Suppress the Dioxin Toxicity through the Aryl Hydrocarbon Receptor Transformation Pathway

6.1. Introduction

Since dioxins invade the body mainly through diet [4], it is critical to search for the natural antagonists from foods for the aryl hydrocarbon receptor (AhR), the initial step of the development of dioxin toxicity. In Chapter 2, the author demonstrated that natural flavonoids, especially flavones and flavonols suppress AhR transformation. In Chapters 4 and 5, the author have isolated and identified lutein [43] and theaflavins (Tfs) as the novel antagonists for the AhR from green and black tealeaves, respectively. Previous studies demonstrated that certain vegetable constituents [35-38, 41, 42, 129] including resveratrol [39] and curcumin [40] act as antagonists for the AhR. However, these studies were performed in cell-free or cell culture-based bioassay, and it is not clear yet whether the natural antagonists reveal the effect in the body.

Tea, which consumed worldwide, abundantly contains flavonoids including catechins and theaflavins. It was demonstrated that drinking green tea modulates both Phase I and Phase II enzymatic activities in the rat liver [127, 128], and black tea components suppress CYP1A1 expression and its activity in the rat intestine [134]. These results suggest that drinking green or black tea can suppress AhR transformation and its downstream events induced by polycyclic aromatic hydrocarbons (PAHs) including dioxins *in vivo*. In this chapter, the author demonstrates that *ad libitum* drinking tea has a potency to suppress the dioxin toxicity through suppressing AhR-dependent transduction induced by 3-methylcholanthrene (MC), a member of PAHs, in the rat liver.

6.2. Materials and methods

6.2.1. Materials

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) and MC were purchased from the same companies as described in Chapter 2 [35]. Dried green or black tea leaves (*Camellia sinensis*) were manufactured in Japan and Sri Lanka, respectively. All other reagents used were of the highest grade available from commercial sources.

6.2.2. Animal treatments

To confirm the conditions to induce AhR transformation and its subsequent downstream events, 27 male Sprague-Dawley rats (six week-old, 140-170 g, purchased from Japan SLC were at random divided into 9 groups of 3 each, and intraperitoneally injected with MC (10 mg/kg body weight) or corn oil (1 ml/kg body weight) as a vehicle control, and these rats were sacrificed at 0, 1, 2, 4, 6, 8, 12, and 24 h after injection. At the time-point of 24 h, the control group was also prepared. The nuclear extract and microsomal fraction of each group were prepared as previously described [138].

To examine the suppressive effects of tea on MC-induced AhR transformation and its downstream *in vivo*, another series of 18 rats were at random divided into green tea (GT), black tea (BT), and water (control) groups (6 each). GT or BT was prepared by subjecting 3 g of each tea leaves to extraction with 150 ml of hot water for 5 min, followed by cooling to room temperature. The rats were given freshly prepared GT, BT, or tap water *ad libitum* daily for 8 days. On day 7, the rats were received MC (10 mg/kg body weight) or corn oil (1 ml/kg body weight) as a vehicle control through the intraperitoneal injection (3 each), and they were then sacrificed 2 or 24 h after MC-injection, and the serum and liver were obtained. For the estimation of AhR transformation by electrophoretic mobility shift assay (EMSA) and southwestern chemistry-based enzyme-linked immunosorbent assay (SW-ELISA) and for the detection of translocated AhR by Western blot analysis, the nuclear extract and post-nuclear fraction was prepared from the rat liver of 2 h-treatment groups as previously described [138]. For the measurement of CYP1A1 expression and 7-ethoxyresorufin-*O*-deethylase (EROD) activity, the microsomal fraction was prepared

from the rat liver of 24 h-treatment groups as previously described [139].

For *ex vivo* experiments, rats were given green tea or tap water *ad libitum* for 2 weeks. The livers from rats were subjected to the preparation of the cytosolic fraction as described in Chapter 2 [35]. The cytosolic fraction was treated with 0, 0.05, 0.1, and 0.5 nM TCDD at 20°C for 2 h and evaluated AhR transformation by EMSA as follows.

6.2.3. EMSA

Transformed AhR was evaluated by EMSA using the hepatic nuclear extract or the incubated cytosolic fraction in the manner described in Chapter 2 [35]. Briefly, 10 μg protein of the nuclear extract or the incubated cytosol fraction was further incubated with 250 ng of poly[dI-dC] in HEDG buffer and the ^{32}P-labeled DRE (30 kcpm, 10 fmol) for 15 min. The mixture was then loaded onto a 6% or 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 detected by autoradiography.

6.2.4. SW-ELISA

To determine the transformed AhR, SW-ELISA was also carried out as described in Chapter 3 [55]. Briefly, the reaction mixture consisted of 12.5 μl of nuclear extract containing 12.5 μg protein and 37.5 μl of HEDG buffer. The reaction mixture was plated into a 96-well microtiter plate (Maxisorp; Nalge Nunc International) coated with anti-FITC antibody (DakoCytomation) as capturing antibody and FITC-labeled DRE probe. After successive incubation with specific antibody against ARNT (anti-ARNT C-19; Santa Cruz Biotechnology), biotinylated secondary antibody (Jackson Immuno Research Lab.), and peroxidase-conjugated streptavidin (DakoCytomation), bound peroxidase activity was visualized with tetramethylbenzidin (DakoCytomation), and color development was stopped by addition of 0.5 M sulfuric acid. Transformed AhR was quantified by measuring absorbance at 450 nm using Wallac ARVO sx multilabel counter (Perkin-Elmer Life Science).

6.2.5. Western blot analysis

For detection of CYP1As in the microsomal fraction, and of AhR in nuclear extract

and the post-nuclear fraction, aliquots of the microsomal fraction (1 μg protein), nuclear extract (7.5 μg protein), or post-nuclear fraction (2.5 μg protein) were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). After electrophoresis at 20 mA for 2 h, the proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane (Hybond-P, Amersham Pharmacia Biotech) at 50 mA for 90 min. Nonspecific binding was blocked with 5% skim milk in Tris-buffered saline containing 0.05% Tween 20 (TBST) for 1 h. The membrane was then washed with TBST 5 times each for 5 min, and incubated with primary anti-CYP1A1 antibody (Daiichi Pure Chemicals, Tokyo, Japan) or anti-AhR antibody (Affinity BioReagents) in TBST containing 1% skim milk for 1 h. The membrane was washed again under the same conditions and treated with secondary antibody conjugated with horseradish peroxidase (Wako Pure Chemical) in TBST containing 1% skim milk for 30 min. Immune complexes were visualized using ECL plus reagent (Amersham Pharmacia Biotech) according to the manufacturer's instructions.

6.2.6. Measurement of EROD activity

The hepatic microsomal fraction was also subjected to measurement of EROD activity according to the method of Burke *et al.* [140]. Briefly, aliquots of 25 μg protein of the microsomal fraction was added to 0.1 M phosphate buffer (pH 7.4) containing 5 μM (final) 7-ethoxyresorufin as a substrate and 250 μM (final) β-NADPH as a coenzyme. After 20 min of incubation at 37°C, fluorescence was measured with excitation and emission wavelengths of 530 and 585 nm, respectively.

6.2.7. Extraction of catechins and Tfs from the rat serum

To determine the contents of catechins and Tfs in the rat serum, polyphenols were extracted as previously described [141]. Briefly, aliquots of 400 μl of the serum was mixed with 40 μl of ascorbic acid-EDTA solution (0.4 M NaH_2PO_4 buffer containing 20% ascorbic acid-0.1% EDTA, pH 3.6), and a half volume of the mixture was incubated at 37°C for 45 min with 1 U of sulfatase (Sigma Chemical Co.) and 250 U of β-glucuronidase (Sigma Chemical Co.) in 0.4 M phosphate buffer. Another half volume of the mixture was incubated without the enzymes under the same conditions. After

stopping the incubation by adding 1 ml of ethyl acetate, mixed for 4 min and extracted polyphenols. The organic phase (750 μl) obtained by centrifugation at 8,000 rpm for 10 min was transferred to another microtube. After second extraction with 750 μl of ethyl acetate, the combined extract was dried up with nitrogen gas, dissolved in 100 μl of 15% acetonitrile, and subjected to detection of catechins and theaflavins by using high-performance liquid chromatograph-tandem mass spectrometer (LC-MS/MS) as previously described [133].

6.2.8. Instrumental analysis

The contents of catechins and Tfs in GT or BT used in this chapter were determined by high-performance liquid chromatography (HPLC) as previously described [141-143], and shown in Table 6.1. To determine the contents of catechins and Tfs, they were extracted with ethyl acetate from the rat serum of 2 h-treatment groups and analyzed by injection of aliquots of 10 μl into LC-MS/MS, the higher sensitive instrument than HPLC, as described previously [133]. Analytical conditions were as follows: for determination of catechins, Waters 1525μ pump (Waters, Milford, MA) with Quattro Premier Tandem mass detector (Waters); a 150- × 2.1-mm i. d., particle size 3 μm, Atlantis C_{18} column (Waters) maintained at 40°C; mobile phase, methanol as solvent A and 0.1 M acetate buffer as solvent B; gradient program, 0-10 min, 10-50% A; 10-15 min, 50-90% A ; 15-17 min, 90-10% A; flow rate, 0.2 ml/min; and for determination of theaflavins, Acquity UPLC™ system (Waters) with Quattro Premier Tandem mass detector was used; a 50- × 2.1-mm i. d., particle size 1.7 μm, Acquity UPLC™ BEH C_{18} column (Waters) maintained at 40°C; mobile phase, methanol as solvent A and 0.1 M acetate buffer as solvent B; gradient program, 0-0.5 min, 30% A; 0.5-2.5 min, 30-40% A; 2.5-5 min, 40-30% A; and flow rate, 0.4 ml/min.

6.3. Results

6.3.1. MC induces AhR transformation and CYP1As expression in the liver

To confirm the conditions to induce AhR transformation and its downstream

Table 6.1. Contents of catechins and Tfs in BT and GT[a]

Compounds	GT	BT
	(mg/l)	
(+)-C[b]	24.6	22.6
EC	227.5	58.6
GC	58.1	14.5
EGC	811.4	39.1
Cg	11.7	35.7
ECg	141.5	132.2
GCg	26.0	7.6
EGCg	609.7	130.0
Tf	4.9	25.0
Tf3g	8.3	26.7
Tf3′g	7.4	17.9
Tfdg	7.6	30.0

[a]Compounds in GT and BT were detected by HPLC analysis and represented as mg/l in each extract. [b](+)-C, (+)-catechin; EC, (−)-epicatechin; GC, (−)-gallocatechin; EGC, (−)-epigallocatechin; Cg, (−)-catechin gallate; ECg, (−)-Epicatechin gallate; GCg, (−)-gallocatechin gallate; EGCg, (−)-epigallocatechin gallate; Tf, theaflavin; Tf3g, theaflavin-3-gallate; Tf3′g, theaflavin-3′-gallate; Tfdg, theaflavin-3,3′-digallate.

CYP1As expression by MC, the author examined the time-dependent induction of AhR transformation by both EMSA and SW-ELISA, and CYP1As expression by Western blot analysis after intraperitoneal injection of MC (10 mg/kg body weight). As the results, MC-induced AhR transformation reached maximum at 2 h after the injection, and decreased to the control level at 4-6 h, remained unchanged by 24 h (Fig. 6.1, panels A & B). On the other hand, both CYP1A1 and 1A2 expression induced by MC gradually increased by 24 h after the injection. The levels of AhR transformation (data not shown) and CYP1As expression (Fig. 6.1.C) in 24-h control did not alter compared to 0-h control. These results indicate that activation (transformation) of the AhR induced by MC was transient, but its downstream expression of CYP1As increased by 24 h after MC-injection.

6.3.2. Tea suppresses MC-induced AhR transformation, CYP1As expression, and EROD activity in the liver

To reveal the suppressive effects of tea on AhR transformation and subsequent AhR-associated downstream events *in vivo*, the rats were given GT or BT instead of tap

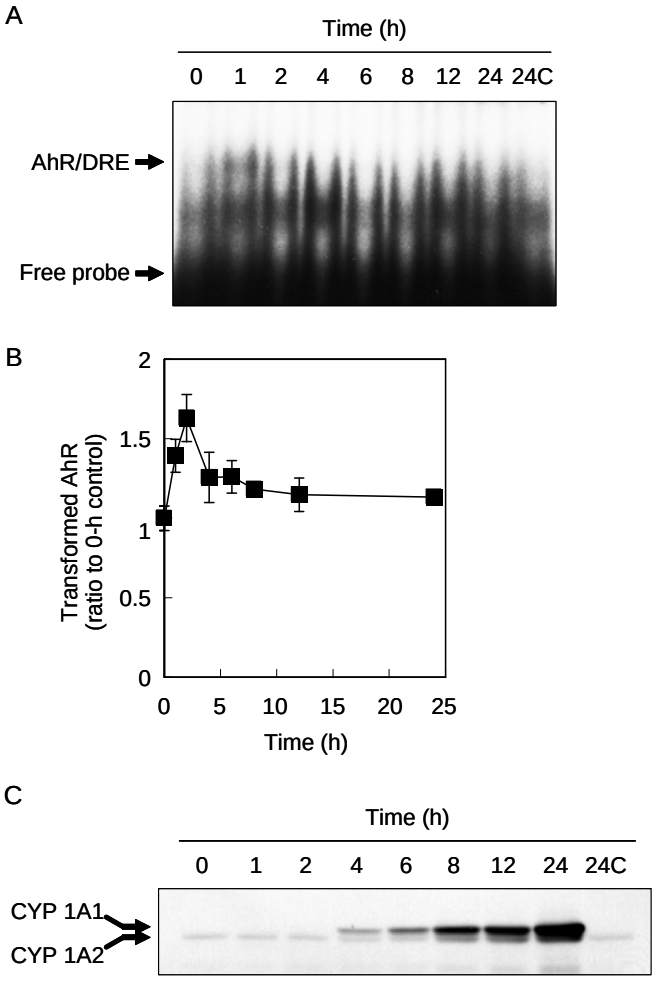


Fig. 6.1. MC induces AhR transformation and CYP1As expression in the rat liver. The livers of the rats 1, 2, 4, 6, 8, 12, and 24 h after intraperitoneal injection of MC (10 mg/kg body weight) or corn oil (1 ml/kg body weight) for 0-h or 24-h controls were subjected to detection of AhR transformation and CYP1As expression as described in Materials and methods. (A) Representative EMSA result, and arrows indicate AhR/DRE complex and free DRE probe, respectively. (B) Transformed AhR was also measured by SW-ELISA, and data are expressed as ratio to 0-h control. (C) CYP1As expression was detected by Western blot analysis.

water for 8 days. There was no significant difference in body weight and the intake amount of diet and beverage during the experimental period (data not shown). As shown in Fig. 6.2.A, the intake of GT or BT almost completely suppressed MC-induced AhR transformation in the liver 2 h after the MC-injection (compare even lanes). To analyze the transformation quantitatively, SW-ELISA was performed and found that the suppressive effects of tea on AhR transformation was statistically significant (Fig. 6.2.B), although tea did not induce the transformation itself. Tea also suppressed AhR-dependent CYP1As expression (Fig. 6.3.A), and significantly suppressed EROD activity, which mainly reflects CYP1A activity, without elevation by tea itself (Fig. 6.3.B). These results indicate that tea contains AhR antagonist(s), and that part of the antagonist(s) is incorporated into the body and suppresses AhR transformation in the liver.

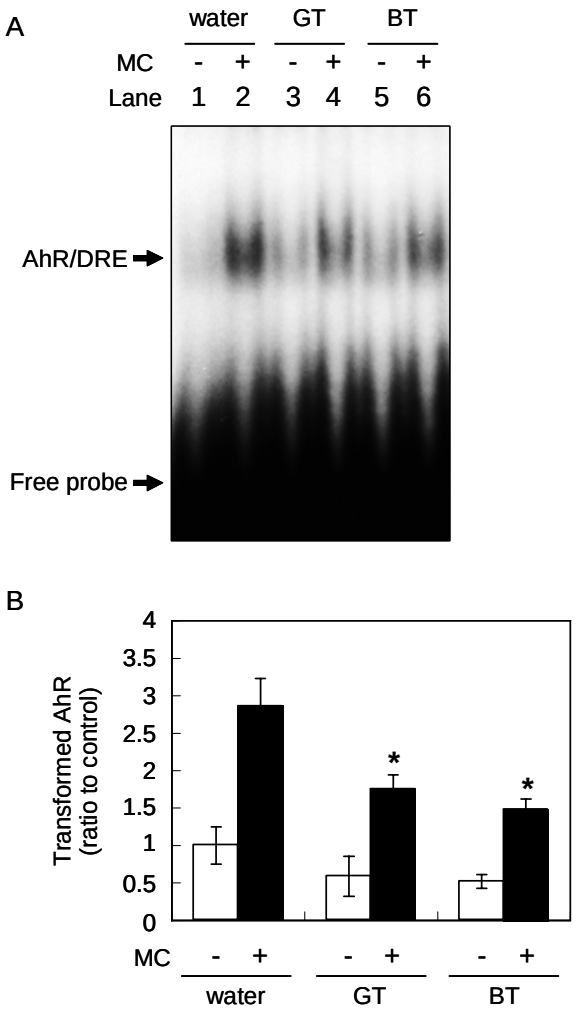


Fig. 6.2. Tea suppresses AhR transformation induced by MC in the rat liver. Rats were given GT, BT, or tap water *ad libitum* for 8 days. On day 7, rats were intraperitoneally injected with MC (10 mg/kg body weight), and the livers 2 h after the injection were subjected to detection of AhR transformation as described in Materials and methods. (A) Representative EMSA result, and arrows indicate AhR/DRE complex and free DRE probe, respectively. (B) Transformed AhR was also measured by SW-ELISA, and data are expressed as ratio to control (open bars) of water groups. Asterisks indicate significant differences ($p < 0.05$, Student's *t*-test) from MC-injected AhR transformation in water groups (n =3).

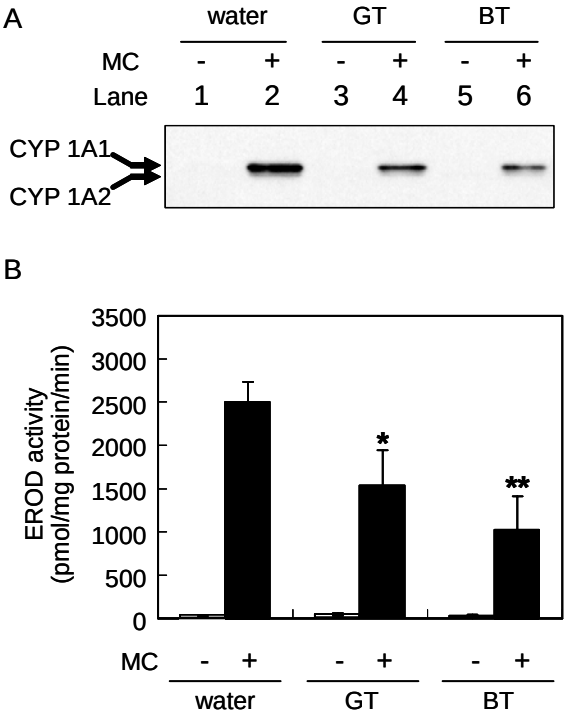


Fig. 6.3. Tea suppresses CYP1As expression (A) and EROD activity (B) induced by MC in the rat liver. Rats were given GT, BT, or tap water *ad libitum* for 8 days. On day 7, rats were intraperitoneally injected with MC (10 mg/kg body weight), and the livers 24 h after the injection were subjected to detection of CYP1As expression and measurement of EROD activity as described in Materials and methods. (A) CYP1As expression was detected by Western blot analysis. (B) Asterisks indicate significant differences ($p < 0.05$ (*), $p < 0.01$ (**), Student's *t*-test) from MC-injected EROD activity in water groups (n =3).

6.3.3. The contents of catechins and Tfs in the serum after ad libitum drinking of tea

In Chapters 4 and 5, the author demonstrates that either GT or BT contains the specific antagonists, catechins and Tfs [43]. Tea catechins were reported to be metabolized to their glucuronated or sulfated forms after absorption [141, 144]. Therefore, the author determined the contents of these compounds as intact and conjugated form in the serum of 2 h-treatment groups. As shown in Table 6.2, a part of catechins and Tfs was absorbed into the body, e.g., 6.139 ± 0.224 ng/ml of (−)-epicatechin (EC) and 0.19 ± 0.009 ng/ml of Tf as total were detected in the serum from GT and BT groups, respectively. Interestingly, the composition of catechins and Tfs in the rat serum did not reflect that in each tea, e.g., (−)-epigallocatechin (EGC), which is the most abundant catechin in GT, exist only total 0.878 ± 0.032 ng/ml in the serum. Moreover, 64% of (−)-epigallocatechin gallate (EGCg) and 74% of Tf were detected as their intact forms, although most of the other catechins exist as their conjugated forms. (−)-Catechin gallate (Cg), theaflavin-3-gallate (Tf3g), theaflavin-3′-gallate (Tf3′g), and theaflavin-3,3′-gallate (Tfdg) in the serum were under the detection limit in this experiment. These results indicate that the components of GT or BT were selectively absorbed into the body, and exist at least in the serum as intact or conjugated form, suggesting that continuous drinking of tea leads elevation of physiological levels of catechins and Tfs, which may contribute to the suppressive effects on AhR transformation and its downstream events in the liver.

6.3.4. Tea suppresses MC-induced AhR translocation into the nucleus in the liver

Before the binding of transformed AhR to DRE site on DNA, the AhR complex with heat shock protein 90 (Hsp90), the X-associated protein 2 (XAP2), and p23 undergoes a conformational change to translocate into the nucleus [18]. In Figs. 6.2 , the author demonstrated that drinking of tea suppressed the binding of transformed AhR to DRE site on DNA. To examine whether tea suppresses AhR translocation into the nucleus, AhR proteins were detected in both the nuclear extract and post-nuclear fraction. As the results, MC induced AhR translocation into nucleus, and this induction was canceled by GT and BT (Fig. 6.4, even lanes). These results indicate that tea component(s) blocks the AhR translocation by inhibiting a conformational change of

the AhR complex in the hepatic cytosol.

Table 6.2. Contents of catechins and Tfs in the rat serum[a]

Compounds	GT			BT		
	total	intact	conjugated	total	intact	conjugated
			(ng/ml)			
(+)-C[b]	3.277 ± 0.119	0.062 ± 0.002	3.215 ± 0.117	ND[c]	ND	ND
EC	6.139 ± 0.224	0.714 ± 0.026	5.425 ± 0.198	ND	ND	ND
GC	0.471 ± 0.017	0.030 ± 0.001	0.441 ± 0.016	ND	ND	ND
EGC	0.878 ± 0.032	0.087 ± 0.003	0.791 ± 0.029	ND	ND	ND
Cg	ud[d]	ud	ud	ND	ND	ND
ECg	1.009 ± 0.037	0.491 ± 0.018	0.518 ± 0.019	ND	ND	ND
GCg	0.140 ± 0.005	0.066 ± 0.003	0.074 ± 0.003	ND	ND	ND
EGCg	5.516 ± 0.201	3.517 ± 0.128	1.999 ± 0.073	ND	ND	ND
Tf	ND	ND	ND	0.190 ± 0.009	0.140 ± 0.018	0.050 ± 0.025
Tf3g	ND	ND	ND	ud	ud	ud
Tf3′g	ND	ND	ND	ud	ud	ud
Tfdg	ND	ND	ND	ud	ud	ud

[a]Contents of catechins and Tfs in the rat serum of GT and BT groups (n =3) were determined by LC-MS/MS and represented as ng/ml in the serum. [b]Abbreviations are the same as shown in Table 6.1. [c]ND, not determined. [d]ud, under the detection limit.

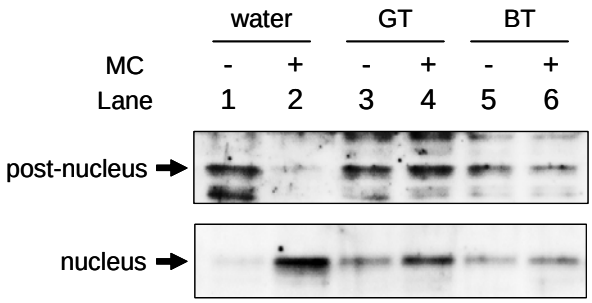


Fig. 6.4. Tea blocks AhR translocation into the nucleus. Rats were given GT, BT, or tap water *ad libitum* for 8 days. On day 7, rats were intraperitoneally injected with MC (10 mg/kg body weight), and the livers 2 h after the injection were subjected to detection of AhR proteins in the post-nucleus (upper panel) and nucleus (lower panel) as described in Materials and methods.

6.3.5. Tea suppresses AhR transformation ex vivo

From *in vivo* experiments, the author revealed that tea suppresses not only AhR transformation but its translocation in the liver. It is, however, not clear that whether active component(s) of tea are absorbed into hepatocytes. The author detected a part of catechins and Tfs in the serum, and it is expected that these antagonistic compounds work in hepatocytes. To prove this hypothesis to be correct, the author examined the suppressive effects of GT on AhR transformation *ex vivo*. The AhR transformation was

not induced by TCDD in the cytosol fraction prepared from the liver of rats of a GT group, although it was induced by TCDD in a dose-dependent manner in that prepared from a water group (Fig. 6.5). These results suggest that a part of the active component(s) was absorbed into hepatocytes, and blocks the binding of ligands to the AhR, leading suppression of AhR translocation and transformation.

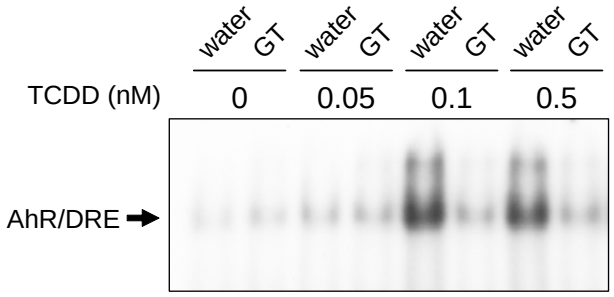


Fig. 6.5. GT suppresses AhR transformation *ex vivo*. Rats were given GT or tap water *ad libitum* for 2 weeks. The liver cytosol fraction was subjected to evaluation of AhR transformation as described in Materials and methods. Arrow indicates AhR/DRE complex.

6.4. Discussion

Since it is important to search for natural antagonists of the AhR from dietary sources, previous reports demonstrated that dietary antagonists show the effects *in vitro* [35-43, 129, 130]. It is, however, not clear yet whether intake of foods containing antagonistic compound(s) suppress AhR transformation *in vivo*. In this chapter, the author clarified that *ad libitum* drinking of tea suppresses AhR transformation, CYP1As expression, and EROD activity induced by MC in the liver. Moreover, a part of catechins and Tfs, which are the antagonists of the AhR exist in GT and BT found in cell-free bioassay, exists in the serum. These results intensively suggest that continuous drinking tea elevates the physiological levels of dietary antagonists of the AhR, leading to suppressing AhR transformation and its downstream events in the liver. However, the actually active components in hepatocytes should be carefully discussed.

The active components contained in GT and BT are catechins and Tfs *in vitro* as described in Chapters 4 and 5 [43]. GT and BT contained 1910.5 and 440.3 mg/l catechins and 28.2 and 99.6 mg/l Tfs, respectively. Other major components in GT and

BT would not contribute to the suppressive effects on AhR transformation. For example, caffeine did not affect the AhR as described in Chapter 4 [43], and thearubigins, which consist 50% of BT polyphenols [57], are seemed to have a low bioavailability due to their high molecules. In this chapter, the author determined the contents of catechins and Tfs in the rat serum, and found that conjugated EC and intact EGCg abundantly exist in the serum of GT groups while only Tf exists in that of BT groups. Previous report demonstrated that *in situ* perfusion of (+)-catechin ((+)-C) into the jejunum and ileum in rats resulted in absorption of 35% of perfused (+)-C into intestinal cells, metabolization into its glucuronide or methylated form in the intestinal cells, and additional methylation or sulfation in the liver [144]. Bharathi *et al.* [145] demonstrated that (−)-epicatechin gallate (ECg) and EGCg rather than EC and (+)-C permeated through human intestinal epithelial Caco-2 cells. The results in this chapter and the previous reports suggest that catechins and Tfs are selected to be absorbed in the intestine, metabolized into its glucuronide, sulfated, or methylated forms in the liver, and a part of these compounds are deconjugated in the liver, and resulted in elevation of the levels of intact or glucuronated/sulfated forms in the serum.

The suppressive effects of catechins and Tfs on the CYP1A1 expression or DRE-driven transcription activity using cell culture-based bioassay have been reported. Tfs suppress CYP1A1 expression induced by omeprazole, a non-AhR ligand, in human hepatoma HepG2 cells [146]. Catechins including EGC, ECg, (−)-gallocatechin (GC), EGCg suppressed TCDD-induced transcription of a DRE-dependent luciferase activity [37]. It was also demonstrated that GT modulates both Phase I and Phase II enzymatic activities in the rat liver [127, 128], and BT components suppress CYP1A1 expression and its activity in the rat intestine [134] *in vivo*. In this chapter, drinking tea suppresses AhR translocation into the nucleus *in vivo* and AhR transformation *ex vivo*. These results indicate that the antagonists of the AhR in tea are absorbed in hepatocytes, and catechins and Tfs are candidates for the dietary antagonists.

In human studies, it was reported that the intake of 525 mg of EGCg resulted in the plasma concentration of 4.4 μM [81] and the intake of 700 mg of Tfs resulted in the plasma concentration of 1.0 μg/l and a concentration of 4.2 μg/l in the urine [133]. Although it was reported that the metabolites of catechins are different between

human and rat [147], continuous drinking tea leads to elevation of the physiological levels of catechins and Tfs as demonstrated in this chapter. In conclusion, continuous drinking tea would suppress the dioxin toxicity through the suppression of the AhR-dependent pathway.

CHAPTER 7

Interaction between the Aryl Hydrocarbon Receptor and its Antagonists, Natural Flavonoids

7.1. Introduction

Since it is important to search for natural antagonists of the aryl hydrocarbon receptor (AhR), the author and others demonstrated that certain pigments exist in plant foods can suppress AhR transformation and its subsequent downstream events [35-43, 129, 130]. In Chapters 2-5, the author demonstrates that flavonoids including catechins and theaflavins, lutein, and chlorophylls act as antagonists of the AhR *in vitro*. In addition, in Chapter 6, the author reveals that tea, which abundantly contains catechins and theaflavins, suppresses 3-methylcholanthrene (MC)-induced AhR translocation and its transformation, and CYP1As expression and its activity, although tea itself does not activate the AhR-dependent transduction *in vivo*. These results suggest that dietary antagonists bind to the AhR, and inhibit specific binding of agonists to the AhR.

Previous reports demonstrated that galangin [103] and synthetic retinoid [115] inhibit binding of 2,3,7,8-tetarachlorodibenzo-*p*-dioxin (TCDD) to the AhR *in vitro*, however, it is not clear yet whether other natural flavonoids including (−)-epigallocatechin gallate (EGCg) bind to the AhR. In this chapter, the author shows that natural flavonoids inhibit specific binding of MC to the AhR in a dose-dependent manner. In addition, the author demonstrates the interaction between EGCg and the AhR by development of new detection system using surface plasmon resonance (SPR) analysis. Findings in this chapter provide the possibility that certain dietary flavonoids can bind to the AhR and competitively inhibit binding of agonists to the receptor protein.

7.2. Materials and methods

7.2.1. Materials

[^{3}H]MC (1.9 Ci/mmol) was obtained from Moravek Biochemicals, Inc. (Brea, CA). Natural flavonoids and 2,3,7,8-tetrachlorodibenzofuran (TCDF) were purchased from the same companies as described in Chapters 2 and 3, respectively [35, 55]. All other reagents used were of the highest grade available from commercial sources.

7.2.2. Preparation of the cytosol fraction from rats

Livers form male Sprague-Dawley rats (6 weeks old, 140-170 g, obtained from Japan SLC) were subjected to preparation of the cytosol fraction as described in Chapter 2 [35]. For AhR ligand binding analysis using hydroxylapatite (HAP), the livers were homogenized with HEDG buffer (25 mM HEPES of pH 7.4, 1.5 mM EDTA, 1.0 mM DTT, 10% glycerol), and buffer A (10 mM HEPES of pH 7.4, 0.15 M NaCl) was used for SPR analysis.

7.2.3. AhR ligand binding analysis

Specific binding of [^{3}H]MC to the AhR was determined by ligand binding assay using HAP as previously described [148]. Briefly, the cytosol fraction (2 mg protein/ml) was incubated with 0.083-2.5 nM [^{3}H]MC at 20^{o}C for 2 h in 2 ml of HEDG buffer. Non-specific binding was defined by incubating the cytosol with [^{3}H]MC in the presence of 200-fold molar excess of TCDF. Aliquots of 250 μl of the reaction mixture was transferred to the scintillation vial to measure total [^{3}H]MC. Remained aliquots of 500 μl of the mixture was incubated with 300 μl of HAP, which was suspended in double volume of HEDG buffer, at 4^{o}C for 30 min. Incubation was stopped by the washing process with 1 ml of HEDG buffer containing 0.5% Tween 80 for 5 times, and the final precipitation of HAP mixture was transferred to the scintillation vials with 2 ml of ethanol and 7 ml of scintillation cocktail to measure [^{3}H]MC-bound AhR.

The binding of natural flavonoids to the AhR in rat liver cytosol was measured by determining the ability of flavonoids to compete with [^{3}H]MC for specific binding. The

cytosol fraction was pretreated with 0.5 nM-1 μM natural flavonoids 10 min prior to addition of 0.25 nM [^{3}H]MC, and the inhibitory effects were represented as % of specific binding.

7.2.4. SPR analysis

To detect the interaction between EGCg and the AhR, SPR analysis was performed by using Biacore 3000 model (Biacore AB, Uppsala, Sweden). A CM5 sensor chip (research grade, Biacore AB) was coated with anti-AhR antibody (Santa Cruz) or Rabbit IgG (Wako Pure Chemicals) as a negative control by using an amine coupling kit following the manufacturer's protocol (Biacore AB). Aliquots of 10 μl of the rat liver cytosol, which was diluted with buffer B (10 mM HEPES of pH 7.4, 3.4 mM EDTA, 0.15 M NaCl, 0.005% Tween 20) to 3.8 mg protein/ml, was injected at a flow of 5 μl/min in buffer B. After the reaction reached to equilibrium, 30 μl of 20-60 μM EGCg dissolved in buffer B was injected at a flow rate of 15 μl/min in the same running buffer. All reaction was carried out at 25^{o}C. Sensorgrams were recorded and normalized to a baseline of 0 resonance units (RU).

7.3. Results

7.3.1. Binding of MC to cytosolic AhR

AhR ligand binding analysis using HAP demonstrated that [^{3}H]MC bound to cytosolic AhR in a dose-dependent manner from 0.083 to 2.5 nM (Fig. 7.1.A). Scatchard plot of the same data indicated that [^{3}H]MC specifically bound to cytosolic AhR, and the dissociation constant (K_d) value and maximum binding capacity (B_{max}) were calculated as 0.14 ± 0.02 nM and 17.3 ± 3.6 fmol/mg protein, respectively (Fig. 7.1.B). The author used 0.25 nM [^{3}H]MC, a median concentration-point of the plot, to determine the competitive effects of natural flavonoids in the following experiments.

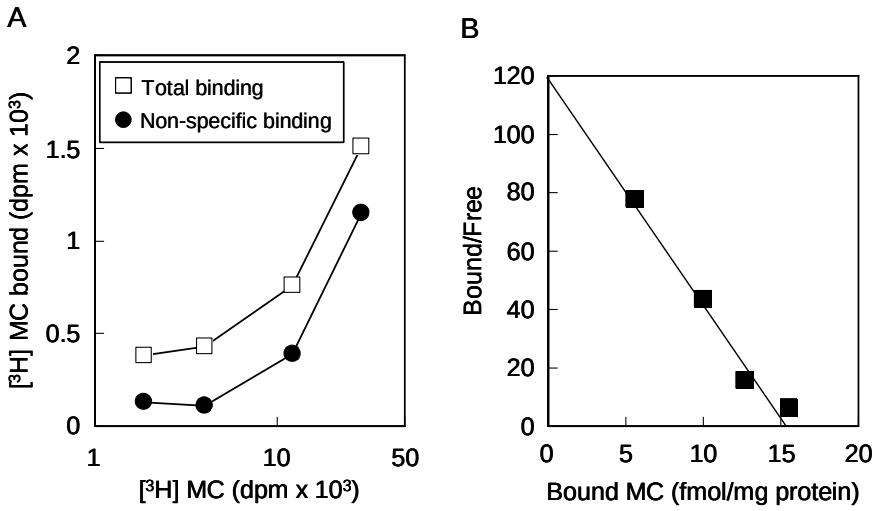


Fig. 7.1. [³H]MC binds to the cytosolic AhR in a dose-dependent manner. Rat hepatic cytosol was incubated with [³H]MC, and total, non-specific, and specific binding of [³H]MC to the AhR were determined as described in Materials and methods. (A) Representative data of total and non-specific [³H]MC binding to the cytosolic AhR. (B) Representative Scatchard plot of [³H]MC binding to the cytosolic AhR from the same data of (A).

7.3.2. Natural flavonoids inhibit specific binding of MC to cytosolic AhR

In Chapters 2 and 3, the author indicates that natural flavonoids suppress AhR transformation in cell-free bioassay, and the effects are suggested to be revealed by inhibition of TCDD binding to the AhR [35, 55]. To clarify this hypothesis, the author examined whether 5 kinds of natural flavonoids, i.e., flavone, apigenin, galangin, kaempferol, and quercetin, inhibit specific binding of MC to the AhR. As shown in Fig. 7.2, all flavonoids at 1 μM inhibited the specific binding, and especially flavone, galangin, and quercetin strongly inhibited to the lower level of non-specific binding. Moreover, kaempferol, quercetin, and galangin inhibited specific binding of 0.25 nM [³H]MC to the AhR in a dose-dependent manner with the IC_{50} values of 295, 25.7, and 20.9 nM, and the inhibitory constant (K_i) values of 106, 9.21, and 7.49 nM, respectively (Fig. 7.3). These results indicate that natural flavonoids have an ability to bind to the AhR, and that they competitively inhibit binding of other agonists to the receptor protein.

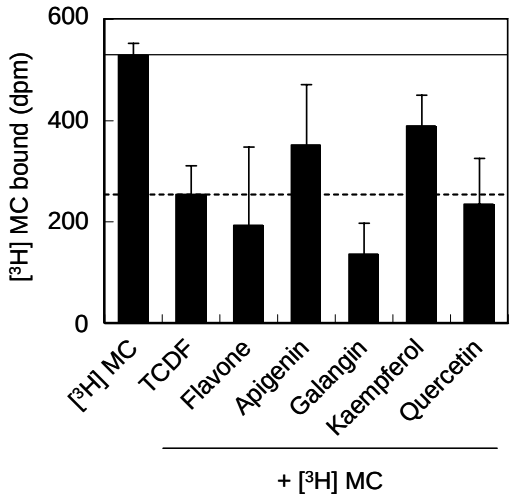


Fig. 7.2. Natural flavonoids inhibit [³H]MC binding to the AhR. Rat hepatic cytosol was pretreated with 1 μM natural flavonoids 10 min prior to addition of 0.25 nM [³H]MC. For determination of non-specific binding, 200-fold molar excess of TCDF was added to the cytosol before addition of [³H]MC. Solid and broken lines indicate total and non-specific binding, respectively.

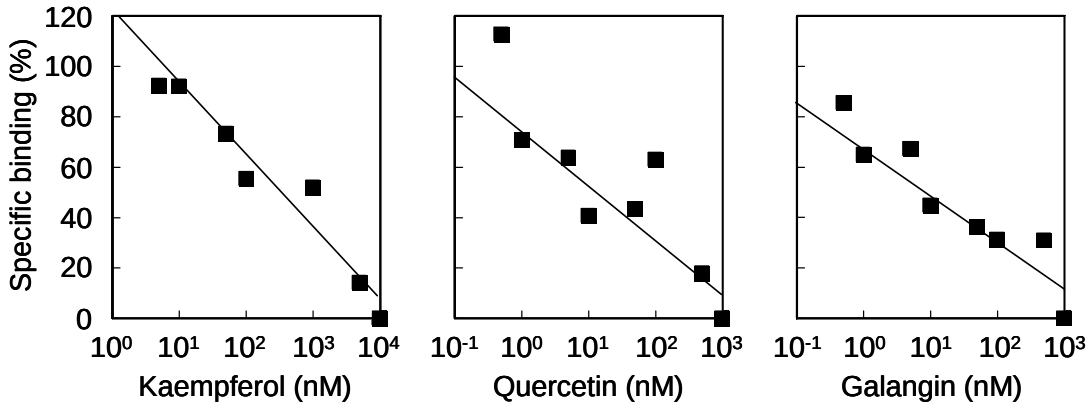


Fig. 7.3. Natural flavonoids inhibit specific binding of [³H]MC to the AhR. Rat hepatic cytosol was pretreated with indicated concentrations of flavonoids, and the inhibitory effects of flavonoids on [³H]MC binding to the AhR were indicated as % of specific binding as described in Materials and methods.

7.3.3. Detection of interaction between EGCg and cytosolic AhR by SPR analysis

In Chapters 2 and 4, the author demonstrates that EGCg also can be a candidate for the dietary antagonist of the AhR [35, 43]. Therefore, the author developed following the detection system for estimation of interaction between EGCg and cytosolic AhR by SPR analysis using anti-AhR antibody or rabbit IgG as negative control coated CM5 sensor chip. As shown Fig. 7.4.A, the component(s) contained in the cytosol fraction (3.8 mg/ml) bound to anti-AhR antibody rather than rabbit IgG. Following injection of 30 μl of 60 μM EGCg resulted in an increase of binding to anti-AhR antibody/cytosol

(Fig. 7.4.B). Binding of EGCg to anti-AhR antibody/cytosol was elevated in a dose-dependent manner (Fig. 7.5).

These results indicate that EGCg interact with the AhR in the cytosol fraction. It is, therefore, suggested that the suppressive effects of EGCg on AhR transformation is due to inhibition of binding of agonists to the AhR by binding of EGCg to the receptor proteins.

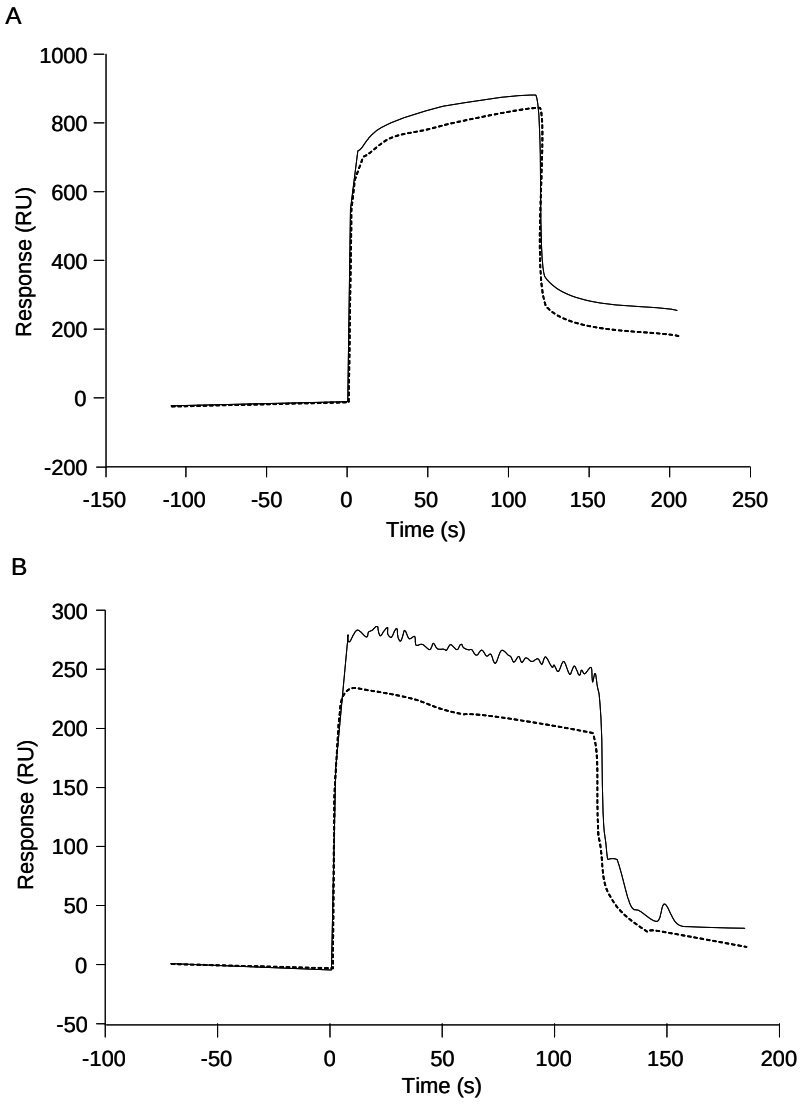


Fig. 7.4. EGCg interacts with cytosolic AhR. Interaction between EGCg and cytosolic AhR was analyzed by SPR using a CM5 sensor chip. Sensorgrams after injection of (A) the rat hepatic cytosol fraction followed by (B) EGCg on the anti-AhR antibody (solid line) or rabbit IgG (broken line) coated CM5 sensor chip.

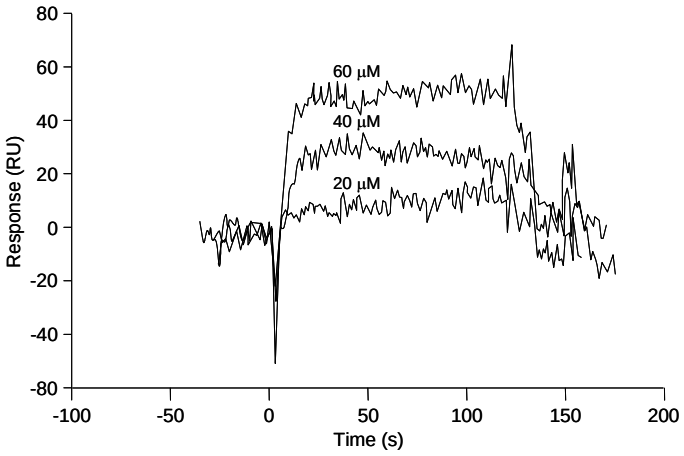


Fig. 7.5. EGCg interacts with the AhR in a dose-dependent manner. Sensorgrams shown are the interaction between indicated concentrations of EGCg and cytosolic AhR.

7.4. Discussion

In Chapter 2, the author demonstrated that natural flavonoids including flavones, flavonols, and catechins suppress AhR transformation in cell-free bioassay using the rat hepatic cytosol fraction [35]. In this chapter, the author investigated whether natural flavonoids inhibit [^{3}H]MC binding to the AhR by ligand binding analysis using HAP. AhR ligand binding analysis performed in this chapter gave K_d value of MC as 0.14 ± 0.02 nM, and this result is consistent with the previous report [148], which determined K_d value of TCDD as 0.12 ± 0.03 nM under the similar experimental conditions. These results indicate that this AhR ligand binding analysis using HAP is available to estimate the inhibitory effects of natural flavonoids on [^{3}H]MC binding to the AhR.

The results in this chapter demonstrate that natural flavonoids inhibit specific [^{3}H]MC binding to the AhR by around 0-50%. The suppressive effects of flavones, flavonols, and flavanones on AhR transformation increase in inverse proportion to their numbers of hydroxyl groups [35]. The numbers of hydroxyl groups of galangin, quercetin, and kaempferol are 3, 4, and 5, respectively. Their inhibitory effects on [^{3}H]MC binding to the AhR were dose-dependent, and ranked in order of galangin > quercetin > kaempferol as the same as that on TCDD-induced AhR transformation as described in Chapter 2 [35]. These results suggest that natural flavonoids suppress AhR transformation by competitively inhibit binding of agonist to the AhR.

The interaction between EGCg and the AhR contained in the cytosol fraction was

also examined by SPR analysis. In this chapter, the author found that 20- 60 μM EGCg binds to anti-AhR antibody/cytosol complex on a CM5 sensor chip in a dose-dependent manner. Recently, it was reported that EGCg binds to 67-kDa laminin receptor (67 LR), which expressed on the surface of cancer cells, with K_d value of 39.9 nM [149]. The binding affinity of EGCg to the receptor proteins differ over 1000-fold, and this difference might be due to the purity of the sample, i.e., the rat hepatic cytosol was used in this chapter, and the purified 67 LR after vector transfection and expression was used in the previous report [149]. It was also demonstrated that EGCg competitively inhibit binding of estradiol to its receptor, estrogen receptor (ER) [150]. Although the binding affinity of EGCg to the ER is significantly weaker than phyto-estrogens such as genistein, daidzein, and coumesterol, the results in this chapter and previous reports suggest that EGCg can interact with various receptors including the AhR.

Natural flavonoids compete with [^{3}H]MC for the AhR in a dose-dependent manner, and the experiments in this chapter are the first approach to indicate that dietary antagonists act as competitive antagonists of the AhR. In conclusion, natural flavonoids suppress AhR transformation by inhibiting agonists binding to the receptor protein.

CHAPTER 8

General Discussion and Conclusion

8.1. Strategy to suppress the dioxin toxicity

Dioxins, environmental contaminants, are serious health concerns because of their resistance to decomposition and toxicological effects [reviewed in 1, 2]. Dioxins are biomagnified through the food chain, and over 90% of human exposure to dioxins is estimated to occur through the diet [4]. It is, therefore, difficult to defense against dioxins by certain medicines, and important to search the steps to reduce the dioxin risk. Previous reports demonstrated that chlorophylls contained in *Chlorella* inhibit absorption and accelerate excretion of dioxins in rats [117] and in human intestinal Caco-2 cells [118]. However, it is not clear whether they show the effects even after dioxins invaded the body.

Dioxins express various adverse effects such as body weight loss, cancer promotion, immunosuppression, and birth defects in animals [reviewed in 5], and chloracne, increasing risk of cancers, and neurodevelopmental delays in humans [4]. These adverse effects of dioxins are mainly mediated by the aryl hydrocarbon receptor (AhR) [10-12], i.e., following binding of agonists to the AhR, the AhR translocates into the nucleus, and act as a transcriptional factor. These sequential actions of the AhR are called as 'transformation', and lead the expression of various proteins including drug metabolizing enzymes such as cytochrome P4501A1 (CYP1A1) [29-31]. Transformed AhR also disrupts intracellular signal transduction by changing the phosphorylation state of several regulatory proteins [32]. Because AhR transformation is the initial step in the development of dioxin toxicity, suppressing the transformation would protect against to the toxicity.

8.2. Flavonoids at dietary levels suppress AhR transformation

The AhR has been found to favor lipophilic compounds which have van der Waals dimensions of 14 × 12 × 5 Å [15-18]. Flavonoids have the similar characteristics, and abundantly exist in plant foods [62, 63]. In Chapter 2, the author determined the IC_{50} values of 34 natural flavonoids against AhR transformation induced by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, the most toxic compound among dioxin congeners, in the cell-free bioassay using rat hepatic cytosol [35]. The results are that the suppressive effects of tested flavonoids are ranked in the order of flavones and flavonols > flavanones > catechins >> isoflavones. Of these, flavones and flavonols suppress AhR transformation at dietary levels, suggesting that they have a potency to protect against the dioxin toxicity through suppression of AhR transformation in the body.

8.3. Development of detection of AhR transformation

To estimate the dioxin risk, the more rapid, low-cost, and sensitive methods to detect AhR transformation are required. In Chapter 3, the author developed a new ELISA system based on southwestern chemical technique (SW-ELISA), which can quantitatively detect AhR transformation [55]. The minimum detection limit of TCDD in SW-ELISA is 2 pM (0.026 pg/assay). This detection limit is almost the same as that of the most sensitive cultured cell-based bioassay [50]. When the correlation coefficient between toxic equivalency factor (TEF) determined by World Health Organization (WHO) [89] and the induction response dose at 50% of that of TCDD was calculated for 12 dioxin-congeners and 6 dibenzofurans, a significant value (r=0.754) was obtained. Moreover, SW-ELISA is also applicable to the screening of antagonists, natural flavonoids. When the antagonistic effects of 9 flavonoids were estimated, the results of SW-ELISA closely correlated with those of gel retardation assay with a significant correlation coefficient (r=0.935). Thus, developed SW-ELISA is a useful tool for estimation of the biological activity of both agonists and antagonists.

8.4. Identification of novel antagonists isolated from tea leaves

Recently, numerous reports by the author and others demonstrated that components exist in plant foods such as flavonoids [35-38], resveratrol [39], curcumin [40], and certain vegetable constituents [41, 42] act as antagonists of the AhR *in vitro*. The results from these reports strongly suggest that plant foods have a possibility to contain novel antagonists of the AhR. In Chapter 4, the author identified lutein and chlorophyll a and b as novel antagonists of the AhR in green tea leaves, and the IC_{50} values against 0.1 nM TCDD-induced AhR transformation were 3.2, 5.0, and 5.9 μM, respectively [43]. These novel antagonists widely distribute in most plants. Thus, intake of plant foods will be effective to suppression of dioxin toxicity. In addition, the author isolated and identified theaflavins (Tfs) from black tea leaves as strong antagonists of the AhR as flavones and flavonols, with the IC_{50} values of 0.7-4.5 μM against 1 nM TCDD-induced transformation in Chapter 5. Tfs are products of oxidation and condensation reactions of catechins, of which antagonistic activity is mentioned in Chapters 2 and 4, and are contained in not only black tea but green tea (see Chapter 6). Therefore, tea is the most intensive and attractive plant food (beverage) for reduction of the dioxin toxicity, because it abundantly contains various types of antagonists.

8.5. Tea suppresses the dioxin toxicity

Numerous antagonists exist in food plants as mentioned above. However, their effects were evaluated in cell-free or cell culture-based bioassays. In Chapter 6, the author demonstrated that tea suppresses AhR translocation into the nucleus, binding of transformed AhR to dioxin responsive element, and its downstream events, CYP1As expression and its activity, which are induced by 3-methylcholanthrene (MC) *in vivo*. Tea also suppresses AhR transformation *ex vivo*, and a part of antagonists of the AhR exists in the serum. These findings indicate that active component(s) contained in tea

are absorbed and show the effects in the body, and that catechins and Tfs are candidates for the dietary antagonists of the AhR. From the findings in this chapter and previous ones, the author would like to make a proposal that continuous drinking tea will suppress the dioxin toxicity.

8.6. Interaction of food factors and the AhR

Finally, the author investigated the mechanistic action of dietary antagonists, natural flavonoids in Chapter 7. AhR ligand binding analysis revealed that natural flavonoids including kaempferol, quercetin, and galangin competitively inhibit binding of [^{3}H]MC to the cytosolic AhR in a dose-dependent manner. Moreover, interaction of (−)-epigallocatechin gallate (EGCg) to the AhR was detected by surface plasmon resonance analysis. These findings indicate natural flavonoids bind to the AhR and act as competitive antagonists. Therefore, it is suggested that these dietary antagonists suppress AhR transformation by inhibiting the binding of agonists to the AhR.

8.7. Conclusion

In this dissertation, the author demonstrates that plant pigments such as flavones, flavonols, catechins, lutein, chlorophylls, and Tfs act as dietary antagonists of the AhR. It is difficult to propose what is the best food for suppression of the dioxin toxicity, because these dietary antagonists abundantly present in common plant foods. However, tea is a typical plant food for suppression of the dioxin toxicity, and the author demonstrates continuous drinking tea suppresses AhR transformation and its downstream events *in vivo* in Chapter 6. However, dietary antagonists have a short life span in the body compared to dioxins, e.g., flavonols are excreted around 24 h after injection [124], while a half-life for TCDD is estimated as 7.5 years [4]. Therefore, ‘continuous’ intake of various plant foods leads to increase in the physiological level of dietary antagonists and contributes to the suppression of the dioxin toxicity.

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List of Publications

(1) Original papers

[1] Hitoshi Ashida, **Itsuko Fukuda**, Takatoshi Yamashita, and Kazuki Kanazawa. Flavones and flavonols at dietary levels inhibit a transformation of aryl hydrocarbon receptor induced by dioxin. *FEBS Lett.* 476(3), 213-217 (Jul 2000).

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