# Studies on suppression mechanism of flavonoid on transformation of an aryl hydrocarbon receptor

Mukai, Rie

# Doctoral Dissertation

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フラボノイドによるアリール炭化水素受容体の形質転換抑制機構に関する研究

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# Contents

## Abbreviations

Abs, absorbance

AhR, aryl hydrocarbon receptor

AhRc, aryl hydrocarbon receptor complex

ARE, antioxidant response element

Arnt, AhR nuclear translocator

bHLH, basic helix-loop-helix

β-NF, β-naphthoflavone

BSA, bovine serum albumin

CA2, cinnamtannin A2

CA, caffeic acid

CPE, cacao polyphenol extract

Cy, cyanidin

Cy 3,5-diglc, cyanidin 3,5-diglucoside

Cy 3-glc-5-Caf-glc, cyanidin 3-glucoside-5-caffeoylglucoside

Cy 3-glc-5-Caf-glc-Caf-glc, cyanidin 3-glucoside-5-caffeoylglucoside-3′-caffeoylglucoside

Cy 3-glu, cyanidin 3-glucoside

Cy 3-rha-glc, cyanidin 3-rhamnosylglucoside

CYP1A1, cytochrome P4501A1

DBA, 3,4-dimethoxybenzoic acid

DEAE, diethyl-aminoethyl cellulose

diHPAc, 3,4-dihydroxyphenylacetic acid

DMSO, dimethyl sulfoxide

Dp, delphinidin

Dp 3-C-glc-5-Ma-glc, delphinidin 3-coumaroylglucoside-5-malonylglucoside

Dp 3-glc-5-Caf-glc-3′-Caf-glc, delphinidin
3-glucoside-5-caffeoylglucoside-3′-caffeoylglucoside

Dp 3-glc-5-Caf-glc-3′-glc, delphinidin 3-glucoside-5-caffeoylglucoside-3′-glucoside

DPPH, 1,1-diphenyl-2-picrylhydrazyl

DRE, dioxin responsive element

DTT, dithiothreitol

EC, (−)-epicatechin

EDTA, ethylenediaminetetraacetic acid

EGb, *ginkgo biloba* extract

EGC, (−)-epigallocatechin

EGCG, (−)-epigallocatechin gallate

EMSA, electrophoretic mobility shift assay

ERK1/2, extracellular signal-regulated kinase

FA, ferulic acid

GA, gallic acid

GST, glutathione $S$-transferase

HAP, hydroxyapatite

HBA, $p$-hydroxybenzoic acid

HBSS, Hanks' balanced salt solution

HPLC, high performance liquid chromatography

hsp90, heat shock protein 90

$IC_{50}$, 50% inhibitory concentration

LBD, ligand binding domain

$LD_{50}$, 50% lethal dose

MAPK, mitogen-activated protein kinase

MC, 3-methylcholanthrene

MCA, 3'-$O$-methyl-(+)-catechin

mCO, $m$-coumaric acid

MEC, 3'-$O$-methyl (−)-epicatechin

MEK, MAPK/ERK kinase

MEM, minimum essential medium

Met-EGCG, (−)-Epigallocatechin 3-(3''-$O$-methyl) gallate

mHBA, $m$-hydroxybenzoic acid

mHPAc, $m$-hydroxyphenylacetic acid

3M4NF, 3'-methoxy-4'-nitroflavone

mHPP, $m$-hydroxyphenylpropionic acid

MS, mass spectrometry

Mv, malvidin

Mv 3-gal, malvidin 3-galactoside

NES, nuclear export signal

NLS, nuclear localization signal

NP-40, nodidet P-40

Nrf2, nuclear factor erythroid 2-related factor 2

PAS, Per-Arnt-Sim

PB2, procyanidin B2

PB5, procyanidin B5

PBS, phosphate-bufferd saline

PBST, PBS containing 0.05% tween20

PC1, procyanidin C1

PCA, protocatechuic acid

PCB, polychlorobiphenyl

PCDD, polychlorinated dibenzo-*p*-dioxin

PCDF, polychlorinated dibenzofuran

pCO, *p*-coumaric acid

Pg, pelargonidin

Pg 3-C-glc-5-glc, pelargonidin 3-coumaroylglucoside-5-glucoside

Pg 3-C-glc-5-Ma-glc, pelargonidin 3-coumaroylglucoside-5-malonylglucoside

Pg 3-glc, pelargonidin 3-glucoside

P-gp, P-glycoprotein

pHHA, *p*-hydroxyhippuric

PI3K, phosphatidylinositol 3-kinase

PKC, protein kinase C

PMSF, phenylmethylsulfonyl fluoride

Pn, peonidin

Pn 3-glc, peonidin 3-glucoside

QR, NAD(P)H: quinone-oxidoreductase

SDS, sodium dodecyl sulfate

SPR, surface plasmon resonance

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

TAD, transactivation domain

TBA, 3,4,5-trimethoxybenzoic acid

TBST, TBS containing 0.05% tween 20

TCDD, tetrachrolodibenzo-*p*-dioxin

TCDF, tetrachlorodibenzofuran

TFA-MeOH, 50% methanol containing 5% trifluoroacetic acid

TGF, transforming growth factor

VA, vanillic acid

XAP2, hepatitis B virus X-associated protein 2

# Chapter 1. General Introduction

## 1-1 Aryl Hydrocarbon Receptor (AhR)

The AhR, also termed dioxin receptor, is a ligand-dependent transcription factor and a basic helix-loop-helix (bHLH) protein belonging to the Per-Arnt-Sim (PAS) family. The AhR resides in various tissues, especially highly in the liver, lung, thymus and kidney (1). Functional domains and regions within the AhR are shown in Fig.1-1 (2-4): The N-terminus half of AhR contains the bHLH-PAS region. The bHLH region includes the nuclear localization signal (NLS) and the nuclear export signal (NES), and binds to DNA. The PAS domain contains two structural repeats, namely PAS A and PAS B, and the former interacts with the PAS domain of AhR nuclear translocator (Arnt). The C-terminus region of PAS domain possesses ligand binding domain (LBD) and interacts with heat shock protein 90 (hsp90) and hepatitis B virus X-associated protein 2 (XAP2). The C-terminus of AhR includes the transactivation domain (TAD), containing the subdomains (the acidic domain, the glutamine (Q)-rich domain, and the proline (P), serine (S), and threonine (T)-rich domain).
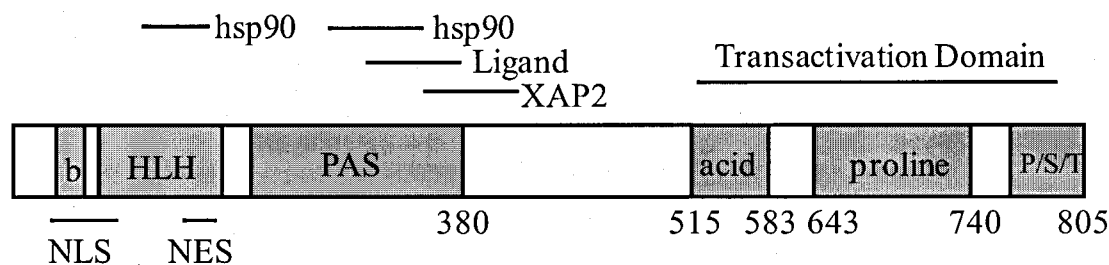


**Fig. 1-1. Functional domains and regions with in the AhR.**
*Abbreviations*; HLH, helix-loop-helix; PAS, Per-Arnt-Sim; hsp90, heat shock protein 90; XAP2, hepatitis B virus X-association protein 2.

## 1-2 The AhR Complex

In the absence of ligand, the AhR located in the cytoplasm forming a complex with two molecules of hsp90 (5,6), XAP2 (7) and p23 (8). Hsp90 plays an important role in the chaperone-associated protein-folding machinery that also prevents degradation of the AhR (9-11). Hsp90 interacts with the AhR in two regions: the bHLH region located in N-terminus and the PAS

domain (12). Since the PAS domain of AhR overlaps ligand binding domain and Arnt dimerization domain, hsp90 is able to suppress the DNA-binding activity of AhR in the absent of ligand (13). XAP2 binds to the PAS domain of AhR (14) and regulates stability of the AhR by preventing its ubiquitination and degradation through the proteasome (15). XAP2 blocks ligand-independent nuclear transport of the AhR (16), by regulates nucleocytoplasmic shuttling by NLS and NES in the AhR. Co-chaperone p23 joins the mature hsp90-AhR complex and is thought to modulate the DNA-binding properties of AhR (8).

**1-3 Physiological Functions of the AhR**

The AhR is essential for maintenance of homeostasis and development of various organs and tissues. The AhR-null mice exhibit the accumulation of retinoids and reduction in the retinoic acid metabolism (17) and TGFβ may be involved in this phenomenon (18). In AhR-deficient animals, the skin lesions (19), progression of cardiac hypertrophy (20), and vascular defects (19,21) were observed. Since the AhR-null female mice have difficulty in maintaining pregnancy, the AhR also affects the development of ovary (22,23). In addition, the liver of AhR-null mice tends to be smaller than that of wild type ones in size and shows portal fibrosis and lipid accumulation (24,25). Thus, the AhR is involved in pleiotropic events, though the conclusive physiological functions have not been defined.

**1-4 AhR Transformation Induced by Ligand**

Ligands of AhR are capable to trigger dissociation of hsp90 and XAP2 from the AhR complex, and nucleartranslocation of the AhR. In the nucleus, the AhR forms heterodimer with Arnt, binds to the specific sites on the chromosome containing an enhancer DNA sequence called DRE, and then activates the transcription of target genes (26-29). These sequential events are combined to be called as the AhR transformation, that leads induction of the various enzymes, such as cytochrome P4501A1 (CYP1A1), glutathione *S*-transferase (GST) Ya subunit, UDP-glucuronocyltransferase, NAD(P)H: quinone-oxidoreductase (QR), and so on (30-32). The AhR transformation also involves phosphorylation of the AhR as well as Arnt (33-37). The heterodimer formation between the AhR and Arnt is shown to require phosphorylation of the Arnt (38). In addition, DNA-binding of the AhR/Arnt heterodimer requires phosphorylation of both

proteins (33,39). It is also known that the phosphorylation statues of some of the tyrosine

residue(s) plays a critical role in the DNA-binding ability of AhR/Arnt heterodimer (37), whereas

phosphorylation at some serine/threonine residue(s) of the AhR and/or Arnt is presumably

involved in transcriptional activation that occur following DNA binding (36).
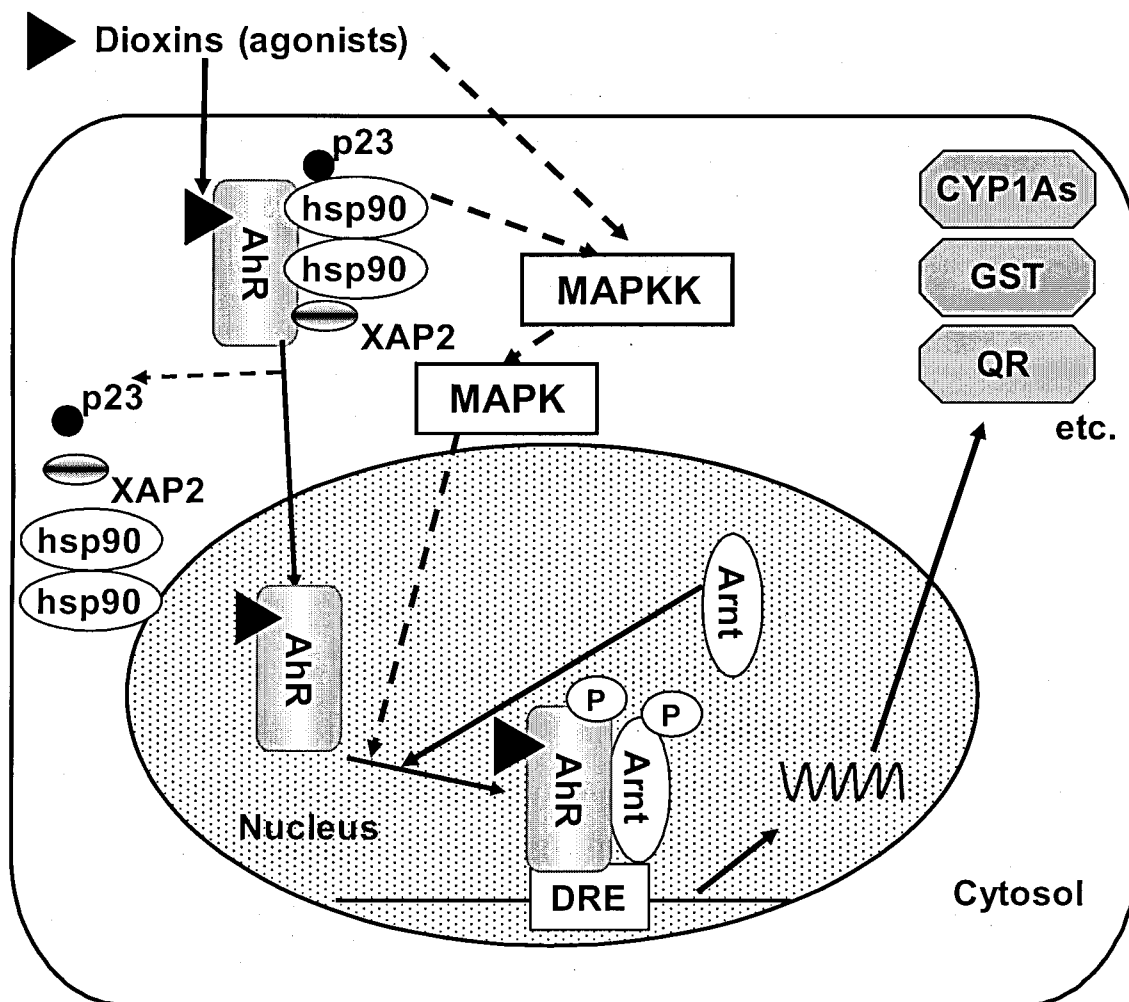


**Fig. 1-2. AhR transformation and its downstream events.**
*Abbreviations*; AhR, aryl hydrocarbon receptor; hsp90, heat shock protein 90; XAP2, hepatitis B virus
X-association protein 2; Arnt, AhR nuclear translocator; DRE, dioxin responsive element; CYP1As,
cytochrome P4501A subfamily; QR, quinone-oxidoreductase; GST, glutathione *S*-transferase; MAPK,
mitogen-activated protein kinase; MAPKK, MAPK kinase

**1-5 Dioxins, the Exogenous Ligands of AhR, and Human Exposure to Dioxins.**

Dioxins are a number of the halogenated aromatic hydrocarbon compounds, comprising

75 polychlorinated dibenzo-*p*-dioxins (PCDDs), 135 polychlorinated dibenzofurans (PCDFs), and

209 polychlorobiphenyls (PCBs) including co-planar PCBs congeners (40,41). Of these, at least 7

PCDDs, 10 PCDFs, and 12 Co-PCBs congeners are known to exhibit toxicological effects (40).

Dioxins are generated as by-products in the incineration of waste, combustion of fossil fuels and wood, manufacturing of chlorophenols and chlorophenoxyl herbicides, and bleaching of paper pulp using free chlorine. They are released into air, soil, and water. Since dioxins are not easily decomposed by acids, basics, heat, and sunlight and are scarcely metabolized by microorganisms, they remain in the environment for decades and accumulated in organisms. After biological concentration through a food chain, dioxins finally invade our body (42). In addition, accidental or occupational exposure also occurs; e.g., an accident at a chemical factory in Seveso in 1976; contaminations in the herbicide during the Vietnam War; and contaminations in food in southern part of the U.S.A. in 1997. In humans, a biological half-life of dioxins is estimated to be over a decade (43).

**1-6 Toxicity of Dioxins**

Some animal studies revealed that susceptibility to 2,3,7,8-tetrachrolodibenzo-*p*-dioxin (TCDD), which is the most toxic compound among dioxins, varies in species, strains, and sexes. Table 1-1 summarizes the 50% lethal dose ($LD_{50}$) values in animals as one of the typical acute toxicities of TCDD (44-47). The $LD_{50}$ values differ for over 8000-fold between male guinea pig (the most sensitive animal strain) and male hamster (the most insensitive one) (44,45), and for over 700-fold between Long-Evans (sensitive strain) and Han/Wister rats (resistant one) (45).

**Table 1-1 The $LD_{50}$ values of TCDD in animals**

| Species | Strain | Sex | $LD_{50}$ value ($\mu$g/kg BW) | Reference |
|---|---|---|---|---|
| Guinea pig | Hartley | Male | 0.6 | (44) |
| Rabbit | New Zealand albino | Mixed | 115.0 | (44) |
| Rat | Long-Evans | Male | 17.7 | (45) |
| Rat | Long-Evans | Female | 9.8 | (45) |
| Rat | Han/Wister | Mixed | >7200.0 | (45) |
| Mouse | C57BL/6J | Male | 182.0 | (46) |
| Mouse | DBA/2J | Male | 2570.0 | (46) |
| Hamster | Golden Syrian | Male | 5051.0 | (47) |

In addition to the lethality, dioxins induce various toxicological effects, such as carcinogenesis, immunosuppression, and body weight loss. Epidemiological studies suggested that concentrations of dioxin in serum are correlated with increment in cancer risk (48). TCDD acts as a carcinogen in various tissues of experimental animals. It was shown either rats, mice, and hamsters that injection of TCDD or other PCDDs caused in various types of cancers in lung, oral/nasal cavities, thyroid, skin and the liver (49,50). Dioxins also influence humoral (51,52) and T-cell mediated immune response including cytokine productions (53) and T-cell differentiation (54). TCDD affects weight of tissues, which concerned with immune response: thymus weight is decreased while spleen increased in mice (55). TCDD causes loss of body weight gain and teratogenicity, such as hydronephrosis and hydrothorax in animals (56). On the other hand, it has been demonstrated that the AhR knockout mice are resistant to these toxic effects of TCDD (57,58). Therefore, the AhR plays an indispensable role in the development of dioxin toxicity (59).

**1-7 The Aim of This Study**

Decomposition of dioxins by the metabolism of microorganisms has been tried for a long time (60), but there obtained few successful results. On the other hand, synthetic and natural compounds that can prevent AhR transformation have been investigated (61-63). Because dioxins invade our bodies mainly through daily diet as mentioned above, the antagonist should be the natural compound from dietary sources. It is now recognized that plant foods are the greatest source of the AhR antagonists (62,64). Various compounds have been identified as the antagonists for AhR (65,66).

It has been demonstrated that certain flavonoids belonging to the subclass of flavone, flavonol, flavanone and catechin, have suppressive effect on the DNA-binding activity of AhR in a cell-free system (63). Anthocyan, a flavonoid subclass, is widely distributed in plant kingdom but its suppressive effect on the DNA-binding activity is unclear yet. In Chapter 2, the author demonstrates that anthocyan does not suppress the DNA-binding activity. The author shows the evidence for the interaction between the AhR and flavonoids using the cell free system in Chapter 3 and that their suppression mechanism on AhR transformation using Hepa-1c1c7 cells in Chapter 4. In the final two chapters, the author confirms the suppressive effect of food extracts containing flavonoids abundantly. Effects of flavonol-rich *ginkgo biloba* extract and its

component kaempferol *in vivo* are shown in Chapter 5, and effects of catechin-rich cacao extract *in vivo* are in Chapter 6.

# Chapter 2. Anthocyans fail to suppress transformation of aryl hydrocarbon receptor induced by dioxin

## 2-1 Introduction

Dioxins, environmental contaminants, induce various adverse effects through the event that they bind to the cytosolic aryl hydrocarbon receptor (AhR), resulting in its transformation (67,68). Transformed AhR works as a transcriptional factor and specifically binds to the dioxin responsive element (DRE), which is located on an enhancer region of drug-metabolizing enzymes, including cytochrome P4501A1, glutathione $S$-transferase (GST), NADP(H): quinone-oxidoreductase (QR) and so on (26,28,32,69). In addition, AhR transformation disrupts intracellular signal transduction by changing the phosphorylation state of several regulatory proteins (70).

Since AhR transformation is the initial step in various adverse effects of dioxins, inhibition of AhR transformation would protect humans from the toxic effects. Because dioxins enter the body mainly through the diet (71,72), it is necessary to search for food factors that possess antagonistic effects against AhR. Previously, others found that flavones and flavonols at dietary levels suppress AhR transformation in a cell-free system (63). Not only flavones and flavonols but also catechins are confirmed as antagonists of AhR (73-75). In addition, other food factors, *viz.*, lutein (66), curcumin(76), resveratrol (77), and certain vegetable constituents (64), act as antagonists of AhR. These results suggest that possible antagonists of AhR are contained in plant-based diets.

Anthocyans are members of the flavonoids widely distributed in vegetables and fruits such as grapes, berries, eggplants, and purple corns, and have been reported to exhibit antioxidative, antimutagenic, and anti-carcinogenic activities (78). The bioavailability of anthocyans has been reported, including their absorption and metabolism in rats and humans (78). After oral intake of cyanidin 3-glucoside (Cy 3-glc), intact Cy 3-glc and/or its metabolite, protocatechuic acid (PCA), was detected in rat plasma (79,80). Acylated anthocyanins are also absorbed, at least in part, into the body after ingestion in rats and humans (81). On the other hand, 2,4-dihydroxybenzoic acid and 2,4,6-trihydroxybenzoic acid, in addition to PCA has been isolated

and identified as degradation products of anthocyans after incubation under physiological conditions (82). These reports suggest that anthocyans are absorbed into the body without hydrolysis by intestinal cells, enter the blood circulation system, and are degraded into PCA in the plasma. PCA is metabolized into related compounds in tissues such as liver. Therefore, both the intact form and metabolites of anthocyans show various biological activities in the body. But it is unclear whether they have suppressive effects on dioxin-induced AhR transformation.

In this chapter, the author examined the stability of anthocyans to determine the conditions for evaluation of their biological effects, and then, the author investigated whether anthocyans can suppress AhR transformation induced by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) in the cell-free system and in mouse hepatoma Hepa-1c1c7 cells.
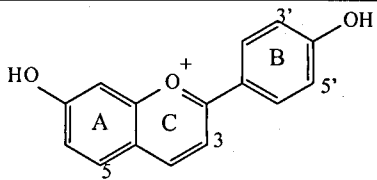
## 2-2 Materials and methods

### 2-2-1 Materials

TCDD was purchased from AccuStandard (New Haven, CT), and dissolved in dimethyl sulfoxide (DMSO). The anthocyans used in this chapter are summarized in Table 2-1. Pelargonidin (Pg), Pg 3-glucoside (Pg 3-glc), cyanidin (Cy), Cy 3-glc, Cy 3-rhamnosylglucoside (Cy 3-rha-glc), Cy 3,5-diglucoside (Cy 3,5-diglc), delphinidin (Dp), peonidin (Pn), Pn 3-glc, malvidin (Mv), Mv 3-glc, Mv 3,5-diglc, and Mv 3-galactoside (Mv 3-gal) were purchased from Extrasynthèse (Genay, France). Pg 3-coumaroylglucoside-5-glucoside (Pg 3-C-glc-5-glc) and Pg 3-coumaroylglucoside-5-malonylglucoside (Pg 3-C-glc-5-Ma-glc) were isolated from *Hyacinthus oreintalis* L. cv. Hollyhock as previously described (83). Cy 3-glucoside-5-caffeoylglucoside (Cy 3-glc-5-Caf-glc), Cy 3-glucoside-5-caffeoylglucoside-3'-caffeoylglucoside (Cy 3-glc-5-Caf-glc-3'-Caf-glc), Dp 3-glucoside-5-caffeoylglucoside-3'-glucoside (Dp 3-glc-5-Caf-glc-3'-glc), and Dp 3-glucoside-5-caffeoylglucoside-3'-caffeoylglucoside (Dp 3-glc-5-Caf-glc-3'-Caf-glc) were isolated from *Gentiana* unnamed cultivar, and Dp 3-coumaroylglucoside-5-malonylglucoside (Dp 3-C-glc-5-Ma-glc) were from *Hyacinthus orientalis* L. cv. Delft Blue, as previously described (83). All anthocyans were dissolved in 50% methanol containing 5% trifluoroacetic acid (TFA-MeOH) or DMSO. All other reagents used were of the highest grade available from commercial sources.

**Table 2-1. Anthocyans used in this chapter**

| Anthocyan | Binding position | | | |
|---|---|---|---|---|
| | 3' | 5' | 3 | 5 |
| Pg | H | H | H | H |
| Pg 3-glc | H | H | glc | H |
| Pg 3-C-glc-5-glc | H | H | C-glc | glc |
| Pg 3-C-glc-5-Ma-glc | H | H | C-glc | Ma-glc |
| Pg 3-glc-5-mMa-glc | H | H | C-glc | mMa-glc |
| Cy | OH | H | H | H |
| Cy 3-glc | OH | H | glc | H |
| Cy 3-rha-glc | OH | H | rha-glc | H |
| Cy 3,5-diglc | OH | H | glc | glc |
| Cy 3-glc-5-Caf-glc | OH | H | glc | Caf-glc |
| Cy 3-glc-5-Caf-glc-3'-Caf-glc | Caf-glc | H | glc | Caf-glc |
| Dp | OH | OH | H | H |
| Dp 3-C-glc-5-Ma-glc | OH | OH | C-glc | Ma-glc |
| Dp 3-glc-5-Caf-glc-3'-glc | OH | OH | glc | Caf-glc |
| Dp 3-glc-5-Caf-glc-3'-Caf-glc | Caf-glc | OH | glc | Caf-glc |
| Pn | Om | OH | H | H |
| Pn 3-glc | Om | OH | glc | H |
| Mv | Om | Om | H | H |
| Mv 3-glc | Om | Om | glc | H |
| Mv 3,5-diglc | Om | Om | glc | glc |
| Mv 3-gal | Om | Om | gal | H |

The structure diagram shows a flavylium skeleton with rings labeled A, C (with $O^+$) and B. Positions marked: 3' and 5' on the B ring, OH at the top of the B ring, HO on the A ring, and positions 3 and 5 labeled.

*Abbreviations*: Pg, pelargonidin; Cy, cyanidin; Dp, delphinidin; Pn, peonidin; Mv, malvidin; glc, glucoside; C, coumaroyl; Ma, malonyl; rha, rhamnosyl; Caf, caffeoyl: m, methyl

## 2-2-2 High performance liquid chromatography (HPLC) analysis

Since anthocyans have two absorption peaks, at 270-280 nm and 510-540 nm, the author analyzed at both wavelengths to detect anthocyans. Cy and Cy 3-glc dissolved in TFA-MeOH or DMSO at a concentration of 5 mM were stored for 4 d at $-20°C$. Each stock solution was diluted with corresponding solvent to 50 µM, and aliquots of 10 µl were immediately injected into the HPLC. Analytical conditions were as follows: Column, Capcell Pak C18, type UG120 (φ 4.6 x 250 mm, Shiseido, Tokyo) maintained at 35°C; mobile phase, 10% formic

acid/0.1% HCl in methanol (80:20, v/v); flow rate, 1 ml/min; monitoring with Hitachi UV-VIS detector L-7420 at 528 nm for detection of Cy and 520 nm for Cy 3-glc. To analyze the other absorption peak at 270 nm, Cy and Cy 3-glc were separately diluted to 250 μM, and aliquots of 10 μl were immediately injected into the HPLC. Elution was performed using 50 mM phosphate buffer (pH 2.1) as solvent A and 0.1% HCl/methanol as solvent B. The gradient system used was as follows: 0-20 min, 95-40% A; 20-25 min, 40-0% A; 25-35 min, 0% A. Other analytical conditions were the same as described above.

### 2-2-3 Measurement of antioxidative activity

To examine the antioxidative activity of anthocyans, the modified 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging method (84) was used. Briefly, 500 μl of 5 μM anthocyans was added to a mixture of 500 μl of 0.1 M acetate buffer (pH 5.5) and 1 ml of ethanol. The reaction mixture was incubated for 30 min with 500 μl of 0.48 mM DPPH solution in ethanol, and absorbance (Abs) at 517 nm was measured. For the positive and negative controls, the same volume of ascorbic acid (50 μM) and distilled water was used instead of anthocyans. To subtract Abs at 517 nm of anthocyans themselves, the reaction mixture without DPPH was also measured. The antioxidative activity of anthocyans was expressed as a percent of that of the positive control.

### 2-2-4 Transformation of the AhR under the cell-free conditions

Animal treatment in this chapter conformed to the "Guidelines for the Care and Use of Experimental Animals, in Rokkodai Campus, Kobe University." The livers of male Sprague-Dawley rats (six weeks old, 140-170 g, purchased from Japan SLC, Shizuoka) were used to prepare the cytosol fraction, as previously described (63). The protein concentration was determined by the Bradford method (85) using bovine serum albumin as a standard protein. Cytosol (15 mg protein/ml) was incubated with 0.1 nM (final) TCDD in HEDG buffer (25 mM HEPES-NaOH, pH 7.4, 1.5 mM ethylenediaminetetraacetic acid [EDTA], 1.0 mM dithiothreitol [DTT], 10% glycerol) at 20°C for 2 h to induce AhR transformation, and with vehicle (10 μl/ml) alone as a negative control. To estimate antagonistic effects, anthocyans were added to the above mixture 10 min prior to the addition of TCDD.

**2-2-5 Culture and treatments of Hepa-1c1c7 cells**

Mouse hepatoma cell line Hepa-1c1c7 cells were grown and maintained at 37°C in α-minimum essential medium (α-MEM, Nissui Pharmaceutical, Tokyo) containing 10% fetal bovine serum (Sigma, St. Louis, MO), 4 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin under a humidified 95% air/5% $CO_2$ condition. The cells were resuspended at a concentration of 2.5 x $10^5$ cells/ml, seeded on 60-mm plastic dishes, and incubated for 48 h. They were washed with phosphate-buffered saline (PBS), incubated in 2 ml of fresh medium, and treated with 0.1 nM (final) TCDD for a further 2 h. To estimate the antagonistic effect, anthocyans were treated to the cells 10 min prior to the addition of TCDD. The cells were washed twice with ice-cold PBS and harvested with ice-cold harvest buffer (20 mM HEPES-NaOH, pH 7.6, 20% glycerol, 10 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.1% nonidet P-40 [NP-40], 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride [PMSF], 5 µg/ml aprotinin, and 20 µg/ml leupeptin) and centrifuged at 1,000 x g for 10 min at 4°C after incubation for 20 min at 4°C. The nuclear pellet was suspended in extraction buffer (20 mM HEPES-NaOH, pH 7.6, 20% glycerol, 500 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.1% NP-40, and the same protease inhibitors as harvest buffer) and rotated for 45 min at 4°C. The nuclear extract was obtained by centrifugation at 20,000 x g for 20 min at 4°C, and stored at −80°C until use. The protein concentration was determined by the Bradford method (85).

**2-2-6 Determination of transformed AhR by southwestern chemistry-based enzyme-linked immunosorbent assay (SW-ELISA)**

To determine the transformed AhR, SW-ELISA was carried out as previously described (86). For SW-ELISA, the reaction mixture consisted of 40 µl of treated cytosol (containing 600 µg protein) and 10 µl of HEDG buffer containing 750 mM KCl (final concentration 150 mM) or 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, Tokyo) coated with anti-FITC antibody (DakoCytomation, Kyoto) as capturing antibody and FITC-labeled DRE probe (5′-GAT CCG GAG TTG CGT GAG AAG AGC CA-3′ and 5′-GAT CTG GCT CTT CTC ACG CAA CTC CG-3′, purchased from Hokkaido System Science,

Sapporo). After successive incubation with specific antibody against ARNT (anti-ARNT C-19, Santa Cruz Biotechnology, Santa Cruz, CA), biotinylated secondary antibody (Jackson Immuno Research Lab., West Grove, PA), and peroxidase-conjugated streptavidin (DakoCytomation), bound peroxidase activity was visualized with tetramethylbenzidin (DakoCytomation), and color development was stopped by the 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 Sciences, Boston, MA). The suppressive effects of anthocyans were evaluated by the ratio of transformed AhR, which was compared to those of both the positive control (0.1 nM TCDD) and the negative one (vehicle alone).

## 2-3 Results

### 2-3-1 Degradation products of cyanidin suppressed AhR transformation

Previously, it was demonstrated that flavones, flavonols (63), and lutein (66) suppress AhR transformation in a cell-free system. To evaluate the suppressive effects of these compounds
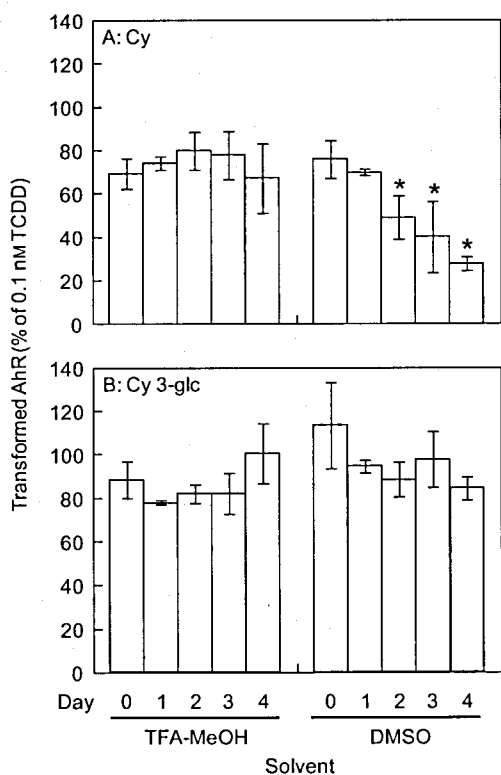
Fig. 2-1. Effect of storage period of Cy and Cy 3-glc on TCDD-induced AhR transformation. Cy (A) and Cy 3-glc (B) were dissolved in TFA-MeOH or DMSO and stored at -20°C for 4 d. The suppressive effect of Cy and Cy 3-glc at 50 $\mu$M on AhR transformation was evaluated by SW-ELISA, as described in Materials and methods. Data are shown as a percent of transformed AhR induced by 0.1 nM TCDD, and represented as the means $\pm$ SD from independent triplicate experiments. Asterisks indicate significant differences ($p < 0.05$, Student's $t$-test) from the corresponding value on day 0.

on transformation, DMSO was used as a solvent. Since anthocyans are sensitive to pH, the suppressive effects of Cy and Cy 3-glc on AhR transformation were examined after they were

dissolved in two solvents, TFA-MeOH (< pH 3) and DMSO, for 4 d. As shown in Fig. 2-1, Cy

and Cy 3-glc dissolved in TFA-MeOH did not suppress transformation. Cy dissolved in DMSO

also did not suppress transformation on day 0, but the suppressive effect became stronger day by

day. On the other hand, the effect of Cy 3-glc dissolved in DMSO on transformation remained

unchanged. These results suggest that Cy dissolved in DMSO was degraded during storage even

at −20°C.

To clarify whether degradation products of Cy exist, HPLC analysis was performed

every day. The HPLC chromatograms obtained at a wavelength of 528 nm revealed that the

content of Cy dissolved in either solvent,

TFA-MeOH (Fig. 2-2A) or DMSO (Fig.

2-2B), decreased day by day. Even on day 0,

the peak area of Cy dissolved in DMSO was

smaller than that in TFA-MeOH. The rest

content of Cy on day 4 was calculated as a

percent of that on day 0, and 64.5% remained

in TFA-MeOH, while only 8.6% remained in

DMSO. The author also monitored the other

absorption peak at 270 nm, and found that the

detectable number of peaks of Cy dissolved in

DMSO increased day by day, compared with

those in TFA-MeOH (Fig. 2-2C and D).

These results suggest that the flavylium

skeleton of anthocyanidins easily broke down

in DMSO. On the other hand, the HPLC

chromatogram of Cy 3-glc dissolved in either

solvent remained unchanged at both 520 nm

and 270 nm (data not shown). These results



Fig. 2-2. HPLC chromatograms of Cy dissolved in
TFA-MeOH or DMSO during the storage period.
Cy dissolved in TFA-MeOH (A, C) or DMSO (B, D)
was analyzed by HPLC at wavelengths of 528 nm (A,
B) and 270 nm (C, D) every day, as described in
Materials and methods.

indicate that Cy, especially when dissolved in DMSO, is unstable for storage even at −20°C,

whereas Cy 3-glc is apparently stable.

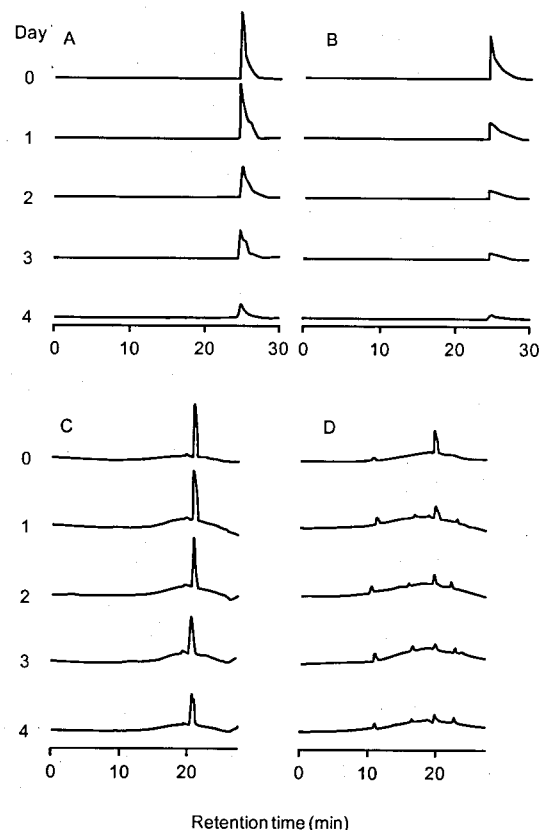Since the degradation products of Cy affected the suppressive effect on AhR

transformation, it is suggested that the bioactivities of anthocyans changed. To confirm the effect of storage period on the bioactivity of anthocyans, the author measured antioxidative activity at the start and end points of the storage period by the DPPH radical scavenging method. On day 4, the antioxidative activity of Cy and Cy 3-glc at 5 mM dissolved in TFA-MeOH decreased significantly to 69% and 84% of the corresponding activity on day 0 respectively, but the activity was still higher than that of 50 μM ascorbic acid (Fig. 2-3). Although no significant difference was observed in the content of Cy 3-glc dissolved in DMSO between day 0 and day 4, the antioxidative activity decreased to 45% on day 4. The antioxidative activity of Cy and Cy 3-glc dissolved in TFA-MeOH was 8.8 and 9.3 times as high respectively as that in DMSO on day 0. These results indicate that the stability of Cy and Cy 3-glc in TFA-MeOH is higher than that in DMSO, and that the stability affects bioactivities, including not only AhR transformation but antioxidative activity. Hence, the author dissolved anthocyans in TFA-MeOH just before use in the following experiments.
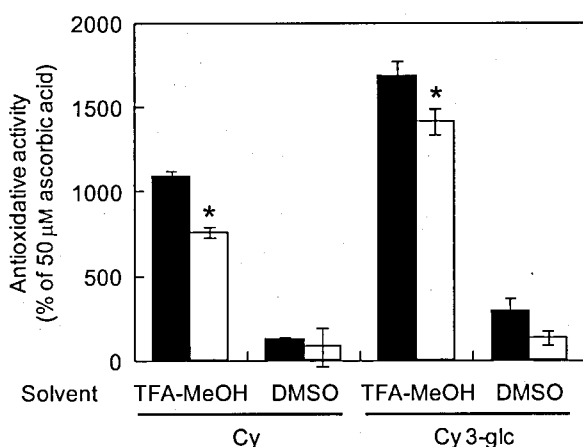


**Fig. 2-3. Effect of storage period of Cy and Cy 3-glc on antioxidative activity.** The antioxidative activity of Cy and Cy 3-glc dissolved in TFA-MeOH or DMSO at 5 mM was measured by the DPPH radical scavenging method on day 0 (closed bars) and day 4 (open bars). Data are shown as a percent of 50 μM ascorbic acid as a positive control, and represented as the means ± SD from independent triplicate experiments. Asterisks indicate significant differences ($p < 0.05$, Student's $t$-test) from the corresponding value on day 0.

### 2-3-2 Suppressive effect of anthocyans on AhR transformation in a cell-free system

Previous reports show that several flavonoids at dietary levels suppress AhR transformation induced by 0.1 or 1 nM TCDD in the cell-free system using rat liver
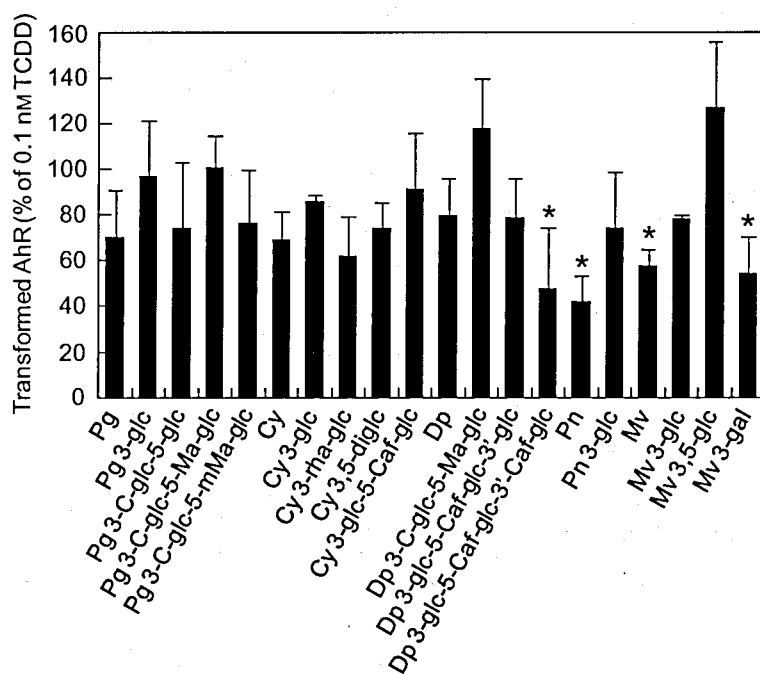
**Fig. 2-4. Suppressive effect of anthocyans on TCDD-induced AhR transformation in the cell-free system.** The suppressive effect of 20 kinds of anthocyans dissolved in TFA-MeOH at 50 μM on TCDD-induced AhR transformation was evaluated in the cell-free system, as described in Fig. 2-1. Data are shown as a percent of transformed AhR induced by 0.1 nM TCDD, and represented as the means ± SD from independent triplicate experiments. Asterisks indicate significant differences ($p < 0.05$, Student's $t$-test) from the value of 0.1 nM TCDD-induced transformation.

cytosol fraction (63,66). To compare the suppressive effects of anthocyans with that of other flavonoids, 20 kinds of anthocyans (50 μM) on AhR transformation induced by 0.1 nM TCDD were measured using the same system as the previous report (63,66). Among the compounds tested here, only 4 anthocyans, Dp 3-glc-5-Caf-glc-3'-Caf-glc, Pn, Mv, and Mv 3-gal significantly suppressed transformation, with suppression levels of 53, 58, 43, and 46% respectively (Fig. 2-4), while no anthocyan itself induced AhR transformation (data not shown). The four efficacious compounds suppressed AhR transformation in a dose-dependent manner, and the 50% inhibitory concentration ($IC_{50}$) values of Dp 3-glc-5-Caf-glc-3'-Caf-glc, Pn, Mv, and Mv 3-gal were determined to be 23, 23, 45, and 51 μM respectively (Fig. 2-5). But no correlation between structure and suppressive effect was observed. These results suggest that the suppressive effect of anthocyans on AhR transformation is not as strong as that of luteolin, quercetin, or kaempferol, whose $IC_{50}$ values against 0.1 nM TCDD-induced AhR transformation were under 1 μM (86).
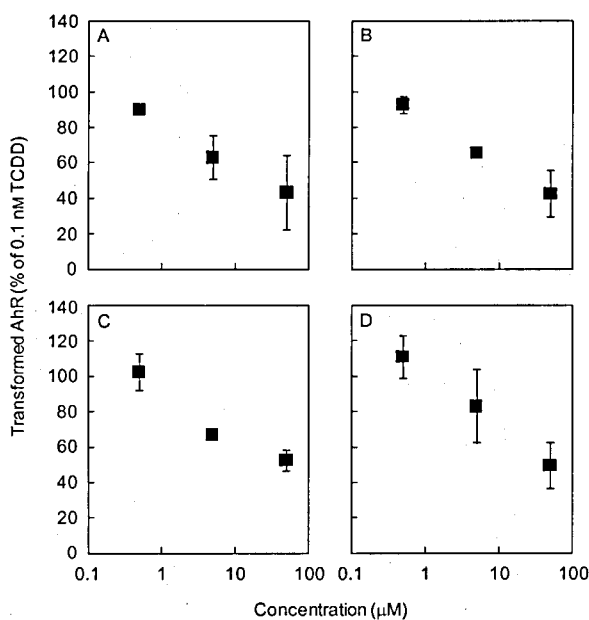
**Fig. 2-5. Dose response of anthocyans on TCDD-induced AhR transformation in the cell-free system.** The suppressive effect of Dp 3-glc-5-Caf-glc-3'-Caf-glc (A), Pn (B), Mv (C), and Mv 3-gal (D) dissolved in TFA-MeOH at the concentrations indicated on TCDD-induced AhR transformation was evaluated as described in Fig. 2-1. Data are shown as a percent of transformed AhR induced by 0.1 nM TCDD, and represented as the means± SD from independent triplicate experiments.

### 2-3-3 Anthocyans do not suppress AhR transformation in Hepa-1c1c7 cells

A small portion of ingested anthocyans can be absorbed in the plasma and liver, and excreted into the urine as a glycosilated form (87,88). To investigate whether anthocyans can suppress AhR transformation in the liver cells, mouse hepatoma cell line Hepa-1c1c7 cells were used. As the results, 50 μM of anthocyans tested here did not significantly suppress AhR transformation induced by 0.1 nM TCDD in Hepa-1c1c7 cells (Fig. 2-6). Previous studies found that anthocyans are degraded into protocatechuic acid (PCA) and related compounds under physiological conditions (79,80). Hence, the author confirmed the suppressive effect of PCA and



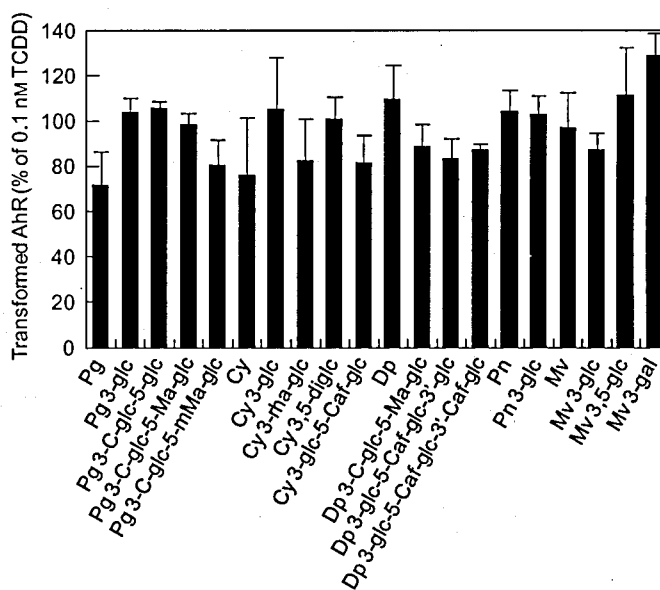**Fig. 2-6. Suppressive effect of anthocyans on TCDD-induced AhR transformation in Hepa-1c1c7 cells.** The suppressive effect of 20 kinds of anthocyans dissolved in TFA-MeOH at 50 μM on TCDD-induced AhR transformation was evaluated in Hepa-1c1c7 cells, as described in Fig. 2-1. Data are shown as a percent of transformed AhR induced by 0.1 nM TCDD, and represented as the means ± SD from independent triplicate experiments.

related compounds, *p*-hydroxybenzoic acid (HBA), vanillic acid (VA), 3,4-dimethoxybenzoic acid

(DBA), gallic acid (GA), and 3,4,5-trimethoxybenzoic acid (TBA), on AhR transformation in the

cell-free system. As shown in Fig. 2-7, no compound at 50 μM suppressed 0.1 nM TCDD-induced

AhR transformation. In addition, 3,5-dihydroxybenzoic acid and 2,4-dihydroxybenzoic acid did

not show any suppressive effect (data not shown). These results indicate that neither ingested

anthocyans nor their metabolites can suppress AhR transformation under physiological conditions,

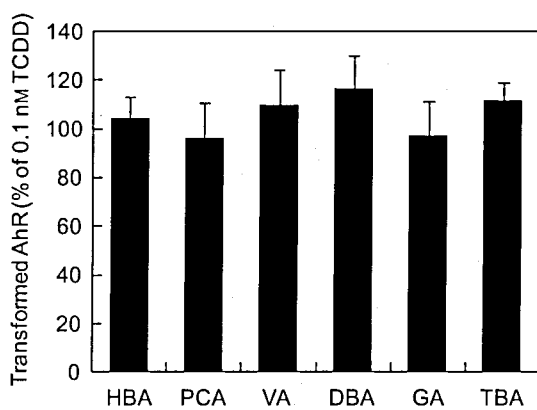suggesting that anthocyans do not affect the AhR.



**Fig. 2-7. Suppressive effect of possible metabolites of anthocyans on TCDD-induced AhR transformation in the cell-free system.** The suppressive effect of possible metabolites and related compounds dissolved in DMSO at 50 μM on TCDD-induced AhR transformation was evaluated in the cell-free system, as described in Fig. 2-1. Data are shown as a percent of transformed AhR induced by 0.1 nM TCDD, and represented as the means ± SD from independent triplicate experiments. Abbreviations are as follows: HBA, p-hydroxybenzoic acid; PCA, protocatechuic acid; VA, vanillic acid; DBA, 3,4-dimethoxybenzoic acid; GA, gallic acid; TBA, 3,4,5-trimethoxybenzoic acid.

**2-4 Discussion**

Dioxins, which are environmental contaminants, induce various adverse effects such as

body weight loss, immunotoxicity, endocrine disruption, cancer promotion, teratogenesis, and

lethality through transformation of AhR (67,68). Since they enter the body mainly through diet

(71,72), it is critical to search for a food factor that is able to suppress AhR transformation.

Previous studies have shown that flavonoids (63), lutein (66), curcumin (76), resveratrol (77), and

certain vegetable constituents (64) act as antagonists of AhR, suggesting that plant-based diets

have the potential to protect humans from dioxin toxicity. Previous study indicates that flavones

and flavonols are stronger antagonists than flavanones, catechins, or isoflavones among the

subclasses of flavonoids (63). It has been reported that AhR favors compounds that have a

hydrophobic coplanar structure as the ligands containing antagonists (3), whereas lutein (66),

curcumin (76), and resveratrol (77) act as antagonists of the AhR, although they do not have a

coplanar structure. In this chapter, the author found that anthocyans and their possible metabolites

neither suppress nor induce AhR transformation in the cell-free system and in Hepa-1c1c7 cells, indicating that anthocyans cannot affect AhR under physiological conditions. Anthocyans do not have a coplanar structure due to the flavylium cation, and they are immediately degraded under physiological conditions (78). These results suggest that anthocyans do not show enough antagonistic effect because of their structure and susceptibility to degradation.

The HPLC chromatograms revealed that Cy, particularly in DMSO, was degraded day by day into at least five products (Fig. 2-2). The degradation products of Cy showed the suppressive effect in this chapter (Fig. 2-1), although the author did not identify the product. It has been reported that anthocyans are metabolized to PCA and related compounds, including 2,4-dihydroxybenzoic acid and 2,4,6-trihydroxybenzoic acid, under physiological conditions (79,80) and some of these compounds are methylated by catechol-$O$-methyltransferase, and exist in the liver. But possible metabolites of anthocyans did not suppress AhR transformation in the cell-free system (Fig. 2-7). These results strongly suggest that the efficacious degraded compounds of Cy in DMSO are not physiological metabolites, but chemically degraded artifacts that have not yet been reported. The antioxidative activity of Cy and Cy 3-glc dissolved in DMSO was clearly lower than that in TFA-MeOH before storage (day 0), although their effect on AhR transformation did not change in either solvent (compare Fig. 2-1 with Fig. 2-3). It is noteworthy that the antioxidative activity of Cy 3-glc significantly decreased during the storage period at $-20°C$ for 4 d even in TFA-MeOH (Fig. 2-3), although neither the HPLC chromatogram nor the effect on AhR transformation changed. Thus, a portion of anthocyans is gradually degraded even in acidic solution at low temperatures.

In the cell-free system, only four of the tested anthocyans (50 μM) dissolved in TFA-MeOH significantly suppressed AhR transformation in a dose-dependent manner with relatively higher $IC_{50}$ values (Fig. 2-4 and Fig. 2-5) than those of certain flavones and flavonols (63). Although the author cannot explain why they suppressed AhR trasformation because of no structure-activity relationship, these efficacious anthocyans had no effect at 0.5 μM (Fig. 2-5). In Hepa-1c1c7 cells, all anthocyans tested were ineffective at 50 μM against 0.1 nM TCDD-induced AhR transformation (Fig. 2-6). Previous studies found that the plasma level of anthocyans in human volunteers ranged from 24 to 97.4 nM after ingestion (79,87,88). The results of this chapter and previous reports indicate that although a portion of ingested anthocyans exists in the plasma in

their intact forms, the plasma concentration is very low, and not enough to show a suppressive effect on AhR transformation in the body. In conclusion, anthocyans are not suitable candidates for protection from dioxin toxicity because they fail to suppress AhR transformation.

**2-5 Summary**

Dioxins induce adverse effects through transformation of the cytosolic AhR. Previous study found that flavones and flavonols at dietary levels suppress AhR transformation. In this chapter, the author investigated whether 20 anthocyans dissolved in TFA-MeOH suppressed AhR transformation in a cell-free system and in Hepa-1c1c7 cells. Although four compounds at 50 μM suppressed 0.1 nM TCDD-induced AhR transformation and their effects were dose-dependent in the cell-free system, they were ineffective at 0.5 μM, which is close to physiological concentration. Moreover, no anthocyan at 50 μM tested here suppressed 0.1 nM TCDD-induced AhR transformation in Hepa-1c1c7 cells. The author also confirmed that protocatechuic acid and related compounds, which are possible metabolites of anthocyans, did not affect the transformation in the cell-free system. It is concluded that anthocyans are not suitable candidates for protection from dioxin toxicity.

# Chapter 3. Interaction between the aryl hydrocarbon receptor and its antagonists, flavonoids

## 3-1 Introduction

Dioxins develop the toxicity through an aryl hydrocarbon receptor (AhR)-dependent pathway (89,90). Because over 90% of dioxin exposures to humans are estimated through the diet, it is important to search for antagonists from food components. It has been reported that flavonoids (63,66,73,74,91-94), resveratrol (77), curcumin (76), lutein (66), and other vegetable constituents or extracts (62,64,95) act as antagonists of the AhR evaluated by DNA-binding activity of the AhR *in vitro*. These results demonstrated that constituents of plant foods have the suppressive effects on AhR transformation and/or downstream events, but the mechanism by which dietary antagonists suppress the transformation is not fully elucidated yet.

In previous reports, it was demonstrated that flavonoids suppress AhR transformation in a subclass-dependent manner in order of flavone = flavonol > flavanone >catechin > isoflavone and anthocyan (63,91). Galangin,quercetin, and kaempferol, which belong to a flavonol subclass are reported to inhibit the specific binding of [$^3$H]2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) to the AhR (92,93). Recently, it was reported that (−)-epigallocatechin gallate (EGCG) suppresses DNA-binding activity of the AhR by its binding to hsp90 but not inhibiting binding of [$^3$H]-TCDD to the AhR (94). These results suggest that the suppression mechanisms of flavonoids on the transformation differ among their subclasses, but the details of the interaction between these compounds and the intact AhR or its complex (AhRc) are not elucidated yet. Here, the author show that flavonoids belong to flavone, flavonol, and flavanone subclasses would directly interact with the AhR and competitively inhibit binding of agonist to the AhR, whereas catechins would interact with the partner protein(s) of AhRc and suppress AhR transformation indirectly.

## 3-2 Materials and methods
### 3-2-1 Materials

[$^3$H]-MC (1.9 Ci/mmol) was obtained from Moravek Biochemicals, Inc. (Brea, CA). 2,3,7,8-Tetrachlorodibenzofuran (TCDF) was purchased from Cambridge Isotope Laboratories

(Andover, MA). Flavone and naringenin were from Nacalai tesque (Kyoto, Japan). Apigenin,

luteolin, galangin, eriodictyol, genistein, and cyanidin were from Extrasynthese (Genay, France).

Kaempferol and quercetin were purchased from Wako Pure Chemical Industries, Ltd. (Osaka,

Japan). ($-$)-Epigallocatechin (EGC) was obtained from Kurita Kogyo (Tokyo, Japan). EGCG and

($-$)-Epigallocatechin 3-(3"-O-methyl) gallate (Met-EGCG) were kindly provided from Dr.

Suong-Hyu Hyon and Dr. Ryoyasu Saijo, respectively. All other reagents used were of the highest

grade available from commercial sources.

### 3-2-2 Preparation of the cytosol fraction from rats

Animal treatments in the present study was approved by 'The Institutional Animal Care

and Use Committee' and carried out according to 'The Guidelines of Animal Experimentation' of

Kobe University. Livers from male Sprague–Dawley rats (six weeks old, 140–170 g; Japan SLC,

Shizuoka, Japan) were subjected to the preparation of a cytosol fraction as previously described

(63). For the AhR ligand analysis using hydroxyapatite (HAP; Bio-Rad Laboratories, CA) and

surface plasmon resonance (SPR) analysis, 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 with Hepes buffer (10

mM Hepes of pH 7.4, 0.15 M NaCl), respectively. Protein content of each cytosol fraction was

determined by the Bradford method (85).

### 3-2-3 Separation of the AhR complex from the cytosol fraction

For SPR analysis, the AhR complex (AhRc)-rich fraction was prepared as previously

described (96). Briefly, an aliquot of 30 ml of the rat liver cytosol fraction was dialyzed with MET

buffer (20 mM Tris of pH 7.5, 20 mM $NaMoO_4$, 1 mM EDTA, 0.05% Triton X-100) after

ammonium sulfate fractionation (80% saturation). The dialyzed protein was applied to a 100- x

20-mm i.d. diethyl-aminoethyl cellulose (DEAE-cellulose, Nacalai tesque) column, which was

equilibrated with MET buffer. The column was washed with 200 ml of MET buffer containing 50

mM NaCl, and the crude AhRc fraction was eluted with 40 ml of MET buffer containing 400 mM

NaCl. The crude AhRc fraction was then dialyzed with HEDG buffer, and applied to a 25- x

15-mm i.d. HAP column. The column was washed with 40 ml of 50 mM sodium phosphate (pH

6.8), and then eluted with 20 ml of 400 mM sodium phosphate (pH 6.8), and the eluate was

collected and used as the AhRc-rich fraction.

### 3-2-4 AhR ligand binding analysis

Specific binding of [³H]-MC to the AhR was determined by a ligand-binding assay using HAP as previously described (97). Briefly, the cytosol fraction (2 mg protein/ml) was incubated with 0.25 nM [³H]-MC at 20 °C for 2 h in 2 ml of HEDG buffer. Non-specific binding was defined by incubation of cytosol with [³H]-MC in the presence of 200-fold molar excess of TCDF. Aliquots of 250 μl of the reaction mixture were transferred to a scintillation vial to measure total radio activity of [³H]-MC. Remained aliquots of 500 μl of the mixture were incubated with 300 μl of HAP, which was suspended in double volume of HEDG buffer, at 4 °C for 30 min. The 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 to measure [³H]-MC-bound AhR. To determine the inhibitory effects of flavonoids on specific binding between the AhR and [³H]-MC, flavonoids at various concentrations (1 nM–100 μM) were pretreated to the cytosol fraction 10 min prior to addition of [³H]-MC.

### 3-2-5 Surface plasmon resonance (SPR) analysis

To detect interaction between flavonoids and the AhRc, SPR analysis was performed using Biacore 3000 model (Biacore AB, Uppsala, Sweden). Anti-AhR antibody (H211; Santa Cruz Biotechnology, Santa Cruz, CA) was immobilized on a CM5 sensor chip (research grade) using the amino coupling kit. Aliquots of 20 μl of the AhRc-rich fraction, which were diluted with Hepes–EDTA buffer (10 mM Hepes of pH 7.4, 3.4 mM EDTA, 0.15M NaCl, 0.005% Tween 20) to 4 mg protein/ml, was injected to the flow cell at a flow rate of 10 μl/min in Hepes–EDTA buffer containing 1% ethanol. After the reaction reached to equilibrium, 5–100 μM flavonoids were injected. Flavonoids were dissolved in ethanol and diluted with the same buffer just before use. All reaction was carried out at 25 °C. Affinity between flavonoids and AhRc was analyzed by BIAevaluation software 3.0.

## 3-3 Results

### 3-3-1 Effects of Flavonoids on binding between the cytosolic AhR and [$^3$H]-MC

Previously it was reported that certain flavonoids suppress DNA binding activity of transformed AhR in a cell-free bioassay (63). However, their suppression mechanisms were not fully clear yet. To examine whether flavonoids inhibit agonist-binding to the AhR, the author carried out the competitive ligand binding assay using HAP. As the results, [$^3$H]-MC bound to the cytosolic AhR in a dose-dependent manner from 0.083 to 2.5 nM, and from Scatchard plot, the dissociation constant (Kd) value and maximum binding capacity were determined as 0.14 ± 0.02 nM and 17.3 ± 3.6 fmol/mg protein, respectively (data not shown). The author used 0.25 nM [$^3$H]-MC, a median concentration-point of the plot, to determine the inhibitory effects of 12 flavonoids on the specific binding of agonist to the AhR. As shown in Fig. 3-1, flavones; flavone, apigenin, and luteolin, and flavonols; galangin, quercetin, and kaempferol at 1 μM inhibited the binding of 0.25 nM [$^3$H]-MC to the AhR. These results indicate that flavonoids belong to the flavone and flavonol subclasses inhibit binding of agonist to the AhR at lower concentration that close to physiological one. Although flavonoids belong to flavone, flavonol, flavanone, and catechin subclasses were demonstrated to suppress DNA binding activity of the AhR (63), flavanones; naringenin and eriodictyol, and catechins; EGC, EGCG, Met-EGCG did not inhibit binding of agonist to the AhR under this experimental condition unexpectedly. Isoflavones; genistein, and anthocyan; cyanidin, which do not suppress AhR transformation (63,91), did not inhibit the binding of agonist to the AhR predictively. To investigate whether the inhibitory effects are competitive or not, the dose-dependency of respective compounds from four subclasses was
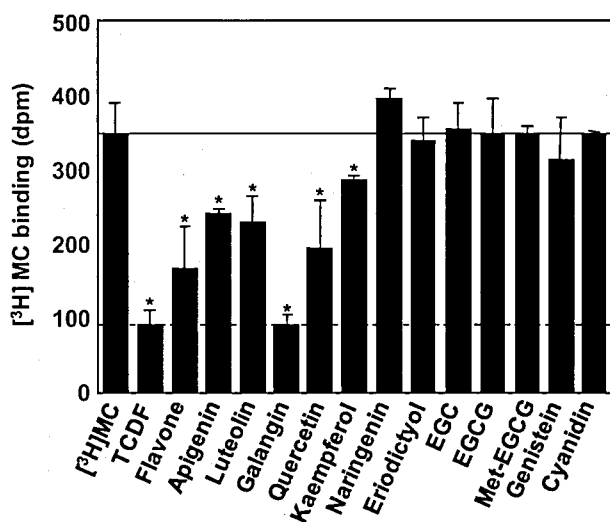


Fig. 3-1. Effects of flavonoids on binding between the AhR and [$^3$H]-MC. Rat liver cytosol was pretreated with 1 mM flavonoids 10 min prior to addition of 0.25 nM [$^3$H]-MC. For determination of non-specific binding, 200-fold excess molar of TCDF was added to the cytosol before addition of [$^3$H]-MC. Solid and broken lines indicate the values of total and nonspecific binding, respectively. Data are represented as means ± SE from independent triplicate experiments. Asterisks indicate significant differences from the total binding ($p < 0.05$, Student's t-test).
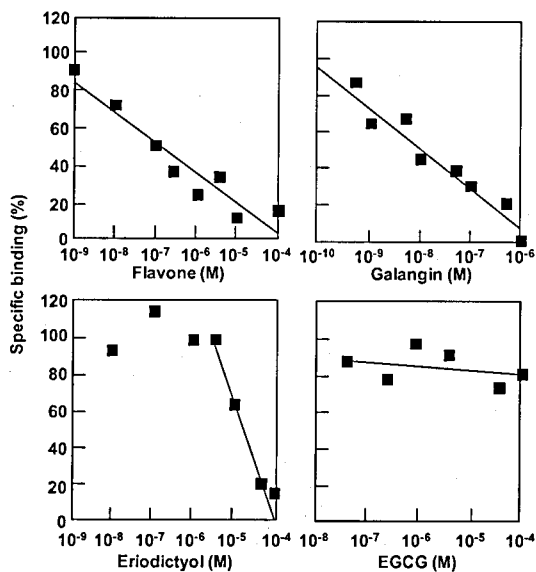
**Fig. 3-2. Dose-dependency of selected flavonoids on specific binding of [$^3$H]-MC to the AhR.** Rat liver cytosol was pretreated with indicated concentrations of flavonoids, and the inhibitory effects of flavonoids on [$^3$H]-MC binding to the AhR were measured as described in Materials and methods. Data are indicated as % of specific binding and represented as means ± SE from triplicate independent experiments.

examined. Flavone and galangin inhibited the specific binding between [$^3$H]-MC and the AhR in a dose-dependent manner with the 50% inhibitory concentration (IC$_{50}$) values of 1.71 x 10$^7$ and 1.19 x 10$^8$, respectively (Fig. 3-2). In addition to flavone and galangin, eriodictyol at higher concentrations also inhibited the [$^3$H]-MC binding to the AhR dose-dependently with the IC$_{50}$ value of 1.90 x 10$^5$ M. On the other hand, EGCG did not inhibit the binding even at 100 μM. These results indicate that flavone, galangin, and eriodictyol act as competitive antagonists of the AhR, but EGCG dose not interact to a ligand-binding site of the AhR.

### 3-3-2 Detection of interaction between the AhRc and flavonoids by SPR analysis

The author, next, analyzed whether these flavonoids directly bind to the intact AhRc by SPR analysis. Recently, it was demonstrated that EGCG interacts with recombinant hsp90 proteins and act as an antagonist of the AhR (94). The author separated the AhRc-rich fraction from the rat liver cytosol, because the crude cytosol fraction is inappropriate to SPR analysis. Components of the AhRc-rich fraction were confirmed by Western blotting analysis after immunoprecipitation using anti-AhR antibody, and hsp90, XAP2, and p23 proteins were detected by immunoblotting using corresponding antibodies (data not shown). To capture the AhRc on a CM5 sensor chip, the anti-AhR antibody was immobilized on a CM5 sensor chip using an amino coupling kit, and then the AhRc fraction was injected to the flow cell. From the sensorgram, the

AhRc was specifically captured on the sensor chip compared to an untreated control flow cell (Fig. 3-3A). After the sensorgram reached to equilibrium, β-naphthoflavone (β-NF), a known agonist of the AhR, was injected to the flow cell at the final concentrations of 50, 75, and 100 μM. β-NF interacted with the AhRc in a dose dependent-manner (Fig. 3-3B), indicating that the constructed sensor chip can detect interaction between the AhRc and its ligands.

**Fig. 3-3. Construction of a sensor chip capturing the AhRc.** (A) The sensorgrams after injection of the AhRc-rich fraction on a CM5 sensor chip immobilized with (solid line) or without (broken line) anti-AhR antibody. (B) Interaction between the AhRc and β-NF at 50, 75, and 100 μM. β-NF was injected at a flow rate of 30 μl/min to the flow cell after the AhRc was captured on the sensor chip. Response of the binding reached to equilibrium at each concentration was shown as a resonance unit (RU). Data are represented as means ± SE from independent triplicate experiments.

Then, the author examined interaction between the AhRc and flavone, galangin, eriodictyol, and EGCG at 100 μM by SPR analysis using the AhRc-captured CM5 sensor chip. As shown in Fig. 3-4, all flavonoids tested directly interacted with the intact AhRc. The Kd values of flavone, apigenin, eriodictyol, and EGCG were determined as $4.99 \times 10^9$, $8.95 \times 10^5$, $2.07 \times 10^4$, and $2.53 \times 10^4$ M, respectively (Fig. 3-4A). Since EGCG did not competitively inhibit [$^3$H]-MC binding to the AhR, the author further examined interaction between the AhRc and EGCG at lower concentrations (5–30 μM), and observed that this compound interacted with the AhRc in a dose-dependent manner (Fig. 3-4B). These results suggest that typical flavonoids belong to flavone, flavonol, flavanone, and catechin subclasses suppress DNA binding activity of the AhR through affecting the ligand-binding site of the AhR or the partner proteins of which AhRc composed.
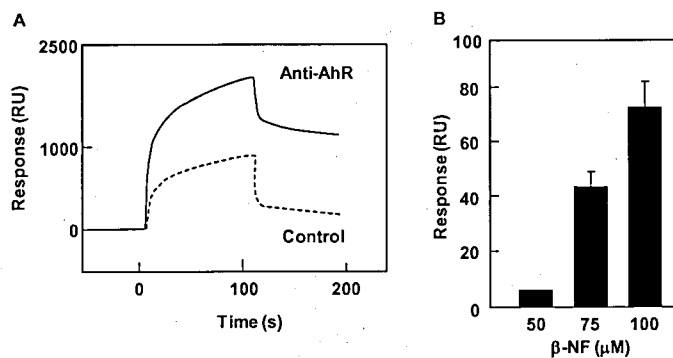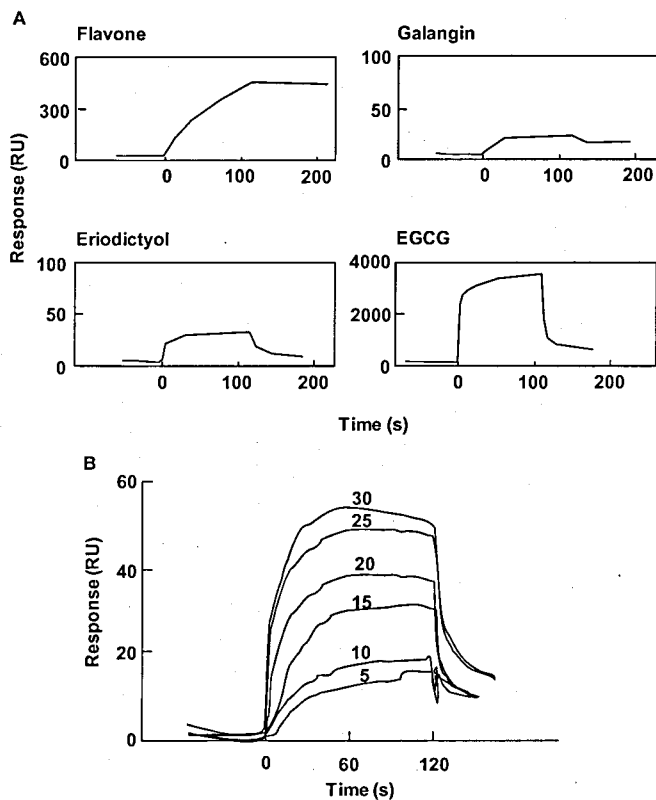
Fig. 3-4. Interaction between the AhRc and flavonoids. (A) The sensorgrams indicated response after injection of 100 µl of 100 µM flavone, galangin, eriodictyol, or EGCG at a flow rate of 50 µl/min to the flow cell of the AhRc-captured sensor chip. (B) The sensorgrams after injection of 100 µl of EGCG. The numerals on the sensorgrams mean concentrations of EGCG (µM).

## 3-4 Discussion

Previous studies demonstrated that flavonoids suppress AhR transformation in a cell-free bioassay (63,91), and their suppressive effects differ from each subclass. From the results in this study, an inhibitory effects of flavonoids on a specific binding of agonist to the receptor also depend on each subclass: Flavone, flavonol, and flavanone inhibit the specific binding of [$^3$H]-MC to the AhR, whereas isoflavone, catechin, and anthocyan do not. Selected compounds in former three subclasses showed a dose-dependent inhibition, indicating that these compounds act as competitive antagonists. It was previously demonstrated that galangin, quercetin, and kaempferol, which belong to flavonol subclass, compete with TCDD for binding to the AhR (92,93). Taken together, these results indicate that flavonoids belong to flavone, flavonol, and flavanone subclasses possibly bind to the ligand binding site of AhR, and they compete the binding of agonist.

EGCG, a catechin, did not inhibit the specific binding of the agonist even at 100 µM in this study, but it suppressed DNA binding activity of the AhR at lower concentration range (63,66). These results indicate that EGCG neither has a competitive nor allosteric inhibition against the AhR. From the results of SPR analysis, EGCG interacts with the intact AhRc

dose-dependently, indicating that it probably affects the partner protein(s) of the AhRc. Indeed, a recent report demonstrates that EGCG is able to interact with recombinant hsp90 (94). However, mechanisms in details of EGCG are unknown yet. It is also demonstrated that EGCG binds to the 67-kDa laminin receptor expressed in various cells, especially in cancer cells, and alters downstream cellular signal transduction (98). Moreover, catechins including EGCG are known to interact to various proteins (94,99,100). These results indicate that EGCG is able to affect the functions of partner protein(s) of the AhRc. In SPR analysis, Rmax value of EGCG is 100 times higher than that of any other flavonoids tested in this study, suggesting that EGCG binds to the AhRc without specificity and/or to the multiple sites of the AhRc and affect to the AhR transformation. Although it needs further experiments to clarify the mechanisms of EGCG on AhR transformation, my findings, at least, indicate that EGCG suppresses AhR transformation indirectly by different mechanism from that of any other flavonoids.

Results in SPR analysis indicate that the Kd value of flavonoids against the AhRc ranked in the order of flavone < galangin < EGCG and eriodictyol. Because smaller Kd value means stronger affinity, flavone showed the highest affinity with the AhRc among 4 flavonoids tested. From the results in this study and previous reports (63,66), the strength of binding affinity between flavonoids and the AhRc is according to the order of suppressive effects of flavonoids on DNA binding activity of the AhR. It was reported that the interaction of flavonols to bovine serum albumin (BSA) is stronger than that of catechins without any structural changes in the BSA molecule by hydrophobic interaction and hydrogen bonding (99). The suppressive effects of AhR transformation also depend on the number of hydroxy and methoxy group in the flavonoid structure, i.e., lesser hydroxy and methoxy group gave the lower $IC_{50}$ value . These results strongly suggest that hydrophobicity of flavonoids is involved in the interaction of the compounds to the target protein(s). Therefore, the structural properties are important to the binding affinity of flavonoids to the AhRc. In conclusion, flavonoids belong to flavone, flavonol, flavanone, and catechin subclass directly bind to the intact AhRc existed in cytosol, and the flavonoids of the former three subclasses suppress AhR transformation by competitive inhibition of binding between the AhR and its agonist.

## 3-5 Summary

Flavonoids have been reported to be dietary antagonists of the AhR. However, little is known about the molecular mechanism on their antagonistic effects. In this study, the inhibitory effect of flavonoids on ligand binding to the AhR and interaction between flavonoids and the AhRc were investigated in each flavonoid subclass. Flavone, flavonol, and flavanone but not catechin inhibited the specific binding between the AhR and MC dose-dependently, indicating that the former three subclasses possibly act as competitive antagonists of the AhR. However, catechin in addition to the former three subclasses directly interacted with the AhRc by surface plasmon resonance analysis. The dissociation constant values showed an inverse correlation with the suppressive effect on the DNA binding activity. These results suggest that flavone, flavonol, and flavanone act as competitive antagonists of the AhR, while catechin associates with the AhRc and indirectly exhibits its antagonistic effects.

# Chapter 4. Suppression mechanisms of flavonoid on the transformation of an aryl hydrocarbon receptor

## 4-1 Introduction

In response to the ligand binding, the AhR dissociates its partner proteins, translocates into nucleus, and forms a heterodimer with an AhR nuclear translocator (Arnt) (101). The AhR/Arnt heterodimer binds to a DNA sequence called dioxin response element (DRE) (30). It was reported that the AhR and/or Arnt was phosphorylated by mitogen-activated protein kinase (MAPK)s (102), tyrosine kinases (103) or protein kinase C (PKC) (104). Formation of the heterodimer requires phosphorylation of the Arnt, while the DNA-binding activity of the heterodimer requires phosphorylation both the AhR and Arnt (33,36-39,105) . Extracellular signal-regulated kinases (ERK1/2) is concerned with phosphorylation of the AhR and its transcriptional activity, since TCDD-induced phosphorylation of ERK is a critical for gene transcription regulated by the AhR/Arnt heterodimer in mouse hepatoma Hepa-1c1c7 cells (102).

In the previous reports (63,91) demonstrated that flavonoids belonging to flavone, flavonol, flavanone and catechin subclasses but not anthocyan and isoflavone subclasses acted as an antagonist of the AhR. Suppressive effects of flavone and flavonol subclasses on binding of [$^3$H]-methylcholanthrene (MC) to the AhR and its DNA-binding activity was stronger than those of flavanone and catechin subclasses in a cell-free system. However, flavonoids in these four subclasses interacted with the AhR complex composed of AhR, hsp90, XAP2 and p23 (106). Moreover, (−)-epigallocatechin gallate (EGCG) inhibited AhR transformation by interacting with hsp90 (94). However, the suppression mechanism of flavonoids on AhR transformation is not fully clarified. In this chapter, the author investigated the effect of flavonoids on nuclear translocation of the AhR, dissociation of its partner proteins, formation of the heterodimer with Arnt, and phosphorylation of the AhR and Arnt in Hepa-1c1c7 cells.

## 4-2 Materials and Methods

### 4-2-1 Materials

TCDD was purchased from AccuStandard (New Haven, CT). Flavone and naringenin

were obtained from Nacalai tesque (Kyoto, Japan). Apigenin, luteolin, galangin and eriodictyol were from Extrasynthèse (Genay, France). Kaempferol was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). (−)-Epigallocatechin (EGC) and EGCG were obtained from Kurita Kogyo (Tokyo, Japan). (−)-Epigallocatechin 3-(3"-*O*-methyl) gallate (Met-EGCG) was generously provided by Dr. Ryoyasu Saijo.

1,4-Diamino-2,3-dicyano-1,4-bis(2-aminophenylthio)butadiene (U0126), an inhibitor of MEK1/2, was obtained from Sigma (St. Louis, MO). For immunoprecipitation, anti-AhR (H211) was obtained from Santa Cruz biotechnology Inc. (Santa Cruz, CA), or anti-phosphoThr was purchased from Sigma. Anti-AhR antibody (MA1-514: Affinity BioReagents, Golden, CO), anti-XAP2 antibody (Affinity BioReagents), anti-hsp90 antibody (Stressgen, Ann Arbor, MI), and phospho-ERK (pERK, pT202/pY204) antibody (Franklin Lakes, NJ), were used for western blot analysis. Anti-Arnt antibody was purchased form Santa Cruz biotechnology Inc. All other reagents used were of the highest grade available from commercial sources.

### 4-2-2 Cell culture

Mouse hepatoma cell line, Hepa-1c1c7 cells were grown and maintained at 37°C in α-minimum essential medium (α-MEM, Nissui Pharmaceutical, Tokyo, Japan) containing 10% fetal bovine serum (Sigma), 4 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin under a humidified 95% air-5% CO2 condition.

### 4-2-3 Construction and Sources of Expression Vectors

cDNA fragments of murine AhR, hsp90 and XAP2 were produced by PCR amplification with murine cDNA for the AhR, hsp90 and XAP2 as the template. The sense and antisense primers used were as follows: For the AhR, 5'-TT AAG CTT ATG AGC AGC GGC GCC AAC (AhR-F) and 5'-TT CCG CGG TCA ACT CTG CAC CTT GCT (AhR-R); for hsp90, 5'-TT GGT ACC ATG CCT GAG GAA ACC (hsp90-F) and 5'-TT TCT AGA GTC TAC TTC TTC CAT GCG (hsp90-R); for XAP2, 5'-TT GAA TTC ATG GCG GAT CTC ATC GC (XAP2-F) and 5'-TT GAT ATC GTG GGA AAA GAT GCC CC (XAP2-R). Underlines indicated the restriction sites in each sequence. These PCR products were first subcloned into a TA cloning vector, pGEM-T Easy (Promega, Madison, WI) and ligated to following vectors after digestion

with restriction enzymes. GFP-AhR was constructed by inserting the AhR into the HindIII and SacII restriction sites in the multiple cloning site of pEGFP-C3 (Clontech, Mountain View, CA). FLAG-hsp90 was constructed by inserting hsp90 into the KpnI and XbaI restriction sites in the multiple cloning site of p3xFLAG-CMV14 (Sigma). FLAG-XAP2 was constructed by inserting XAP2 into the EcoRI and EcoRV restriction sites in the multiple cloning site of p3xFLAG-CMV14 (Sigma). The ligand binding domain mutant of GFP-AhR (GFP-AhRF345A) was generated using the Quick Change Site-directed Mutagenesis kit (Stratagene, La Jolla, CA).

### 4-2-4 Transient expression

Hepa-1c1c7 cells were seeded onto a glass-bottomed culture dish (MatTek Corp., Ashland, MA) and incubated for 24 h before transfection. Transfection mixtures were supplemented with 200 μl of Opti-MEM (Invitrogen, Carlsbad, CA), 5 μl of FuGENETM 6 Transfection Reagent (Roche, Basel, Switzerland) and total 4 μg of DNA, and the cells were incubated with the mixture for 4 h at 37°C.

### 4-2-5 Confocal microscopy analysis of nuclear translocation of the AhR

Transiently transfected cells were cultured for 24 h at 37°C prior to the experiment. To evaluate the effects of flavonoid on the AhR nuclear translocation, the cells were pre-treated with flavonoids at concentrations indicated in each figure or dimethylsulfoxide (DMSO, 1 μl/ml medium) as a vehicle control for 10 min. After pre-treatment, the cells were treated with 0.5 nM TCDD or DMSO (1 μl/ml) for 1 h at 37°C, rinsed twice with ice-cold phosphate-buffered saline (PBS), and fixed for over night in 100 mM phosphate buffer containing 4% paraformaldehyde and 0.2% picric acid. Nuclear stain was performed with propidium iodide (Molecular Probes, Carlsbad, CA) according to the manufacturer's protocol. The fluorescence of GFP and propidium iodide were observed under a confocal laser scanning fluorescent microscopy (LSM 510 invert, Carl Zeiss, Jena, Germany). The fluorescence of GFP was monitored at 488-nm argon laser excitation with 515−535 nm band pass barrier filter and that of the propidium iodide was monitored at 543-nm HeNe excitation with a 560-615 nm band pass filter. To quantify the translocation level of the GFP-AhR as follows: i) The cells were divided into two types. The cells in which the ratio of GFP-fluorescence intensity of cytoplasm and nucleus was under 1 were defined as "GFP-AhR in

the cytoplasm," and the cells with the ratio of 1 or over 1 were defined as "GFP-AhR in the nucleus." ii) The number of cells in each type was counted. iii) The percentage of the number of cells in each type to the number of cells expressing GFP-AhR was calculated. iv) Translocation level was calculated as the ratio of the percentage of each cell "GFP-AhR existed in the nucleus" to "GFP-AhR existed in the cytoplasm."

**4-2-6 Western blotting analysis and Immunoprecipitation**

Hepa-1c1c7 cells were treated with flavonoids and TCDD as described above 'Confocal microscopy analysis of nuclear translocation of the AhR'. To detect the translocated AhR, the nuclear protein was prepared from the cells and Western blotting analysis was performed according to the previous report (107). For immunoprecipitation, the cells seeded on 100-mm dish were harvested and homogenized with 100 μl of RIPA buffer [50 mM Tris-HCl of pH 8.0, 150 mM NaCl, 1.0 % Nonidet P-40 (NP-40), and 0.5 % deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 1.0 mM dithiothreitol (DTT)] containing protease and phosphatase inhibitors [1 mM phenylmethylsulfonyl fluoride (PMSF), 10 μg/ml leupeptin, 5 μg/ml aprotinin, 10 mM sodium fluoride and 1 mM orthovanadate] followed by incubation for 1 h on ice and then centrifuged at 20,000 x g for 20 min at 4°C. The supernatant was collected as the cell lysate and incubated with anti-AhR antibody (H211), anti-Arnt antibody or anti-phosphoThr for over night at 4°C, and then with protein A/G agarose beads (Santa Cruz biotechnology Inc.), followed by incubation with rotation for 2 h at 4°C. The precipitate was washed 5 times with 1 ml of lysis buffer (20 mM HEPES of pH 7.5, 150 mM NaCl, 1 mM EDTA, 1.0% Triton-X 100, and 0.5% deoxycholate, 0.1% SDS), containing the same protease and phosphatase inhibitors as described above. Obtained beads pellet and cell lysate were subjected to Western blot analysis. The samples were separated by 7.5% (for detection of AhR, Arnt or hsp90) and 12% (for detection of XAP2 or ERK1/2) sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were transferred onto a polyvinylidene difluoride membrane (GE Healthcare Bio-Sciences Corp., NJ), followed by blocking of non-specific binding with 1% skim milk in PBS containing 0.05% tween20 (PBST) for the XAP2 immunoprecipitated by anti-AhR antibody and the AhR immunoprecipitated by anti-Arnt antibody, 5% skim milk in PBST for the hsp90 immunoprecipitated by anti-AhR antibody, 100 % commercial TBS-blocking buffer (Nacalai

Tesque, Kyoto, Japan) for the phosphorylated ERK1/2, or 50% commercial TBS-blocking buffer in PBST for others, respectively, for 1 h. The membrane was incubated with anti-AhR antibody (1:1,000), anti-XAP2 antibody (1:3,000), anti-hsp90 antibody (1:1,000), anti-Arnt antibody (1:1,000), and phospho-ERK (pERK, pT202/pY204) antibody (1:1,000), respectively, for 1 h at room temperature. After washing with TBS containing 0.05% tween 20 (TBST, for pERK) or PBST (for other proteins), the membrane was incubated with peroxidase-labeled secondary antibody (1:30000) for 1h. After three times washing with TBST (for pERK) or PBST (for other proteins), the immunocomplex was visualized using a chemiluminescence detection kit (ECL plus, GE Healthcare Bio-Science) and analyzed by Image J software (National Institutes of Health, Bethesda, MD).

### 4-2-7 DNA-binding activity of the AhR

Hepa-1c1c7 cells were seeded on 60-mm dishes (approximately 80% confluent). The cells were starved for 24 h in α-MEM containing 0.2% bovine serum albumin (Fraction-V, Nacalai tesque), 4 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml of streptomysin, and then treated with U0126 at 10 μM (final concentration) for 30 min. The cells were treated with TCDD at 0.5 nM (final concentration) or DMSO (1 μl/ml) as a vehicle control for further 1 h at 37°C. The nuclear extract was prepared from the cells, and used for measurement of DNA-binding activity by southwestern chemistry-based enzyme-linked immunosorbent assay (SW-ELISA) according to the previous report (91).

### 4-2-8 Phosphorylation of ERK

To detect phosphorylation of ERK1/2, Hepa-1c1c7 cells were starved for 24 h as described above and pre-treated with U0126 or flavonoid for 30 min. After incubation, the cells were treated with TCDD at 0.5 nM (final concentration) or DMSO (1 μl/ml) as a vehicle control for further 30 min at 37°C. Lysate was prepared from the cells, and used for detect phosphorylation of ERK1/2 by Western blot analysis as described above.

### 4-2-9 Statistical analysis

Data are expressed as the means ± SE of at least three independent determinations for

each experiment. Statistical significance was analyzed by using Student's t-test or Tukey's

multiple comparisons.


**4-3 Results**

**4-3-1 Estimation of AhR nuclear translocation induced by TCDD**

To estimate subsellular localization of the AhR, the GFP-AhR was transiently

co-expressed with FLAG-hsp90, FLAG-XAP2 or p3xFLAG-CMV14 in Hepa-1c1c7. As shown

in Fig. 4-1A, 75% of the cells co-expressed FLAG-XAP2 kept the GFP-AhR in cytoplasm

whereas only 15 % or 20 % cells kept it in cytoplasm in co-expressed with p3xFLAG-CMV14 or

FLAG-hsp90, respectively. To estimate TCDD-dependent-translocation of AhR, the GFP-AhR or

GFP-AhRF345A, a point mutation in ligand binding domain of the AhR, was transiently

expressed with FLAG-XAP2 in Hepa-1c1c7cells. The GFP-AhR translocated into nucleus

induced by TCDD in a dose-dependent manner, although the GFP-AhRF345A abolished the

translocation (Fig. 4-1B). These results indicated that the cells co-expressing the GFP-AhR and

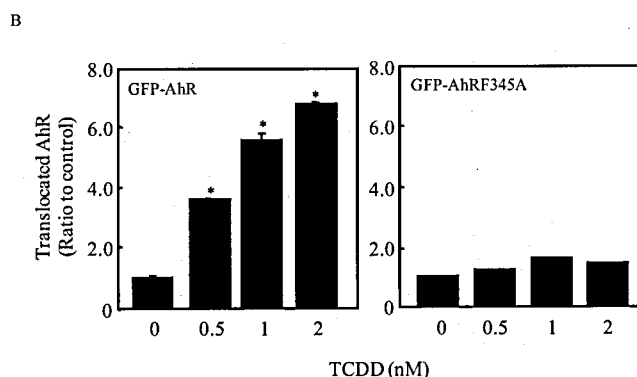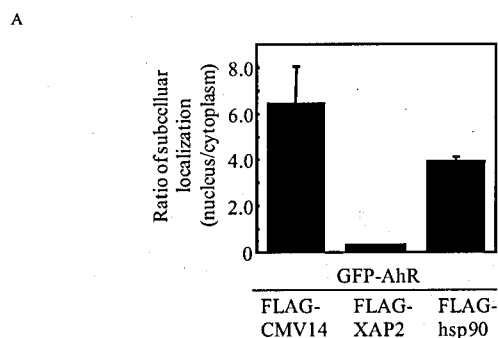FLAG-XAP2 were appropriate to evaluate nuclear translocation of the AhR by TCDD.

A



B



TCDD (nM)

**Fig. 4-1. Subcelluar localization and nuclear translocation of the GFP-AhR.** A, Hepa-1c1c7 cells on glass bottom dishes were co-transfected with the GFP-AhR and either p3xFLAG-CMV14, FLAG- XAP2 or FLAG- hsp90. The GFP-AhR was observed by a confocal microscopy. The ratio of GFP-AhR in nucleus was calculated as compared with GFP-AhR in cytoplasm. Data was represented as the mean ± SE (n=3). B, Hepa-1c1c7 cells were co-transfected with GFP-AhR (left) or GFP-AhRF345A (right) and FLAG-XAP2. Cells were treated with indicated concentrations of TCDD for 60 min and fixed. The GFP fluorescence was observed by the confocal microscopy. The ratio of translocated AhR in the cells expressing the GFP-AhR was calculated as described in Materials and Methods. Data were represented as the mean ± SE (n=3) and asterisks indicate a significant differences from control ($p<0.05$, Student's $t$-test).


**4-3-2 Effect of flavonoid on nuclear translocation of the AhR**

The effect of flavonoid on AhR nuclear translocation was evaluated (Fig. 4-2A and B).

Flavonoid at 10 μM belonging to a flavone subclass; flavone, apigenin and luteolin, and a flavonol

subclass; kaempferol and galangin, significantly suppressed nuclear translocation of GFP-AhR

induced by 0.5 nM TCDD. In contrast, flavanone subclass; eriodictyol and naringenin, and a

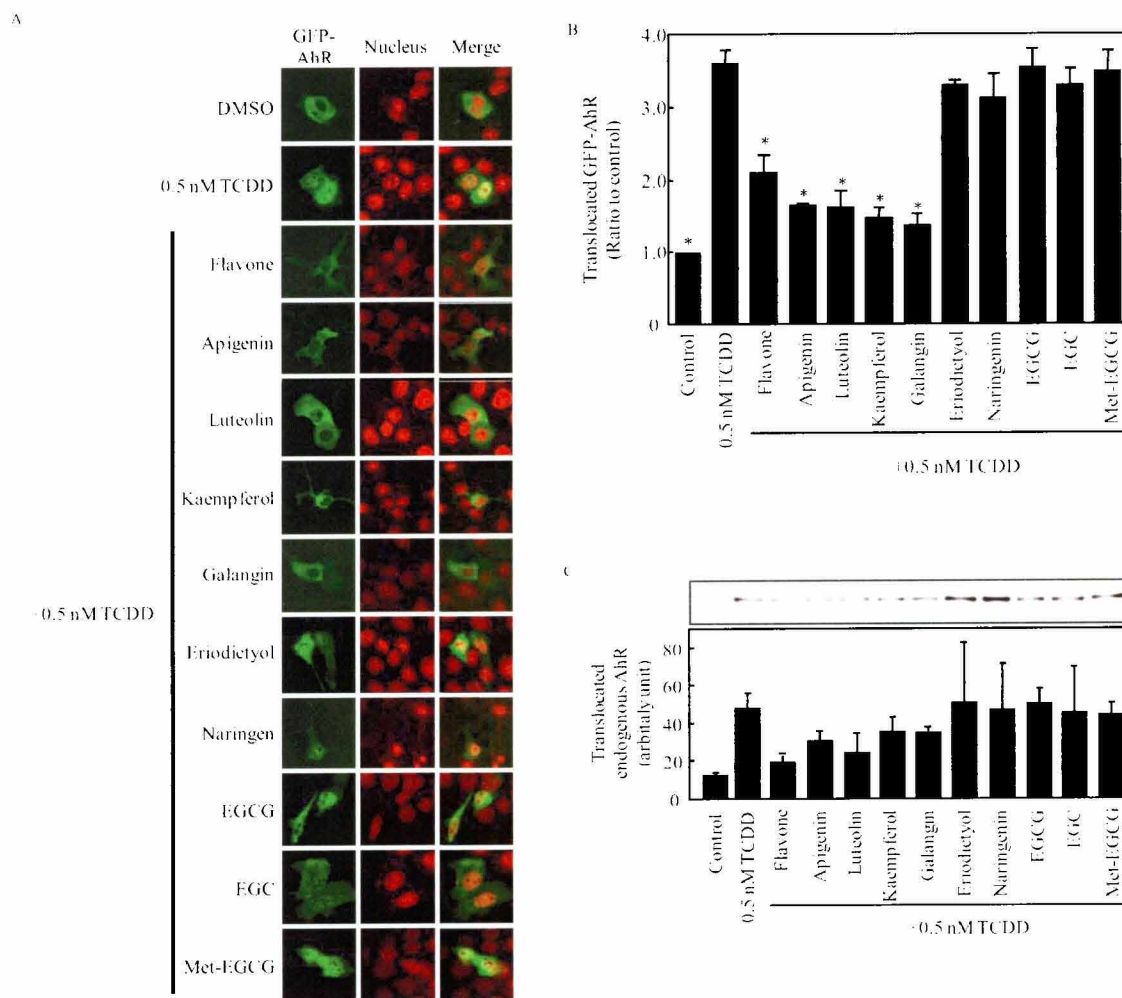catechin subclass; EGCG, EGC, and Met-EGCG did not suppress the translocation. These effects



**Fig. 4-2. Effect of flavonoids on translocation of the GFP-AhR and endogenous AhR.** A and B, Hepa-1c1c7 cells on glass bottom dishes were transfected with the GFP-AhR with FLAG-XAP2. The cells were treated with flavonoids at 10 μM for 10 min prior to treatment of 0.5 nM TCDD for 60 min and fixed. Nucleus was stained with propidiumiodide, and the fluorescence was detected by the confocal microscopy. Typical pictures were shown (A), and ratio of translocated AhR in the cells expressing the GFP-AhR was quantified as described in Materials and Methods (B). Data were represented as the mean ± SE (n=3) and asterisks indicate a significant differences from the data treated with TCDD alone (*p*<0.05, Student's *t*-test). C, Hepa-1c1c7 cells were treated with flavonoids at 10 μM prior to treatment of 0.5 nM TCDD for 60 min. The endogenous AhR in the nucleus was detected by Western blot analysis. Typical picture was shown from at least triplicate determinations (upper). The intensity of each band for AhR was quantified (bottom).

of flavonoids on the endogenous AhR were also confirmed by western blot analysis (Fig. 4-2C), and obtained the same results as the GFP-AhR.

To further investigate the differences of the effect on AhR transformation among flavonoid subclasses, the author selected apigenin, kaempferol, naringenin and EGCG on behalf of each subclass. Apigenin and kaempferol suppressed nuclear translocation of GFP-AhR in a dose-dependent manner, whereas naringenin and EGCG showed no effect on the translocation even at the high concentration, 50 μM (Fig. 4-3). It was also confirmed that these flavonoids did not induced nuclear translocation of GFP-AhR by themselves (Fig. 4-3). These results indicate that apigenin and kaempferol inhibited TCDD-induced nuclear translocation, and naringenin and EGCG unaffected the translocation and subcellular localization of the AhR.
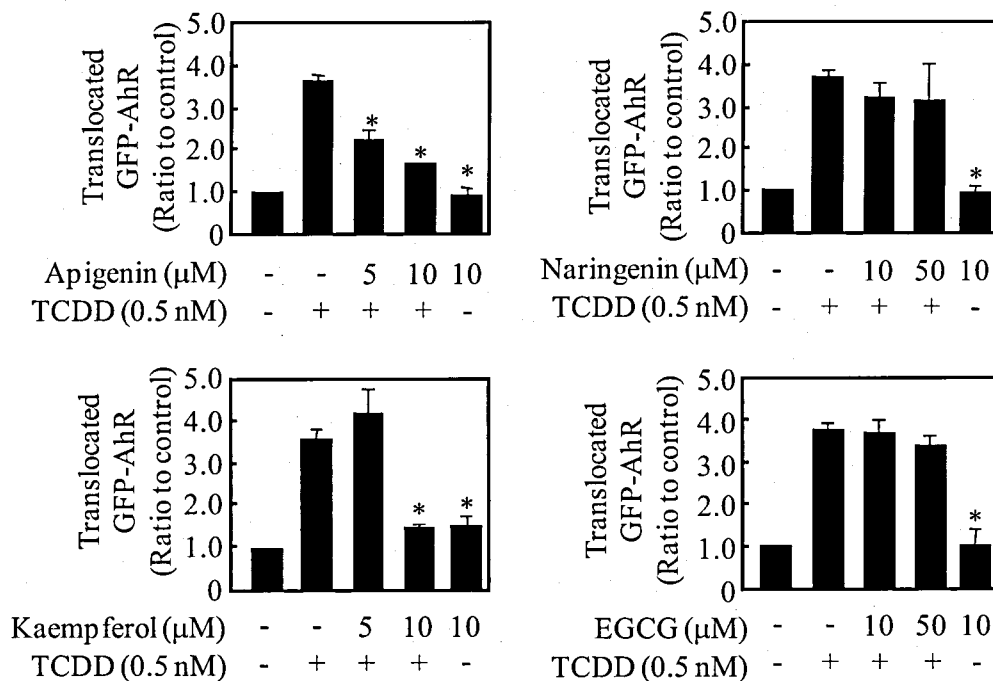


Fig. 4-3. Effect of flavonoids on translocation and subcelluar localization of the GFP-AhR in Hepa-1c1c7 cells. The cells on glass bottom dishes were transfected with the GFP-AhR with FLAG-XAP2 and treated with each flavonoid at indicated concentrations for 10 min prior to addition of 0.5 nM TCDD and fixed. The GFP-AhR was observed by the confocal microscopy. Ratio of translocated AhR in the cells expressing the GFP-AhR was calculated as described in Materials and Methods. Data were represented as the mean ± SE (n=3) and asterisks indicate a significant differences from the data treated with TCDD alone ($p<0.05$, Student's $t$-test).

**4-3-3 Effects of flavonoid on dissociation of proteins composed of AhR complex**

The cytoplasmic unliganded AhR exists as complex consisting of the AhR, two

molecules of hsp90 and XAP2. Since apigenin and kaempferol suppressed nuclear translocation of

AhR in the presence of TCDD, it was hypothesized that these flavonoids kept the complex in

cytoplasm. To detect the members of complex in Hepa-1c1c7 cells, the author carried out

immunoprecipitation method. TCDD caused dissociation of hsp90 and XAP2 from the AhR

complex as expected. Apigenin and kaempferol suppressed the dissociation of these proteins but

naringenin and EGCG did not (Fig. 4-4). This result was identical with that of the suppressive

effects of these flavonoids on nuclear translocation, and suggested that naringenin and EGCG
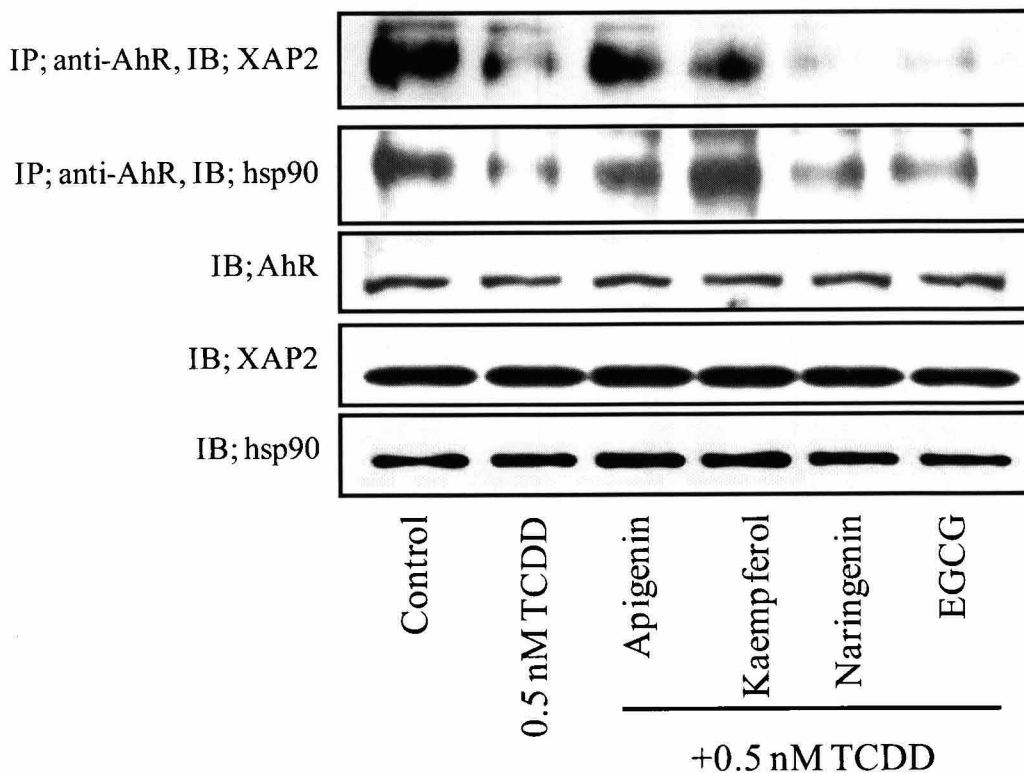
exhibited their suppressive effect in nucleus.



**Fig. 4-4. Effect of flavonoids on partner proteins of the AhR complex.** Hepa-1c1c7 cells were treated with flavonoids at 10 μM prior to treatment of 0.5 nM TCDD for 60 min, and the cell lysate was collected. To detect each protein associated with the AhR in cell lysate, immunoprecipitations (IP) and Western blot analysis (IB) were performed using indicated antibody. Expression level of each protein was also detected by Western blot analysis.

### 4-3-4 Phosphorylation level of the AhR and Arnt, and formation of the AhR/Arnt heterodimer were decreased by flavonoid

In nucleus, the AhR and Arnt undergo phosphorylation to associate with each other, and forms heterodimer works as a transcription factor. The author examined the effects of flavonoid on phosphorylation level of the AhR and Arnt, and formation of heterodimer. Apigenin, kaempferol, naringenin, and EGCG inhibited phosphorylation of Thr residues in the AhR and Arnt, and formation of heterodimer induced by TCDD (Fig. 4-5A and B). These results suggested that EGCG and naringenin suppressed AhR transformation through inhibiting the phosphorylation of the AhR and Arnt and formation of their heterodimer in nucleus.

A
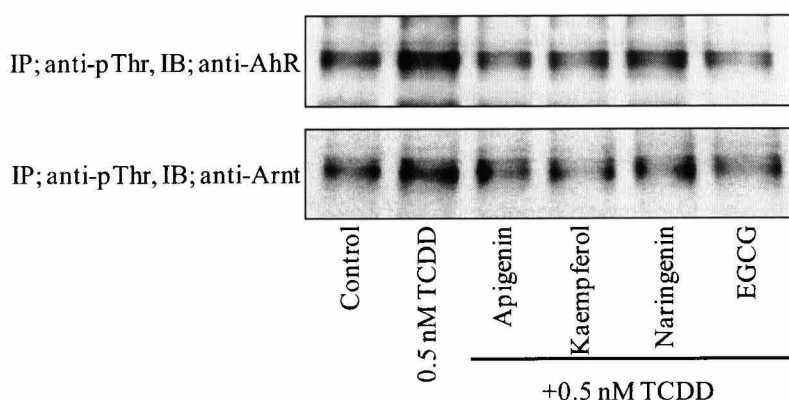


B
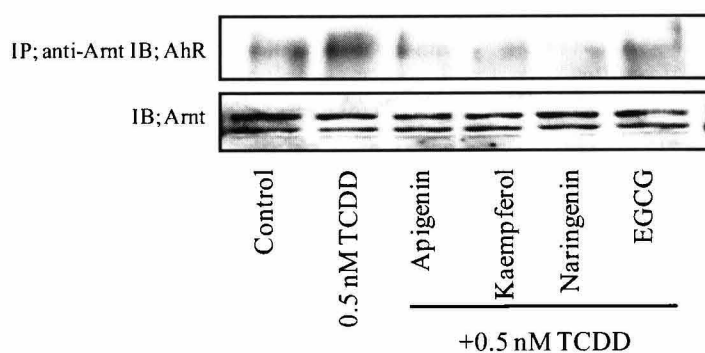


**Fig. 4-5. Effect of flavonoids on phosphorylation of the AhR and Arnt and formation of their heterdimer.** The cells were cultured in serum-starved MEM and treated with 10 μM flavonoid for 10 min and then 0.5 nM TCDD for another 30 min. A, Phosphorylated AhR and Arnt were detected by pThr immunoprecipitation (IP) followed by Western blotting (IB) with anti-AhR and anti-Arnt antibodies. B, Formation of heterodimer between the AhR and Arnt was detected by IP with anti-Arnt antibody and IB by anti-AhR antibody (upper). Expression level of Arnt was confirmed by IB (bottom).

**4-3-5 MEK1/2 was involved in AhR transformation and phosphorylation of ERK1/2 was suppressed by EGCG**

Previous studies demonstrated that that MEK1/2 is involved in phosphorylation of the AhR in Hepa-1c1c7 cells (102). The author confirmed the actions of MEK1/2 on phosphorylation of the Arnt and the DNA-binding activity of AhR. U0126, a MEK1/2 inhibitor, inhibited phosphorylation of Thr residues of not only the AhR but also Arnt (Fig. 4-6A), decreased the DNA-binding activity of AhR to about 60 % of the TCDD-induced level (Fig. 4-6B), and reduced phosphorylation level of ERK1/2, a substrate of MEK (Fig. 4-6C). These results suggested that the phosphorylated MEK1/2 directly and/or indirectly phosphorylated Thr residues of the AhR and Arnt. The author confirmed that EGCG decreased phosphorylation level of ERK1/2 induced by TCDD (Fig. 4-6C), suggesting that inhibition of MEK1/2 pathway by EGCG might be contributed to decrease the phosphorylation level of the AhR and Arnt.
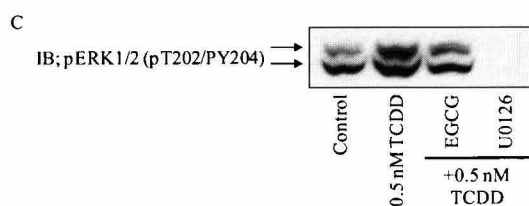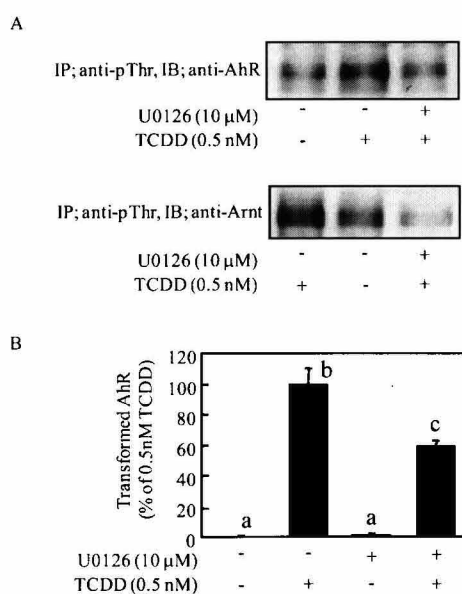


**Fig. 4-6. Effects of U0126 on AhR transformation.** The cells were cultured in serum-starved MEM containing 5 mg/ml BSA for 24 h. These cells were pre-treated with 10 μM U0126 for 30 min and treated with 0.5 nM TCDD for further 30 min. A, Phosphorylated AhR (upper) and Arnt (bottom) were detected by IP with pThr followed by IB with anti-AhR and Arnt antibodies. B, DNA-binding activity of the AhR was measured by SW-ELISA. Data were represented as the mean ± SE (n=3) and different letters indicate statistically significant differences evaluated by Tukey multiple comparison test. C, The cells were treated with treated with EGCG or U0126 at 10 μM for 30 min before adding 0.5 nM TCDD. Phosphorylated ERK1/2 was detected in cell lysate by Western blot analysis.

**4-4 Discussion**

Previously it was reported that flavonoids suppressed AhR transformation in a subclass-dependent manner in order of flavone=flavonol>flavanone>catechin (63,108), and suggested that the interaction of these flavonoids with the AhR complex was different among their subclasses (106). In this chapter, the author demonstrated that the suppression mechanism of flavonoids on AhR transformation in detail using mouse hepatoma Hepa-1c1c7 cells. To assess nuclear translocation level of the AhR, the GFP-AhR was co-expressed with FLAG-XAP2 in the cells. As expected the GFP-AhR translocated into nucleus in a ligand-dependent manner, while the GFP-AhRF345A was abolished ligand-dependency. Nuclear translocation of the GFP-AhR and dissociation of partner proteins were inhibited by flavone and flavonol subclasses but not flavanone and catechin subclasses (Fig. 4-2, 3 and 4), phosphorylation of the AhR and Arnt and formation of their heterodimer were suppressed by not only flavone and flavonol but also flavanone and catechin subclasses (Fig. 4-5). This is the first report that flavonoid showed different suppression mechanisms on AhR transformation; nuclear translocation and dissociation of partner proteins. In addition, the author demonstrated that phosphorylation level of the AhR and Arnt was dominated by ERK1/2, and EGCG reduced phosphorylation of ERK1/2 (Fig. 4-6), and subsequently it suppressed AhR transformation.

The AhR, which consists of the nuclear localization signal (NLS) and nuclear export signal (NES), is nucleocytoplasmic shuttling protein. In the absence of ligand, the AhR predominantly locates in cytoplasm (109,110), whereas ligand-binding triggers dissociation of hsp90 and XAP2 from the AhR (6,13,16) and nuclear translocation of AhR as a function of NLS (109). My finding demonstrated that XAP2 modulated subcellular localization of the AhR as the same as result in the previous report (16), suggesting the suppressive effects of flavone and flavonol subclasses on the translocation are, at least in part, due to the inhibition of dissociation of XAP2. As to hsp90, dissociation of this chaperon protein from the AhR induces conformational change in the AhR, and this change is responsible for formation of heterodimer between the AhR and Arnt (13). This suggests flavone and flavonol inhibited the conformational change by suppressing dissociation of hsp90. From the results in the cell-free experiments flavanone subclass also inhibits binding of agonist competitively (61,92,106). However, naringenin, flavanone subclass, did not suppress nuclear translocation and dissociation of hsp90 and XAP2 in

Hepa-1c1c7 cells unexpectedly in this chapter (Fig. 4-2, 3 and 4). Binding affinity of compound to

the AhR appears to depend on its structural constraints: co-planer structure with the van der Waals

dimensions of 14x12x5Å (111). The affinity of compounds in flavanone subclass to the AhR is

lower than that of flavone and flavonol subclasses (106). In addition, cellular permeability of

flavanone is lower than that of flavone and flavonol (112-114). These results suggest that both

affinity to the AhR and cellular permeability of the compounds in flavanone subclass are not

enough to suppress nuclear translocation and dissociation of partner proteins. As to catechin

subclass, it is demonstrated that EGCG does not inhibit binding of agonist to the AhR in rat and

mouse cytosol fraction (94,106) but it has weaker binding affinity to the AhR complex than

flavone, flavonol and flavanone subclasses (106). It is suggested that compounds in catechin

subclass bind to multiple sites of the AhR complex without specificity. The difference of

interacting with the AhR in catechin subclass from others might be lead to the difference effect on

nuclear translocation of the AhR and dissociation of its partner proteins. Previous report showed

that EGCG suppressed dissociation of hsp90 and XAP2 by interacting with recombinant hsp90

(94). Since the AhR function is also regulated by not only the partner proteins in complex but also

other proteins such as retinoblastoma protein (115) and RelB (116), and also by related

phosphorylation cascades (102-104). It is, therefore, suggested that these regulatory factors may

affect EGCG-caused dissociation of the partner proteins of AhR. Further study is needed to clarify

this issue in various cell linages in detail.

The phosphorylation states of proteins including ligand-activated transcription factors

play an important role in their DNA-binding properties (117). Phosphorylation of the AhR and/or

Arnt is essential for their dimerization and DNA-binding activity (102-104). Finding in this study

demonstrated that flavonoids suppressed phosphorylation of the AhR and Arnt and formation of

their heterodimer (Fig. 4-5). It is suggested that inactivated AhR could not undergo

phosphorylation by kinases because the partner proteins of AhR concealed the phosphorylation

sites. In addition, the AhR dissociating hsp90 is received conformational change that is

responsible for formation of the heterodimer between the AhR and Arnt, since the PAS domain of

AhR overlaps the ligand-binding and Arnt-dimerization domains (13). Taken together, in the case

of flavanone and catechin, they suppress phosphorylation of the AhR and Arnt (Fig. 4-5), whereas

flavone and flavonol retain the AhR as the inactive form and protect its conformational change by

inhibiting the binding of agonist. Catechin is reported as an inhibitor of MAPKs because it reduces the phosphorylation level of ERK1/2 by inhibiting MEK1/2 in cultured cells (118,119). EGCG binds to the 67-kDa laminin receptor expressed in various cancer cells and alters downstream signal transductions (98,120): Methylated catechin inhibits phosphorylation of ERK through the 67-kDa laminin receptor in KU-812 cells (121). Taken together, the author hypothesized that EGCG reduced phosphorylation level of the AhR and Arnt by inhibiting TCDD-induced phosphorylation of ERK1/2. Indeed, the author found EGCG reduced the phosphorylation level of ERK1/2 (Fig. 4-6) and confirmed U0126, a specific inhibitor of MEK1/2, suppressed the DNA-binding activity of AhR and inhibited phosphorylation of the AhR and Arnt (Fig. 4-6). This is the first report that the ERK1/2 phosphorylation cascade is involved in phosphorylation of Thr residues of Arnt. It is presumed that phosphorylation state of Thr residues of either the AhR or Arnt regulates DNA binding step resulting in a transcriptional activity (36). It is, therefore, suggested that inhibition of MEK/ERK pathway by EGCG, at least one part, is contributed to suppress AhR transformation in Hepa-1c1c7 cells.

**4-5 Summary**

The AhR is a ligand activated transcription factor. AhR transformation is induced by its agonist such as dioxin and mediates biological and toxicological effects. Previously study demonstrated that flavonoids suppressed AhR transformation induced by its typical agonist, TCDD in a subclass-dependent manner. In this study, the author investigated the suppression mechanisms of each subclass on AhR transformation in mouse hepatoma Hepa-1c1c7 cells. Flavone and flavonol subclasses suppressed nuclear translocation of AhR and dissociation of hsp90 and XAP 2 from the AhR caused by TCDD, whereas flavanone and catechin did not. These four flavonoid subclasses decreased the phosphorylation level of the AhR and Arnt and formation of heterodimer between these proteins. Since certain flavonoids are also known to inhibit of MAPKs, the author confirmed contribution of MEK/ERK cascade in AhR transformation by using U0126, an inhibitor of MEK1/2. U0126 suppressed TCDD-caused phosphorylation of the AhR and Arnt followed by DNA-binding activity of the AhR. Catechin reduced TCDD-caused phosphorylation of ERK1/2. It is, therefore, suggested that inhibition of MEK/ERK pathway is one of the suppression mechanisms of catechin on AhR transformation in Hepa-1c1c7 cells. In conclusion flavone and flavonol suppressed AhR transformation by inhibiting nuclear translocation of AhR, whereas flavanone and catechin do it by inhibiting phosphorylation of the AhR and Arnt.

# Chapter 5. Inhibition of P-glycoprotein increased the suppressive effect of kaempferol on transformation of an aryl hydrocarbon receptor

### 5-1 Introduction

Flavonols such as kaempferol and galangin are reported to suppress the AhR transformation *in vitro* (122). Recently, it was reported that these flavonols also suppressed transcriptional activity of the AhR after permeating human colon adenocarcinoma Caco-2 cells, a model for enterocytes (108). It is also reported that the kaempferol was substrates for the ATP-dependent efflux transporter P-glycoprotein (P-gp) (123). P-gp is a 170 kDa plasma membrane protein encoded by the human *MDR1* and *MDR3* genes, the murine *mdr1a, mdr1b,* and *mdr* genes and rat *pgp1, pgp2,* and *pgp3* genes (124). P-gp is highly expressed in many tumor cell lines and also found in many normal tissues including the intestinal epithelium, brain capillary endothelial cells, hepatocytes, and renal tubular cells, indicating that it plays an important role in drug absorption, excretion, and tissue distribution (124). Bioavailability of glabridin, a prenyl flavonoid, in rats was increased by orally injected verapamil, a specific inhibitor of P-gp (125). Therefore, an inhibition of P-gp will affect the suppressive effect of flavonoid on the AhR transformation. In this chapter, the author investigated suppressive effect of kaempferol and *ginkgo biloba* extract (EGb) containing 24% flavonol on AhR transformation *in vivo* and examined whether the inhibition of P-gp by verapamil enhanced the effect of kaempferol *ex vivo* and *in vitro*.

### 5-2 Materials and Method

### 5-2-1 Materials

MC and corn oil were purchased from Nacalai Tesque (Kyoto, Japan). TCDD was obtained from AccuStandard (New Haven, CT), and dissolved in dimethylsulfoxide (DMSO). [$^3$H]-Kaempferol was obtained from Moravek Biochemicals, Inc. (Brea, CA). Kaempferol and propylene glycol were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). EGb, which contained 24 % flavonoids including kaempferol, quercetin, and isorhamnetin and 6 % terpenes, was a product of Asahi Food and Healthcare Co., Ltd. (Tokyo, Japan). Rhodamine-123

was obtained from Sigma (St. Louis, MO).

### 5-2-2 Animal treatments

Animal treatment in this study conformed to the "Guidelines for the care and use of Experimental Animals, in Rokkodai Campus, Kobe University." The author carried out following two experiments.

Experiment I: Eighteen male C57BL/6 mice (6 weeks old, 20-25 g, purchased from Japan SLC, Shizuoka, Japan) were divided at random into 3 groups of 6 each. After diet was withheld for 18 h, mice in these groups were received orally kaempferol (100 mg/kg body weight), EGb (100 mg/kg body weight), and propylene glycol (10 ml/kg body weight) as a vehicle control. The mice in each group were further divided into two subgroups of three each. One subgroup was intraperitoneally injected MC (10 mg/kg body weight) 2 h after the first administration, whereas the other was injected corn oil (5 ml/kg of body weight) alone as a vehicle control. These mice were killed 2h after the MC-injection. The liver was removed and used for the *in vivo* experiment.

Experiment II: For the *ex vivo* experiment, eighteen male C57BL/6 mice (6 weeks old were divided at random into 3 groups of 6 each. After diet was withheld for 18 h, one group was orally received verapamil (100 mg/kg body weight) and the other groups were received deionized water (10 ml/kg body weight) as a vehicle control. After 2 h, verapamil-dosed, and one water-dosed group were orally given kaempferol (100 mg/kg body weight), and the rest water-dosed group was given propylene glycol (10 ml/kg body weight) as a vehicle control. These mice were killed 4 h after the kaempferol-injection. The liver was removed, prepared a cytosolic fraction, and used for following *ex vivo* experiment.

### 5-2-3 *Ex vivo* experiment

Ex vivo experiment was carried out as described previously (126). The cytosolic fraction (12 mg protein/ml) was incubated with TCDD at 1.0 nM in HEDG buffer at 20°C for 2 h. After incubation, the mixture was subjected to SW-ELISA for measurement of the AhR transformation as follows.

**5-2-4 SW-ELISA**

To evaluate AhR transformation, the DNA-binding activity of AhR was measured by SW-ELISA (62). For the *in vivo* and cultured cell experiments, the reaction mixture consisted of 12.5 µl of nuclear extract (12.5 mg proteins/assay) from the liver of mice or Hepa-1c1c7 cells (126) and 37.5 µl of HEDG buffer. For the *ex vivo* experiments, the reaction mixture consisted of 10 µl of HEDG buffer containing 0.75 M KCl and 40 µl of the cytosolic fraction. These reaction mixtures were subjected to SW-ELISA described in previously (86). For animal samples, a specific antibody against AhR (Anti-AhR, MA1-514, Affinity BioReagents, Golden, CO) was used as the primary antibody.

**5-2-5 Cell culture and treatments**

Mouse hepatoma cell line, Hepa-1c1c7 cells were grown and maintained at 37°C in α-minimum essential medium (α-MEM, Nissui Pharmaceutical, Tokyo, Japan) containing 10% fetal bovine serum (Sigma, St. Louis, MO), 4 mM L-glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin under a humidified 95% air-5% $CO_2$ condition.

The cells were seeded onto a 24-well culture plate at a density of $4 \times 10^5$ cells/well and incubated for 24 h. To measure the uptake of rhodamine-123 or kaempferol, the cells were treated with rhodamine-123 at 3.75 µM or [³H]-kaempferol at 1, 10 and 20 µM in Krebs-Ringer HEPES buffer (50 mM HEPES, pH 7.4, 137 mM NaCl, 4.8 mM KCl, 1.85 mM $CaCl_2$ and 1.3 mM $MgSO_4$) for 70 min, and washed three times with ice-cold phosphate buffered saline. To estimate the effect of verapamil, it was treated to the cells 10 min prior to the addition of rhodamine-123 or [³H]-kaempferol. The uptake of rhodamine-123 was quantified by measuring fluorescent intensity (excitation wavelength at 485 nm and emission one at 535 nm) using Wallac ARVO sx multilabel counter (Perkin-Elmer Life Sciences, Boston, MA), or the uptake of kaempferol was quantified by measuring the radioactivity in the cell lysate by a liquid scintillation counter.

To measure DNA-binding activity of AhR, verapamil was incubated with the cells seeded on 60-mm dishes (approximately 80% confluent) for 10 min and then kaempferol (10 µM) was incubated for another 10 min at 37°C. Thereafter, the cells were treated with TCDD at 0.5 nM (final concentration) or DMSO (1 µl/ml) as a vehicle control for further 1 h at 37°C. The nuclear extract was prepared from the cells (126) and subjected to SW-ELISA.

To measure cell viability, various concentrations of verapamil (0-100 μM) was added to the cells seeded on a 96-well plate (approximately 60% confluent) and incubated for 80 min at 37°C. After the cells were treated with 10 μl 4-[3-(4-Iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate solution (WST-1, Roche Diagnostics GmbH, Mannheim, Germany) for further 4 h, absorbance at 450 nm for the formazan product and 630 nm for reference were measured.

### 5-2-6 Statistical analysis

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

### 5-3 Results

### 5-3-1 Kaempferol and EGb inhibit MC-induced AhR transformation in the liver of mice

First, the author investigated the effect of kaempferol and EGb containing flavonol on the AhR transformation *in vivo*. As shown in Fig. 5-1, MC at 10 mg/kg body weight significantly promoted the transformation in the liver of mice as expected. Kaempferol and EGb at 100 mg/kg body weight completely suppressed the MC-induced transformation to a control level, but they did not affect the transformation in the liver of control animals.
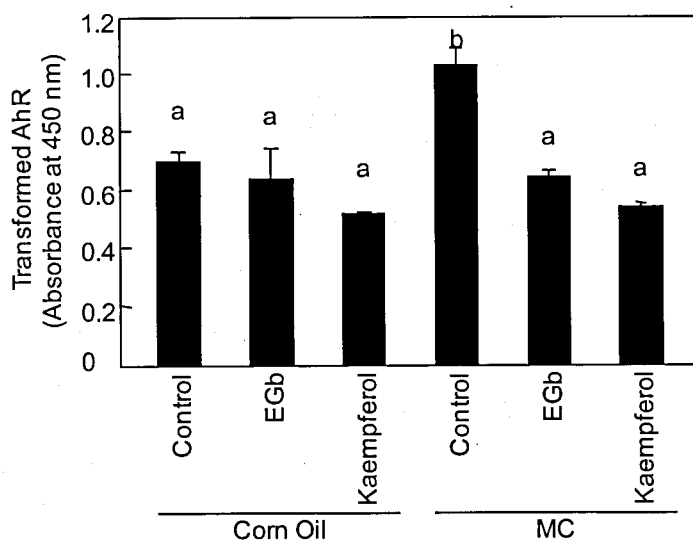


**Fig. 5-1. EGb and kaempferol suppress the DNA-binding activity of AhR induced by MC in the liver of mice.** Mice were orally administered kaempferol or EGb at 100 mg/kg body weight, followed by an intraperitoneal injection of MC at 10 mg/kg body weight. Hepatic nuclear protein was prepared and subjected to measure of AhR transformation by SW-ELISA as described in Materials and methods. Data are represented as the mean ± SE (n=3). Different letters indicate statistically significant differences evaluated by Tukey multiple comparison test.

## 5-3-2 Verapamil enhances suppressive effect of kaempferol on AhR transformation *ex vivo*

To confirm the effect of verapamil, *ex vivo* experiment was carried out (Fig. 5-2). TCDD at 1.0 nM induced the AhR transformation in a cytosolic fraction prepared from the liver of vehicle-dosed control mice. The cytosolic fraction from the liver of kaempferol-dosed mice tended to reduce the AhR transformation compared with that of vehicle-dosed control mice without significance. It was noteworthy that the cytosolic fraction from both verapamil and kaempferol-dosed mice significantly suppressed TCDD-induced AhR transformation compared with that from control mice. Therefore, pre-administration of verapamil may enhance the suppressive effect of kaempferol in the liver of mice.
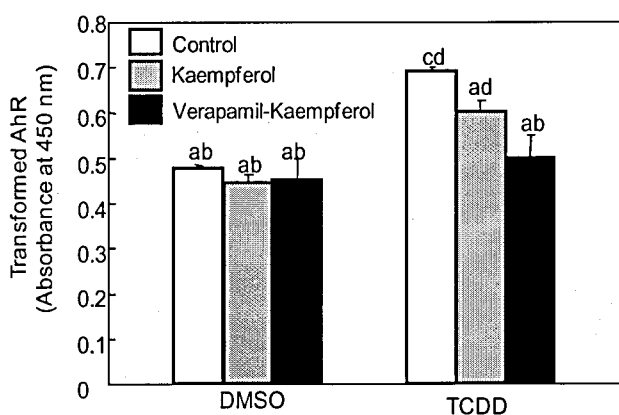


**Fig. 5-2. Kaempferol suppresses the DNA-binding activity of AhR and the effect is enhanced by verapamil *ex vivo*.** Mice were orally administered verapamil at 100 mg/kg body weight, followed by an intraperitoneal injection of kaempferol at 100 mg/kg body weight. A hepatic cytosolic fraction prepared from mice and subjected to measure of AhR transformation by SW-ELISA. Data are represented as the mean ± SE (n=3). Different letters indicate statistically significant differences evaluated by Tukey multiple comparison test.

## 5-3-3 The effect of verapamil on the uptake of kaempferol and its suppressive effect on the AhR transformation in Hepa-1c1c7 cells

It was confirmed that the uptake of [3H]-kaempferol was increased in a dose-dependent manner in Hepa-1c1c7 cells with almost the same percent of uptake in the treatments (Table 5-1). Uptake of rhodamine-123, a substrate of P-gp (127), was increased by verapamil (Fig 5-3A), indicating that verapamil could inhibit P-gp in Hepa-1c1c7 cells.Under this experimental condition, verapamil increased the uptake of kaempferol dose-dependently (Fig 5-3B). The uptake reached maximum at $10^2$ nM and decreased at higher concentrations. In addition, verapamil enhanced suppressive effect of kaempferol on TCDD-induced AhR transformation, and $10^2$ nM verapamil showed the strongest effect (Fig. 5-3C). Verapamil itself neither suppressed AhR transformation (Fig. 5-3D) nor decreased cell viability (Fig. 5-3E). Thus, verapamil enhanced suppressive effect of kaempferol on AhR transformation accompanied with the increase in the uptake of this flavonol without cytotoxicity in hepatocytes.

**Table 5-1 Cellular uptake of kaempferol in Hepa-1c1c7 cells**

| | Kaempferol ($\mu$M) | | |
| --- | --- | --- | --- |
| | 1 | 10 | 20 |
| Incorporated amount [a] (pmols/4x10^5 cells) | 21.4±1.83 | 227±12.5 | 427±33.1 |
| Percent of uptake | 7.13±0.61 | 7.58±0.42 | 7.13±0.55 |

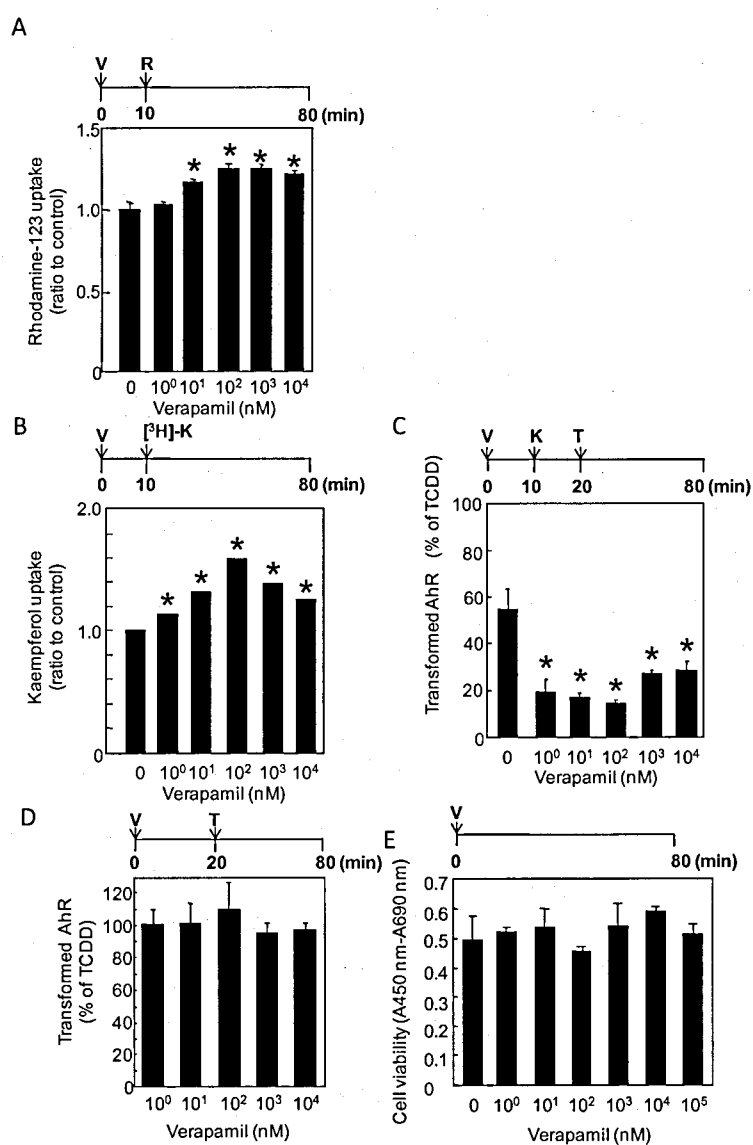[a] Each value represents the means ± SE from at least three independent experiments.



**Fig. 5-3 Cellular uptake and suppressive effect on AhR transformation of kaempferol were increased by verapamil.** Verapamil (V) at various concentrations, rhodamine-123 (R) at 3.75 $\mu$M, kaempferol (K) at 10 $\mu$M, and TCDD (T) at 0.5 nM were treated to the cells as illustrated in the upper part of each panel. A, Fluorescence intensity of rhodamine-123 incorporated into the cells was measured. B, [$^3$H]-kaempferol was used and radioactivity incorporated into the cells was measured. C and D, The nuclear extracts from cells were subjected to SW-ELISA. E, Cell viability was measured by WST-1 method described in Material and methods. Data was represented as the mean ± SE from three independent experiments. Asterisks indicate significant differences from the value of control (A and B), and from the value of TCDD-alone (C and D), ($p$<0.05, Student's $t$-test).

**5-4 Discussion**

The toxicological effects of dioxins are mainly induced by AhR transformation (128), and the liver is one of the major target organs of dioxins. Previously, kaempferol was reported to suppress AhR transformation in a cell-free system using rat liver cytosolic fraction (122) and in mouse hepatoma Hepa-1c1c7 cells (108). In this study, the author demonstrated that kaempferol and EGb suppressed AhR transformation induced by MC *in vivo* (Fig. 5-1), and verapamil, an inhibitor of P-gp, enhanced suppressive effect of kaempferol in *ex vivo* (Fig. 5-2) and cultured cells (Fig. 5-3). These findings indicate verapamil increased the amount of kaempferol in the liver through the inhibition of P-gp and enhanced the suppressive effect of kaempferol on AhR transformation. This is the first report that flavonoid suppressed AhR transformation *in vivo* and the inhibition of P-gp enhanced this suppressive effect.

Flavonol aglycone but not glycoside showed suppressive effect on AhR transformation *in vitro* (122). In this study, the author used kaempferol aglycone for estimation of the suppressive effect on AhR transformation *in vivo*. It is known that flavonol undergoes metabolism; i. e., glucuronidation and/or sulfation in the body (129). It is reported that a conjugation position-specificity of quercetin metabolites on AhR transformation (130). Therefore, not only kaempferol aglycone but its metabolites may suppress AhR transformation *in vivo*. Further study is needed to accumulate the results of the effect of metabolites of flavonoid. As to EGb, it contains flavonol such as kaempferol. After intake of EGb, kaempferol and phenolic acids, such as 4-hydroxyhippuric acid, 3-methoxy-4-hydroxyhippuric acid, 3,4-dihydroxybenzoic acid, and 4-hydroxybenzoic acid, are detected in urine or plasma (131,132). In previous report demonstrated that certain phenolic acids also suppressed AhR transformation in the cell-free system (126). Therefore, these metabolites, in addition to kaempferol, might be existed in liver and contributed to the suppressive effect *in vivo*.

P-gp is expressed in many normal tissues including small intestine, liver, brain and so on, and has important role in drug absorption, elimination, and distribution (124). Previously, it was reported that flavonols are excreted from cell through an efflux transporter such as P-gp (133). Verapamil is able to inhibit the P-gp-mediated efflux of its substrate by competitively (134), and kaempferol is reported as substrate for P-gp (123,133). From these reports, it is hypothesized that the inhibition of P-gp reduced excretion of kaempferol from the body, and this could enhance the

biological activity in *vivo*. In the present study, treatment with verapamil enhanced suppressive effect of AhR transformation in *ex vivo* (Fig. 5-2) and cultured cell experiments (Fig. 5-3) accompanied by the increment of cellular level of kaempferol. This is the first report that the inhibition of P-gp enhanced suppressive effect of flavonoid on AhR transformation. Previously, it is reported that bioavailability of glabridin, a prenyl flavonoid, is increased by oral intake of verapamil through inhibition of P-gp *in vivo* (125,135). Uptake of quinine, one of drug for malaria and excreted by P-gp, into the brain was increased by verapamil (136). These reports suggested that verapamil inhibited P-gp both in small intestine and in the liver, resulted in the increased kaempferol lead to enhance of the suppressive effect on AhR transformation *in vivo*. It is necessary to investigate the effect of verapamil on bioavailability, plasma concentration and tissue distribution, of kaempferol in future. Since certain flavonoid were reported as a substrate for P-gp (137), it is also interesting issue to investigate the measurement of the suppressive effect by combination of flavonoids.

**5-5 Summary**

Dioxins enter the body mainly through diet, bind to the AhR, and cause various toxicological effects. In this study, the author showed that oral administration of kaempferol or EGb containing 24% flavonol at 100mg/kg body weight suppressed MC (10 mg/kg body weight)-induced AhR transformation in the liver. This suppressive effect of kaempferol was increased by verapamil, an inhibitor of P-gp in *ex vivo* experiment using a cytosol fraction of mice liver and TCDD. An increase in the suppressive effect of kaempferol by verapamil was also observed in mouse hepatoma Hepa-1c1c7 cells accompanied with an increase in the uptake of kaempferol into the cells. In conclusion, an inhibition of P-gp enhanced suppressive effect of kaempferol on AhR transformation through the increase in the intracellular kaempferol level.

# Chapter 6. Cacao polyphenol extract suppresses transformation of an aryl hydrocarbon receptor in C57BL/6 mice

### 6-1 Introduction

Cacao polyphenol extract (CPE), an ingredient of cocoa and chocolate, has various beneficial effects such as anti-oxidative effect (138), inhibition of diabetes-induced cataract (139), prevention of atherosclerosis (140), and reduction of hyperglycemia (141). CPE is rich in polyphenols including catechins and their oligomers, procyanidins (142). After the administration of cocoa, conjugated forms of (−)-epicatechin (EC) to glucuronide and sulfate and a methylated form, 3′-*O*-methyl (−)-epicatechin (MEC), are detected in the plasma in addition to the intact form (143). EC and (−)-epicatechin-(4β-8)-(−)-epicatechin, procyanidin B2 (PB2), reach the colon without absorption or metabolism in the small intestine, where they are catabolized by colonic microflora into low-molecular weight phenolic acids (144,145). It was also reported that components of cacao and their metabolites were excreted into urine (146). These reports suggest that the beneficial effects of CPE are, at least in part, due to polyphenols and their metabolites.

Previous reports demonstrated that green and black tea extracts suppressed the AhR transformation *in vitro* (66,75). Polyphenols derived from plant foods, such as flavonoids including catechins, curcumin and resveratrol, are reported to suppress the transformation *in vitro* (63,66,76,147). Recently, it was demonstrated that flavonoids also suppressed the transformation after permeating human colon adenocarcinoma Caco-2 cells, a model for enterocytes (108). These findings suggest that polyphenol-rich plant foods may suppress the AhR transformation and downstream signal transduction events, such as the induction of drug-metabolizing enzymes, *in vivo*. Previously, the author demonstrated that an intake of molokhia (*Corchorus olitorius* L.) extract inhibited the translocation of AhR into the nucleus and its transformation in the liver of SD rats (62). However, data demonstrating the inhibitory effect of dietary agents on the AhR transformation *in vivo* is scare. In this study, the author examined whether the intake of CPE suppressed the AhR transformation induced by 3-methylcholanthrene (MC), an agonist of the AhR, and modulated the induction of drug-metabolizing enzymes.

**6-2 Materials and methods**

**6-2-1 Materials**

MC and corn oil were purchased from Nacalai Tesque (Kyoto, Japan). TCDD was obtained from AccuStandard (New Haven, CT), and dissolved in dimethylsulfoxide (DMSO). CPE, procyanidins, 3'-*O*-methyl-(+)-catechin (MCA), and MEC were prepared according to a previous report (142). The CPE used in this chapter contains 2.5% (+)-catechin, 6.6% EC, 3.8% PB2, 2.9% procyanidin C1 (PC1) and 1.8% cinnamtannin A2 (CA2) (142). EC and (+)-catechin were from Kurita Kogyo (Tokyo, Japan). Hippuric acid, ferulic acid (FA), caffeic acid (CA), 3,4-dihydroxyphenylacetic acid (diHPAc), and *m*-hydroxyphenylacetic acid (mHPAc) were obtained from Sigma Aldrich (St. Louis, MO). *m*-Coumaric acid (mCO) and *p*-coumaric acid (pCO) were purchased from Extrasynthèse (Genay, France). *m*-Hydroxybenzoic acid (mHBA), *m*-hydroxyphenylpropionic acid (mHPP), and *p*-hydroxyhippuric acid (pHHA) were obtained from Wako Pure chemical Industries, Ltd. (Osaka, Japan), Lancaster Synthesis Ltd (Morecambe, UK) and Bachem Holding AG (Bubendorf, Switzerland), respectively. All other reagents were of the highest grade available from commercial sources.

**6-2-2 Cell-free experiment**

Measurement of DNA-binding activity of AhR in cell-free experiments using rat liver cytosol was described in Chapter2. Compounds used in this study were listed in Table 6-1.

**6-2-3 Cultured cell experiment**

An intestinal permeability system using human colon adenocarcinoma Caco-2 cells and mouse hepatoma Hepa-1c1c7 cells was constructed according to a previous report (62) with some modifications. Briefly, the differentiated Caco-2 cells on the inserts were washed with fresh medium, kept in Hanks' balanced salt solution (HBSS) for 30 min at 37°C, and used for transport experiments. An aliquot of 100 µl of CPE dissolved in HBSS (100 µg/ml) was added to the apical side of inserts. The same volume of HBSS containing DMSO (1 µl/ml) was used as a vehicle control. The basorateral side was filled with 600 µl of HBSS. After incubation for 2 h at 37°C, the basolateral solution was collected. Caco-2-permeated CPE or intact CPE itself (100 µg/ml) was added to Hepa-1c1c7 cells seeded on 60-mm dishes (approximately 80% confluent) for 2h at 37°C.

Thereafter, the cells were treated with TCDD at 0.1 nM (final concentration) or DMSO as a vehicle control for a further 2 h at 37°C. The nuclear extract was prepared from Hepa-1c1c7 cells (91), and used to measure the DNA-binding activity as follows.

**Table 6-1. Polyphenols in CPE and their related chemicals used in this chapter**

| Compounds | Abbreviations |
|---|---|
| Cacao extract | CPE |
| *Procyanidins* | |
| procyanidin B2, [epicatechin-(4β-8)-epicatechin] | PB2 |
| procyanidin B5; [epicatechin-(4β-6)-epicatechin] | PB5 |
| procyanidin C1 | PC1 |
| cinnamtannin A2 | CA2 |
| *Catechins and its metabolites* | |
| (+)-catechin | |
| (−)-epicatechin | EC |
| 3'-*O*-methyl-(+)-catechin | MCA |
| 3'-*O*-methyl-(−)-epicatechin | MEC |
| *Phenolic acids* | |
| hippuric acid | HA |
| ferulic acid | FE |
| caffeic acid | CA |
| *m*-coumaric acid | mCO |
| *p*-coumaric acid | pCO |
| *m*-hydroxybenzoic acid | mHBA |
| 3,4-dihydroxyphenylacetic acid | diHPAc |
| *m*-hydroxyphenylacetic acid | mHPAc |
| *m*-hydroxyphenylpropionic acid | mHPP |
| *p*-hydroxyhippuric acid | pHHA |

**6-2-4 Animal treatments**

Animal treatment in this study conformed to the "Guidelines for the care and use of Experimental Animals, in Rokkodai Campus, Kobe University." Twenty-four male C57BL/6 mice (6 weeks old, 20-25 g, purchased from Japan SLC, Shizuoka, Japan) were divided at random into two groups of 12 each (CPE- or water-group). All mice were withheld diet for 18 h, and received orally a CPE (100 mg/kg body weight) suspension in deionized water or vehicle alone (10 ml/kg body weight) as a control. These mice were further divided into two sub-groups of 6 each. One sub-group was given MC (10 mg/kg body weight) in corn oil through an intraperitoneal injection

1 h after the administration of CPE or water, while the other was given corn oil (5 ml/kg body weight) as a vehicle control. Half of the mice in each sub-group (3 each) were killed 3 h after the CPE-injection and the rest, 25 h after. The liver was removed and used for experiments.

### 6-2-5 *Ex vivo* experiment

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

### 6-2-6 SW-ELISA

The DNA-binding activity of AhR was measured by SW-ELISA (62,86) described in Chapter 2. For the cell-free system and *ex vivo* experiments, the reaction mixture consisted of 10 μl of and cultured cell experiments, the reaction mixture consisted of 12.5 μl of nuclear extract (12.5 μg proteins/assay) from the liver of mice or Hepa-1c1c7 cells, and 37.5 μl of HEDG buffer. The reaction mixture was subjected to SW-ELISA. For *in vivo* samples, a specific antibody against AhR (Anti-AhR, MA1-514, Affinity BioReagents, Golden, CO) was used as the primary antibody.

### 6-2-7 EMSA

EMSA was also performed to determine the DNA-binding activity of AhR (66). Briefly, nuclear extract (10 μg protein) from the liver of mice was incubated with 500 ng of poly [dI-dC], and the $^{32}$P-labeled DRE probe (30 kcpm, 10 fmols) in HEDG buffer for 15 min. The mixture was then loaded onto a 4% nonstacking polyacrylamide gel in TBE buffer (25 mM Tris, 22.5 mM borate, and 0.25 mM EDTA). After electrophoresis, the AhR/DRE complex was visualized by autoradiography, and quantitatively analyzed by Gel-Pro Analyzer (Media cybernetics, Bethesda, MD).

### 6-2-8 Western blot analysis

A post-nuclear fraction (5 μg protein) for the detection of CYP1A1 and the nuclear

extract (3 μg protein) for the AhR and Arnt were separated by 10% and 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, respectively. The proteins were transferred onto a polyvinylidene difluoride membrane (GE Healthcare Bio-Sciences Corp., Piscataway, NJ). This was followed by the blocking of non-specific binding with 1% skim milk in PBST for CYP1A1 or commercial blocking buffer (Nacalai Tesque, Kyoto, Japan) for the others for 1 h. The membrane was incubated with primary antibody for CYP1A1 (Anti-CYP1A1, 1:5000, Daiichi Pure Chemicals, Tokyo, Japan), AhR (Anti-AhR, MA1-514, 1:1500), and Arnt (Anti-Arnt, C-19, 1:3000, Santa Cruz Biotechnology, Santa Cruz, CA), and horseradish peroxidase-conjugated secondary antibodies. Immune complexes were visualized using ECL plus reagent (GE Healthcare Bio-Sciences Corp.) according to the manufacturer's instructions, and quantitatively analyzed by Gel-Pro Analyzer.

### 6-2-9 Immunoprecipitation

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

### 6-2-10 Measurement of QR and GST activities

QR and GST activities were measured in the cytosolic fraction according to the methods of Lind *et al.* (148) and Habig *et al.* (149), respectively. Briefly, the reaction mixture for QR activity consisted of 10 μl of the cytosolic fraction (0.11-0.22 μg protein), 50 mM Tris-HCl (pH 7.5) containing 0.08% Triton-X100, 0.5 mM NADPH, and 0.077 mM cytochrome *c*. To start the reaction, 0.1 ml of 0.3 mM menadione was added to the mixture, and the change in absorbance at 550 nm was monitored for 3 min. For the measurement of GST activity, the reaction mixture consisted of 1 μl of the cytosolic fraction (0.011-0.022 μg protein), 2.7 ml of 111 mM potassium phosphate buffer, pH 7.5, and 0.1 ml of 30 mM glutathione (reduced form). To start the reaction,

0.1 ml of 30 mM 1-chloro-2,4-dinitrobenzen was added to the reaction mixture, and the increase

in absorbance at 340 nm caused by the formation of 2,4-dinitrophenylglutathione was monitored

for 3 min. The activities of QR and GST were represented as μmol/min/mg protein.

### 6-2-11 Statistical analysis

Data are expressed as the mean ± SE of at least three independent determinations for

each experiment. Statistical significance was analyzed using Student's *t*-test or Duncun's method.

### 6-3 Results

### 6-3-1 Effect of CPE and its components on the DNA-binding activity of AhR in a cell-free

### system and cultured cells

The author examined the suppressive effect of CPE on the DNA-binding activity of AhR

by SW-ELISA in the cell-free system. As shown in Fig. 6-1A, CPE suppressed the activity in a

dose-dependent manner with a 50% inhibitory concentration ($IC_{50}$) value of 98 μg/ml. CPE

contains procyanidins, catechins, and other polyphenols (142). The effect of these polyphenols on

the AhR transformation was measured. (+)-Catechin, PB2, PB5 and CA2 suppressed the

transformation at 50 μg/ml (Fig. 6-1B). Methylated catechins and phenolic acids are detected as

metabolites and decomposed products of CPE, respectively (143-146). The suppressive effects of

these metabolites on the AhR transformation were also examined (Fig. 6-1C). MCA and MEC

suppressed the transformation (Fig. 6-1C) and their effect was stronger than that of the

corresponding original compound. Regarding the decomposed products, most of the phenolic

acids tested here showed a significant effect in the cell-free system.

In cultured cells, CPE (100 μg/ml) did not have a suppressive effect on Hepa-1c1c7 cells,

but it revealed a suppressive effect in Hepa-1c1c7 cells after permeation through the Caco-2 cells

(Fig. 6-2). This result indicated that metabolites of CPE could suppress the AhR transformation.
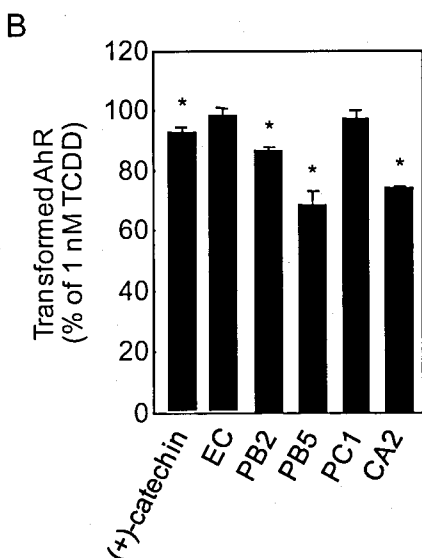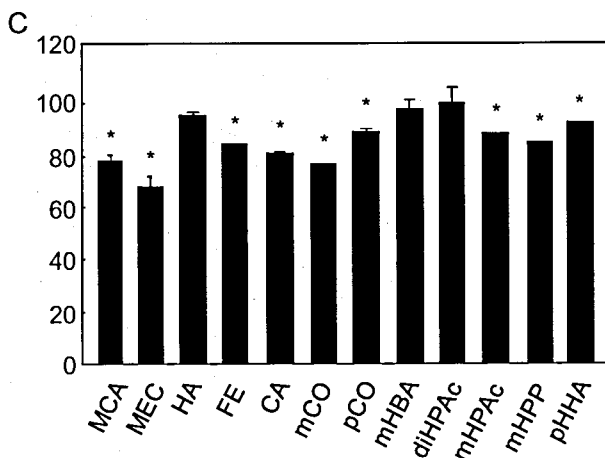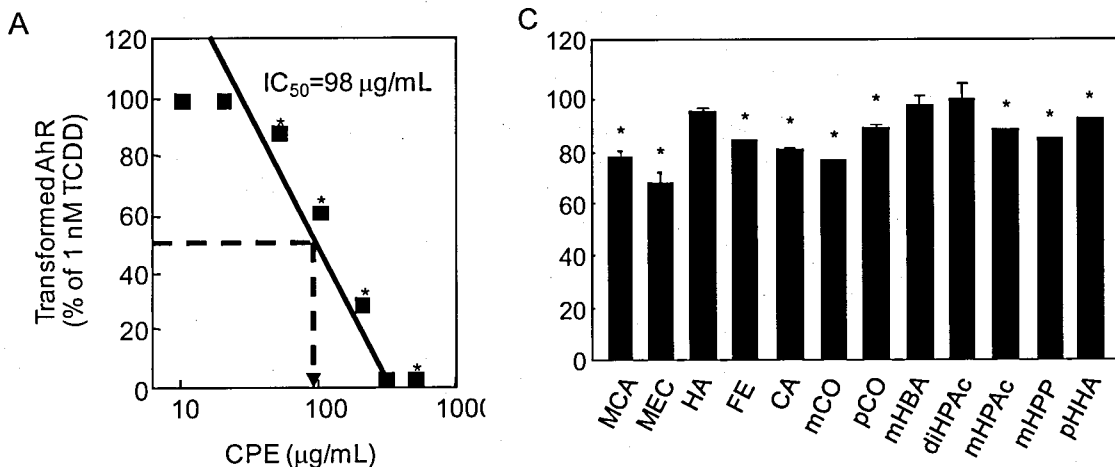
Fig. 6-1. Suppressive effects of CPE and its components and metabolites on TCDD-induced AhR transformation in the cell-free system. The suppressive effect of (A) CPE at indicated concentrations, (B) its components at 50 μg/ml and (C) metabolites at 50 μg/ml on TCDD-induced AhR transformation was evaluated by SW-ELISA. Data are shown as % of transformed AhR induced by 1 nM TCDD and represented as the mean ± SE from three independent experiments. Asterisks indicate significant differences from the value of TCDD-induced transformation ($p<0.05$, Student's $t$-test).
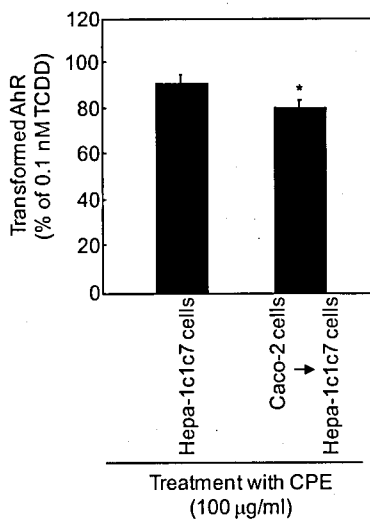


Fig. 6-2. Suppressive effects of CPE on TCDD-induced AhR transformation in cultured cells. Hepa-1c1c7 cells were treated with 100 μg/ml of CPE or CPE permeated through Caco-2 cells 10 min prior to 0.1 nM TCDD. Nuclear extracts from cells were subjected to SW-ELISA. Data are shown as % of transformed AhR induced by 0.1 nM TCDD and represented as the mean ± SE from three independent experiments. Asterisks indicate significant differences from the value of TCDD-induced transformation ($p<0.05$, Student's t-test).

**6-3-2 CPE inhibits MC-induced AhR transformation and regulates induction of drug-metabolizing enzymes in the liver of mice**

Next, the author investigated the effect of CPE on the AhR transformation and downstream events *in vivo*. After the mice were orally administered CPE at 100 mg/kg body weight followed by an intraperitoneal injection of MC at 10 mg/kg body weight, AhR transformation was evaluated by EMSA and SW-ELISA. EMSA results were shown that MC significantly promoted the transformation as expected (Fig. 6-3). CPE completely suppressed the MC-induced transformation to the control level in the liver 3 h and 25 h after the injection. The suppressive effect of CPE was confirmed by SW-ELISA (Table 6-2). The results were similar to the EMSA (Fig. 6-3).

**Table 6-2. Suppressive effects of CPE on the AhR transformation in the liver of mice**

| Time after MC-injection | Corn oil | | MC | |
|---|---|---|---|---|
| | Water | CPE | Water | CPE |
| 3 h | $0.332\pm0.012^a$ | $0.316\pm0.017^a$ | $0.392\pm0.013^b$ | $0.332\pm0.031^a$ |
| 25 h | $0.319\pm0.006^a$ | $0.291\pm0.019^a$ | $0.374\pm0.008^b$ | $0.312\pm0.013^a$ |

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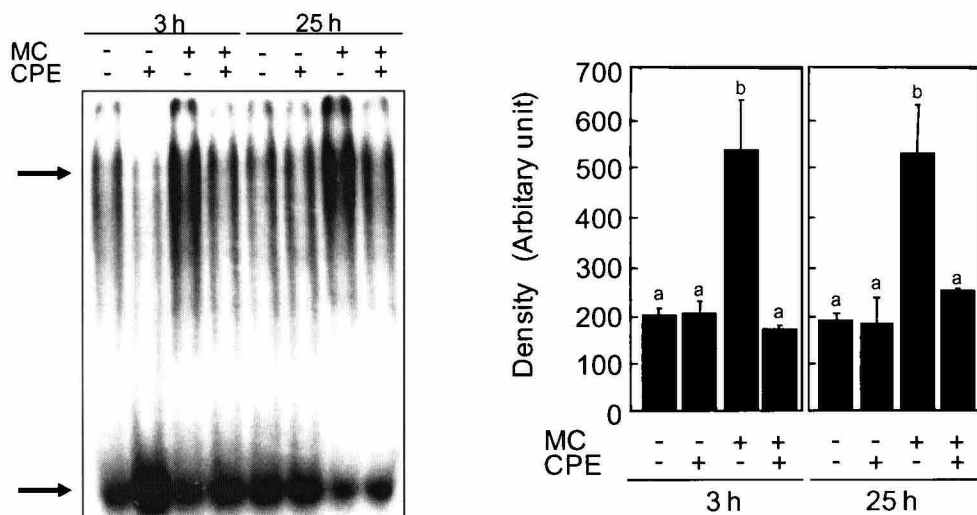


**Fig. 6-3. CPE suppresses the AhR transformation induced by MC in the liver of mice.** Mice were orally administered with CPE at 100 mg/kg body weight, followed by an intraperitoneal injection of MC at 10 mg/kg body weight, and sacrificed 3 or 25 h later. A representative result of EMSA is shown in the upper panel, and the quantified density of the AhR/DRE complex is shown in the lower panel. Data are represented as the mean ± SE (n=3). Different letters indicate statistically significant differences evaluated by multiple comparison tests using Duncun's method.

A



CPE also suppressed MC-induced CYP1A1 expression, downstream event of the AhR transformation, without a change in the constitutive level (Fig. 6-4A). In the case of QR and GST, MC enhanced both enzymatic activities in the liver (Fig. 6-4B and 4C). CPE significantly lowered the level of QR activity in both corn oil- and MC-dosed mice (Fig. 6-4B). On the other hand, CPE enhanced the GST activity in both groups additively (Fig. 6-4C).

**6-3-3 CPE inhibits the MC-induced dimerization between the AhR and Arnt but not nuclear translocation of the AhR into the nucleus in the liver**

**Fig. 6-4. Effects of CPE on CYP1A1 expression, QR activity, and GST activity in the liver of mice dosed with MC.** Mice were orally administered CPE at 100 mg/kg body weight the given an intraperitoneal injection of MC at 10 mg/kg body weight. (A) CYP1A1 was detected in the liver 25 h after the CPE-injection by Western blot analysis. Representative results (upper) and the quantified density (bottom) are shown. (B) QR and (C) GST activities were measured in the liver as described in Materials and methods. Data are represented as the mean ± SE (n=3). Different letters indicate statistically significant differences evaluated by multiple comparison tests using Duncun's method.
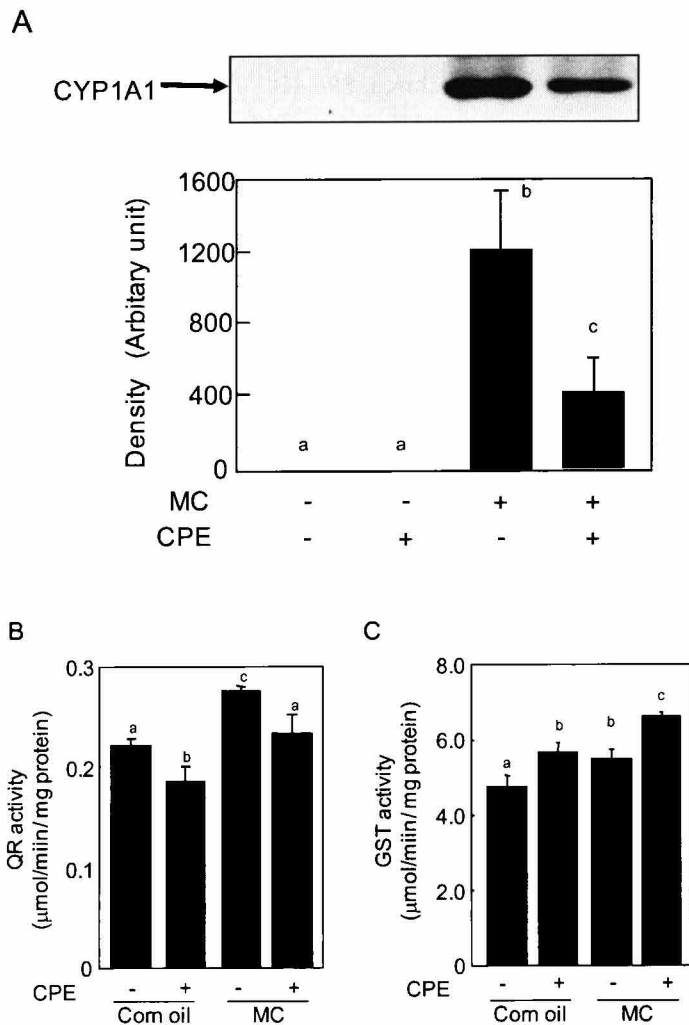
Possible inhibition of nuclear translocation of the AhR and/or heterodimerization of

AhR and Arnt were investigated. As expected, MC induced the translocation of the AhR and formation of the heterodimer without affecting the Arnt level (Fig. 6-5). CPE significantly inhibited the formation of the AhR/Arnt heterodimer, although it accelerated the translocation of AhR in MC-dosed mice.
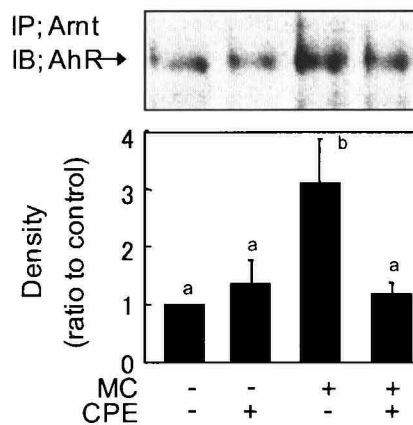
A
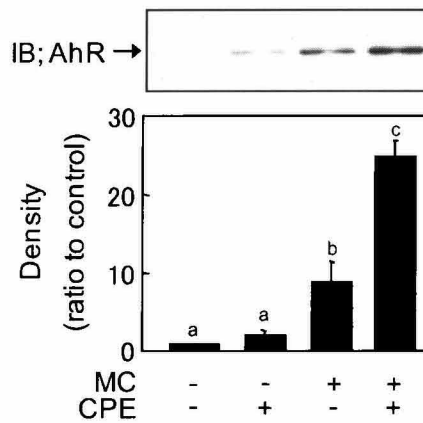


B
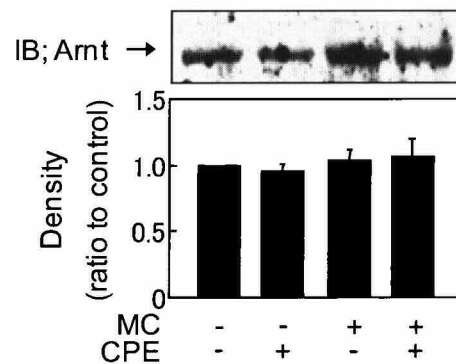


C



**Fig. 6-5. CPE suppresses formation of AhR/Arnt heterodimer but not AhR nuclear translocation in the liver of mice dosed with MC.** A hepatic nuclear fraction was prepared and (A) AhR and (B) Arnt were examined by Western blot analysis. (C) The hepatic nuclear fraction was immunoprecipitated with anti-Arnt antibody and the AhR/Arnt heterodimer was determined by immunoblot using anti-AhR antibody. Representative blotting results are shown (upper) and quantified density was calculated as a ratio to the control and represented (bottom) as the mean ± SE (n=3). Different letters indicate statistically significant differences evaluated by multiple comparison tests using Duncun's method.

**6-3-4 CPE suppresses the AhR transformation *ex vivo***

From the *in vivo* experiments, it was observed that orally dosed CPE suppressed the AhR transformation by inhibiting the formation of the AhR/Arnt heterodimer in the liver. To confirm the suppressive effect of CPE, an *ex vivo* experiment was carried out (Fig. 6-6). TCDD induced the AhR transformation at 0.1 or 1.0 nM in the cytosolic fraction prepared from the liver of mice given water in a dose-dependent manner. The cytosolic fraction from the liver of CPE-injected mice showed significantly reduced the AhR transformation compared with that of water-dosed mice. The suppressive effect did not disappear even 25 h after the CPE-injection, suggesting that active compound(s) reached the liver and remained there, at least, by 25 h.
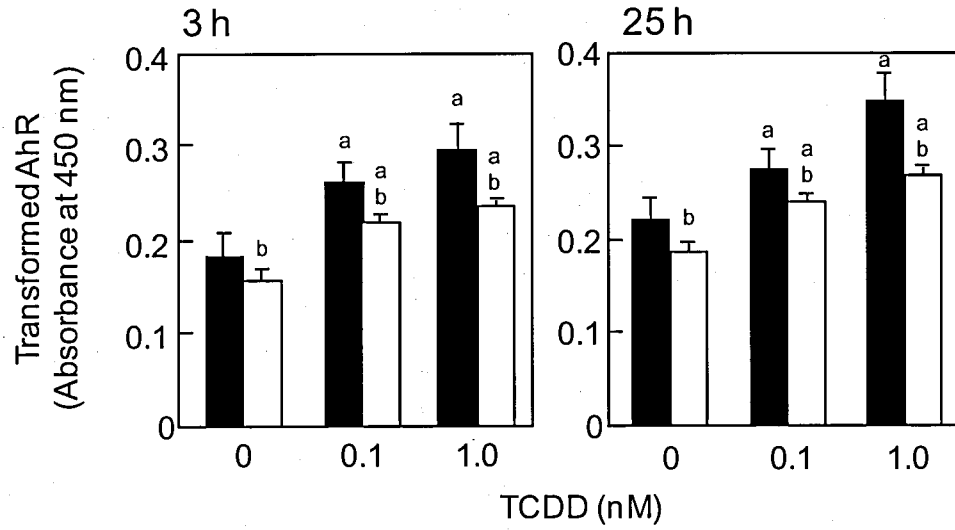
**Fig. 6-6. CPE suppresses the AhR transformation *ex vivo*.** A hepatic cytosolic fraction prepared from mice dosed with CPE at 100 mg/kg body weight or water was subjected to an evaluation of the AhR transformation induced by 0.1 or 1.0 nM TCDD *ex vivo*. The level of transformed AhR in the liver (A) 3 h and (B) 25 h after the CPE-injection was determined by SW-ELISA as described in Materials and methods. Data are shown as absorbance at 450 nm that reflects the transformed AhR and represented as the mean ± SE from three independent triplicate experiments (n=3). a, significant difference from each negative control (vehicle alone); b, from the corresponding water group ($p<0.05$, Student's $t$-test).

**6-4 Discussion**

Dioxins enter the body mainly through diet and accumulate in fatty tissues such as the liver and adipose tissue (67). The toxicological effects of dioxins are mainly induced by the transformed AhR (128), and the liver is one of the major organs of toxicological effects. In the present study, the author demonstrated that CPE suppressed the DNA-binding activity of AhR (Fig. 6-3) and subsequent induction of CYP1A1 expression (Fig. 6-4A) by inhibiting the formation of a heterodimer between the AhR and Arnt (Fig. 6-5) *in vivo*. The result from *ex vivo* experiments confirmed that the liver from the CPE-dosed mice tolerated the TCDD-increased DNA-binding activity of AhR (Fig. 6-6). Therefore, certain active compound(s) in CPE would be, at least in part, absorbed in the body and incorporated into hepatocytes and revealed the suppressive effects.

Many previous reports demonstrated that food stuffs such as green tea, black tea and vegetable extracts suppressed the AhR transformation and/or CYP1A1 expression *in vitro* (62,66,75), but *in vivo* experiments are scarce and inconclusive. The author reported that an orally administered molokhia extract suppressed the AhR transformation by inhibiting the nuclear translocation of the AhR in the liver of rats (62). In the present study, the administration of CPE suppressed the AhR transformation by inhibiting formation of the AhR/Arnt heterodimer in the liver of mice, although CPE accelerated the nuclear translocation of AhR (Fig. 6-5). The mechanism of action of CPE is different from that of molokhia, possibly be due to a difference in active component(s): CPE contains polyphenols abundantly (142) and these polyphenols and their putative metabolites exhibited suppressive effect in the cell-free system (Fig. 6-1). In the case of molokhia, lutein and chlorophylls are identified as the active compounds (65). Recently curcumin, a polyphenol in turmeric, was reported to inhibit formation of the AhR/Arnt heterodimer by inhibiting phosphorylation of the AhR and Arnt in Hepa-1c1c7 cells (107). In the same report, curcumin accelerated the nuclear translocation of AhR the same as CPE in this study. Therefore, polyphenols and/or their metabolites are candidates for the active compounds in CPE.

The CPE used in this study contains 2.5% (+)-catechin, 6.6% EC, 3.8% PB2, 2.9% PC1 and 1.8% CA2 (142). These compounds showed suppressive effects on the AhR transformation in the cell-free system (Fig. 6-1B). The bioavailability of catechins and procyanidins has been described as follows: Polyphenols undergo glucuronidation by UDP-glucuronosyl transferase,

sulfation by phenyl sulfotransferase and/or methylation by catechol-*O*-metyltransferase in enterocytes and/or hepatocytes (146,150-152). When cacao powder was administered orally to rats at 50 mg/kg body weight, 0.1 µM of MEC was detected in plasma (153), in addition to the detection of the glucuronid and/or sulfate form of catechin in urine (143). Moreover, over 1 µM of methylated catechin was detected in human plasma after the intake of cocoa powder (154). In the case of aglycone forms, EC and PB2 were detected at 0.1-1.1 µM and 0.5 µM, respectively, in rat serum after the oral administration of cacao powder at 50 mg/ kg body weight (153), 0.05-0.34 µM EC was detected in plasma 1 h after the oral administration of cocoa powder to rats at 150-1500 mg/kg body weight (143), and 1.13 µM EC and 5.23 µM (+)-catechin were detected in rat urine 24 h after the p.o.-injection of these catechins at 172 µmol/kg body weight each (146). These reports indicate that polyphenols in CPE, at least in part, are incorporated into the body. Methylated catechins are candidates for the active compounds in CPE, because their antagonistic effect was stronger than that of aglycones (Fig. 6-1). Procyanidins, which reach the large intestine without being absorbed in the small intestine, undergo biotransformation to phenolic acids by microflora (144,145). After the consumption of a polyphenol-rich meal, FE was detected in plasma, and other phenolic acids such as mHPP and pHHA were present in urine (144). In this study, these phenolic acids also suppressed the AhR transformation in the cell-free system (Fig. 6-1C). Moreover, CPE had a significant suppressive effect on the DNA-binding activity of AhR after its permeation through Caco-2 cells (Fig. 6-2). These results suggest that metabolites of polyphenols in CPE contributed to the suppression of AhR transformation.

The structure-activity relationships of polyphenols and phenolic acids tested in this study are as follows. Regarding the catechins and procyanidins, the activity of procyanidines is stronger than that of catechins (Fig. 6-1B), and the activity of CPE fractions separated by molecular size is tetramer- > trimmer- > dimmer- > monomer-fraction (data not shown). These results suggest that the higher degree of polymerization reveals the stronger suppressive effect on the DNA-binding activity of AhR. As to the phenolic acids, phenol propanoid derivatives with the less hydroxyl group in phenolic moiety show stronger activity, and binding of hydroxyl group to the meta-position also important to reveal the activity. In addition, propenoic acid has stronger activity than propanoic acid.

After ligand-binding, the AhR translocates into the nucleus and forms a heterodimer

with Arnt (101,155). Phosphorylation of AhR and/or Arnt was considered to be essential for the transcriptional activity of the heterodimer (102-104). In a previous study, curcumin inhibited the phosphorylation of AhR and Arnt (107). It was also reported that (–)-epigallocatechin-3-gallate suppressed the DNA-binding activity of AhR by interacting with hsp90 (94) without inhibiting the nuclear translocation of AhR. It is, therefore, suggested that the active component(s) in CPE interacts with partner proteins of the AhR and/or inhibits certain kinase activity, which is involved in the AhR transformation. Regarding the nuclear translocation, CPE accelerated nuclear translocation of the AhR in the liver of MC-dosed mice (Fig. 6-5). The author speculates the following reason on this point: After the conformational change of AhR by MC (13), the active component in CPE interacts with the transactivation domain in the AhR resulting in acceleration of nuclear translocation, since the transactivation domain is reported to regulate subcellular localization of the AhR without agonist (2). Further study is needed to clarify the mechanism of action of CPE and its components in detail.

Regarding drug-metabolizing enzymes, CYP1A1 protein, a phase I enzyme, is recognized as a typical gene product of the AhR transformation (30). The AhR also regulates the expression of QR and GST Ya (156,157). In this study, CPE suppressed MC-induced CYP1A1 expression and QR activity, whereas it promoted GST activity in the liver. GST and QR expression are regulated by not only the AhR/DRE but also nuclear factor erythroid 2-related factor 2 (Nrf2)/antioxidant response element (ARE) (158). Previous studies demonstrated that polyphenols enhanced GST activity *in vivo* (159,160). Therefore, the active component in CPE has a possibility to regulate expression of GST by Nrf2/ARE, while it may regulate expression of QR by AhR/DRE. Further experiments are needed to investigate the effect of CPE on Nrf2/ARE pathway in future. Since GST plays an important role in the conjugation of certain chemicals including carcinogens (161), the induction of GST activity by CPE, in addition to the suppression of MC-induced CYP1A1 expression may prevent the toxicological effects including carcinogenicity of polyaromatic hydrocarbons.

**6-5 Summary**

Dioxins enter the body through diet, and cause various toxicological effects through transformation of the AhR. Plant extracts and phytochemicals including flavonoids are reported to suppress this transformation. Here the author investigated the suppression by a CPE on the AhR transformation *in vivo*. The CPE was administered orally to C57BL/6 mice at 100 mg/kg body weight, followed 1 h later by MC, an AhR agonist, injected intraperitoneally at 10 mg/kg body weight. CPE suppressed the MC-induced transformation to the control level by inhibiting the formation of a heterodimer between AhR and Arnt in the liver at 3 h post-administration. It also suppressed MC-induced CYP1A1 expression and QR activity, while it increased GST activity at 25 h. CPE constituents and their metabolites might contribute, at least in part, to the suppression of the AhR transformation. The results indicate that the intake of CPE suppressed the toxicological effects of dioxins in the body

# Chapter 7. General Discussion

The AhR is an orphan receptor whose endogenous ligand and physiological functions have not been fully elucidated at the moment, although it exists in the cytoplasm where it forms a complex with a dimer of hsp90 (5,6), XAP2 (7), and p23 (8). It has been found that halogenated and polycyclic aromatic hydrocarbons (HAHs and PAHs) including dioxins bind to the AhR as exogenous ligands (67,162-164), and binding of the these compounds to the AhR result in the conformational change of AhR to its DNA-binding form through sequential steps, termed AhR transformation. Since the toxicity of HAHs and PAHs is expressed through the transformation (165,166), it has been suggested that suppression of this transformation would be reduce dioxin toxicity.

The author demonstrated that anthocyans, one of the flavonoid subclasses, did not suppress the DNA-binding activity of AhR *in vitro* (see Chapter 2). Together this result, with previous one (63), leads to the suppressive effect of flavonoid was in a subclass-dependent manner in order of flavone= flavonol >flavanone> catechin >isoflavone and anthocyan. Major biological effect of flavonoid is anti-oxidative activity (167), but it is also reported that anti-oxidative activity of plant extracts do not correlate to antagonistic effect on the AhR (168), suggesting that the active site of phytochemicals for the AhR might be different from that for the anti-oxidative activity. Moreover, there is no correlation between the suppressive effect of flavonoids and their number of hydroxyl group (63). It was reported that structure-activity analysis of synthesized flavone derivatives, 3′-methoxy-4′-substituted flavones, and the most potent structure for suppression of the DNA-binding activity of AhR was the electron withdrawing group such as $NO_2$ or N=N=N at the 4′ position (61). However, suppression mechanisms of flavonoid on AhR transformation have not defined yet. It is, therefore, meaningful for elucidation of the suppression mechanisms of flavonoids on AhR transformation.

The author demonstrated that flavonoids belonging to flavone, flavonol, and flavanone but not catechin subclasses inhibited binding of agonist to the AhR in Chapter 3. The rank order of the AhR binding affinity for ligand appears to be dependent on its structural characteristics; e. g., co-planar aromatic structure with approximate van der Waals dimension of 14 x 12 x 5 Å and with

few substituent group (111,169). Since flavonoids such as flavone, flavonol and flavanone have these chemical properties, these flavonoids will interact with LBD in the AhR. Moreover, the author analyzed interaction between the AhRc and flavonoids and found that catechins in addition to flavones, flavonols, and flavanones interacted with the AhRc, suggesting that catechin suppresses AhR transformation by through associating with the partner proteins of AhR. Indeed, catechins are reported to interact with hsp90 and suppress AhR transformation (94). Suppression mechanisms of flavonoids on TCDD-induced AhR transformation were different among their subclasses: flavone and flavonol inhibited at a nuclear translocation step and consequently suppressed downstream events such as dissociation of partner proteins and formation of heterodimer with Arnt (Chapter 4). From the results in Chapter 3 and 4, it is suggested that flavone and flavonol are incorporated into the cells, and compete binding of agonist to the AhR. Incorporation of flavonol into the cells was shown in Appendix.

Previously, curcumin is reported to inhibit PKC, resulting in inhibiting phosphorylation of the AhR and Arnt (107). This report suggests that inhibition of phosphorylation pathway is one of the candidates for suppression mechanism of phytochemicals. The author demonstrated in Chapter 4 that compounds in four flavonoid-subclasses inhibited phosphorylation of the AhR and Arnt, and catechin inhibited ERK1/2 phosphorylation induced by TCDD. AhR transformation requires phosphorylation of the AhR by MAPKs such as ERK1/2 and JNK (102), and tyrosine kinases (103). This is the first report for the mode of action of phytochemicals on suppression of AhR transformation through the reduction in the phosphorylation level of ERK1/2.

The aim of this thesis was reduction of dioxin toxicity by flavonoid contained in daily food, thus the *in vivo* investigation was important and meaningful. Result from *in vivo* experiment, kaempferol, and EGb containing rich flavonol suppressed the DNA-binding activity induced by MC (see Chapter 5), and this effect was identical with the result of *in vitro* experiment. Since verapamil, an inhibitor of P-gp (134), caused the increment of cellular uptake of kaempferol, resulting in the enhancement suppressive effect on AhR transformation. This indicates kaempferol would work after absorption into the cells. Therefore, increment of bioavailability of flavonoid is effective for suppression of AhR transformation. Previously, certain flavonoids such as genistein and catechin were reported as an inhibitor of P-gp (137,170-172). The results in Chapter 5 can propose that the combined intake of flavonoids may enhance the suppressive effect of flavonoid

on AhR transformation.

In Chapter 6, the author demonstrated that CPE suppressed AhR transformation by inhibiting formation of heterodimer between the AhR and Arnt. Since this mechanism was the same manner as that of catechin shown in Chapter 4, the active compound(s) might inhibit phosphorylation of the AhR and/or Arnt. Moreover, the effect of CPE was enhanced after permeating Caco-2 cells, indicating that metabolism in enterocyte was an important for the suppressive effect of flavonoids. Certain metabolite(s) of polyphenol, phenolic acids also had the suppressive effect in a cell-free system. Therefore, in the body, both flavonoids and their metabolites contribute to the suppressive effect on AhR transformation.

**Conclusions**

This thesis demonstrates the suppressive effect of flavonoids on AhR transformation, indicating that flavonoids are effective food components to reduce dioxin toxicity. Flavonoids reveal different mechanisms among their subclasses; i. e., flavone and flavonol inhibit binding of agonist to the AhR, while flavanone and catechin inhibit phosphorylation of the AhR and Arnt.

# Appendix. Subcellular localization of flavonol aglycones in hepatocytes visualized by confocal laser scanning fluorescent microscope

### 1 Introduction

Flavonoids are widely distributed in the plant kingdom including edible plants such as vegetables and fruits. They are divided into seven subclasses; flavone, flavonol, flavanone, catechin, anthocyan, isoflavone and chalcone, according to their basic skeletal structure. Flavonoids have various beneficial effects such as anti-cancer, anti-obesity, anti-inflammatory and anti-oxidative activities (173-178). To evaluate the cellular absorption and metabolism of flavonoids, human colon adenocarcinoma Caco-2 cells were used as an intestinal model. It was reported that flavonoids are incorporated into Caco-2 cells by passive diffusion (114) and/or an active transporter, sodium-dependent glucose transporter 1 (179), and excreted through an efflux transporter such as multidrug resistance-associated protein-2 (180). In addition, the bioavailability of flavonoids has also been investigated in animals and human subjects. In mucosa and the liver, flavonoids undergo glucuronidation, sulfation and methylation, and the metabolites produced are detected in plasma, urine and the liver (181,182). Minute amounts of intact flavonoids are also detected in these tissues (183,184).

To determine the physiological concentrations and chemical structure of flavonoid metabolites *in vivo* and in cultured cells, high-performance liquid chromatography (HPLC) or HPLC combined with mass spectrometry (MS) is usually used (131,183,184). To examine the subcellular localization of flavonoids, it is necessary to prepare the subcellular fractions from tissues or cultured cells. Moreover, cross contamination can occur among the fractions. In this study, we attempted to visualize the incorporation and subcellular localization of flavonoids in mouse hepatoma Hepa-1c1c7 cells with a fluorescence microscope.

## 2 Materials and methods

### 2-1 Materials

[3H]-Kaempferol was obtained from Moravek Biochemicals, Inc. (Brea, CA), flavone, morin and naringenin from Nacalai tesque (Kyoto, Japan), isorhamnetin, kaempferol-3-glucoside, kaempferol-3-rutinoside, quercetin, apigenin, luteolin, galangin and eriodictyol from Extrasynthèse (Genay, France), kaempferol and rutin from Wako Pure Chemical Industries, Ltd. (Osaka, Japan), and (−)-epigallocatechin (EGC) and (−)-Epigallocatechin gallate (EGCG) from Kurita Kogyo (Tokyo, Japan). (−)-Epigallocatechin 3-(3″-O-methyl) gallate (Met-EGCG) was generously provided by Dr. Ryoyasu Saijo. Propidium iodide was purchased from Molecular Probes, Inc (Carlsbad, CA).

### 2-2 Cell culture

The mouse hepatoma cell line, Hepa-1c1c7 cells were grown and maintained at 37°C in α-minimum essential medium (α-MEM, Nissui Pharmaceutical, Tokyo, Japan) containing 10% fetal bovine serum (Sigma, St. Louis, MO), 4 mM L-glutamine, 100 U/ml of penicillin, and 100 mg/ml of streptomycin under a humidified atmosphere containing 5% $CO_2$.

### 2-3 Uptake of [3H]-kaempferol

Hepa-1c1c7 cells were seeded onto 24-well culture plates at a density of $2 \times 10^5$ cells/well, incubated for 24 h, and then treated with [3H]-kaempferol at 1, 10 and 20 μM in Krebs-Ringer HEPES buffer (50 mM HEPES, pH 7.4, 137 mM NaCl, 4.8 mM KCl, 1.85 mM $CaCl_2$, and 1.3 mM $MgSO_4$) for 70 min. The cells were washed three times with ice-cold phosphate-buffered saline and lysed by adding 250 μL of 0.05 N NaOH for 4 h. The lysate was transferred to a vial with scintillation cocktail, and the radioactivity incorporated into the cells was measured by a liquid scintillation counter.

### 2-4 Fluorescence microscopic analysis

Hepa-1c1c7 cells were seeded onto a glass-bottomed culture dish (MatTek Corp., Ashland, MA) and incubated with fresh α-MEM supplemented with 5 % feral bovine serum (biowest, Nuaillé France) for 24 h prior to treatment. The cells were treated with a flavonoid in the

medium. The concentration and incubation time are given in the figure captions. After the

incubation, the cells were fixed with 4% paraformaldehyde and 0.2% picric acid in 100 mM

sodium phosphate buffer (pH 7.2) overnight. Autofluorescence from the flavonoid was observed

under a confocal laser scanning fluorescence microscope (LSM 510 invert, Carl Zeiss, Jena,

Germany) with excitation by an argon laser at 488 nm and a 515–535 nm band pass filter. The

nucleus was counter-stained with propidium iodide (PI: Molecular Probes, Carlsbad, CA)

according to the manufacturer's directions, and its fluorescence was monitored at 543 nm (HeNe

excitation) with a 560-615 nm band pass filter.

## 3 Results

### 3-1 Incorporation of [$^3$H]-kaempferol into Hepa-1c1c7 cells

To investigate the uptake of flavonoids by hepatocytes, [$^3$H]-kaempferol was employed

as a model compound and treated to Hepa-1c1c7 cells. The radioactivity, which is reflecting to the

amount of kaempferol, in the cells increased in a dose-dependent manner (Fig. 1), though percent

uptake was almost the same (Data not shown). This result indicated that kaempferol was

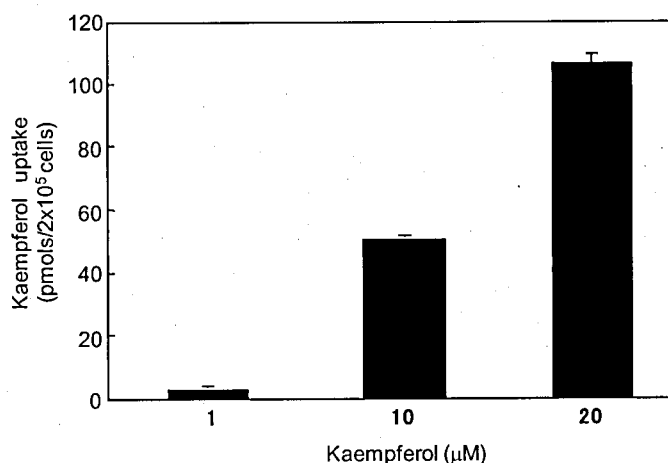incorporated into Hepa-1c1c7 cells without saturation under the experimental conditions.



**Fig. 1 Determination of Cellular uptake of [$^3$H]-kaempferol in Hepa-1c1c7 cells.** Hepa-1c1c7 cells were treated with [$^3$H]-kaempferol at 1, 10 and 20 μM for 70 min. Cell lysate was transferred to vials with scintillation cocktail, and the radioactivity incorporated into the cells was measured by a liquid scintillation counter. Data are represented as the mean ± SE (n=3).

### 3-2 Detection of flavonol aglycones in hepatocytes by fluorescence microscopy

Next, we examined the autofluorescence from kaempferol in Hepa-1c1c7 cells under the

confocal scanning microscope. Green autofluorescence was detected at over 50 μM of kaempferol

(Fig. 2). When the time-dependent uptake of kaempferol at 50 μM was also monitored, the

intensity of fluorescence increased time-dependently (Fig. 3). After 15 min, apparent fluorescence was observed into the cells. Taken together, the fluorescence microscope is able to monitor the uptake of kaempferol in Hepa-1c1c7 cells.
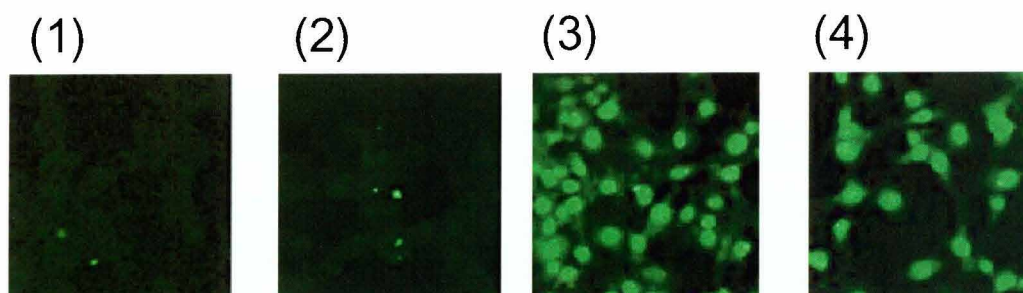


**Fig. 2 Cellular uptake of kaempferol visualized by a fluorescence microscope in Hepa-1c1c7 cells.** The cells were seeded onto glass bottom dish and treated with kaempferol at (1) 0 $\mu M$, (2) 10 $\mu M$, (3) 50 $\mu M$ and (4) 100 $\mu M$ for 70 min. Green fluorescence was observed under a confocal microscope (Ex. 488 nm-Em. 515-535 nm).



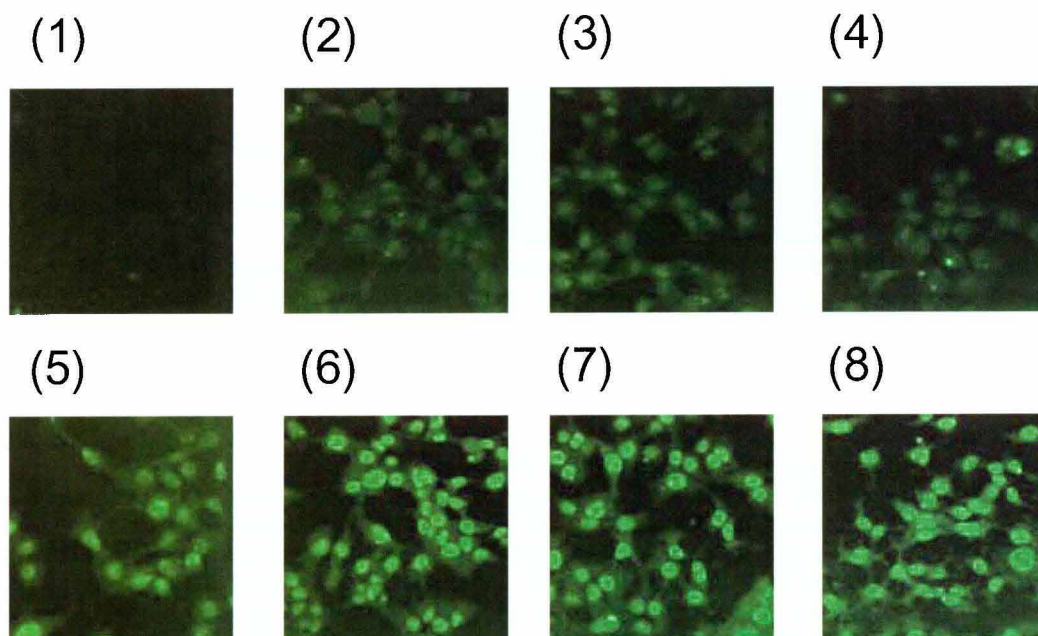**Fig. 3 Time-dependent cellular uptake of kaempferol in Hepa-1c1c7 cells.** The cells on glass bottom dish were treated with kaempferol at 50 $\mu M$ for (1) 0, (2) 1, (3) 5, (4) 10, (5) 15, (6) 30, (7) 45, and (8) 70 min. Green fluorescence was observed under a confocal microscope.

We applied this method to the uptake of other flavonoids. When the cells were treated with each flavonoid at 50 $\mu M$ for 70min, four flavonol aglycones, namely galangin (Fig. 4-1), isorhamnetin (Fig. 4-2), morin (Fig. 4-3), and quercetin (Fig. 4-4), showed green autofluorescence in the cells. In contrast, three flavonol glycosides, i.e. kaempferol-3-glucoside (Fig. 4-5),

kaempferol-3-rutinoside (Fig. 4-6) and rutin (Fig. 4-7), did not show autofluorescence. We further examined 8 aglycones; flavone, apigenin, luteolin, naringenin, eriodictyol, EGC, EGCG and Met-EGCG, belonging to other flavonoid subclasses, but did not detect any autofluorescence (data not shown). These results indicated that the uptake of flavonol aglycones in Hepa-1c1c7 cells can be observed with a confocal microscope.
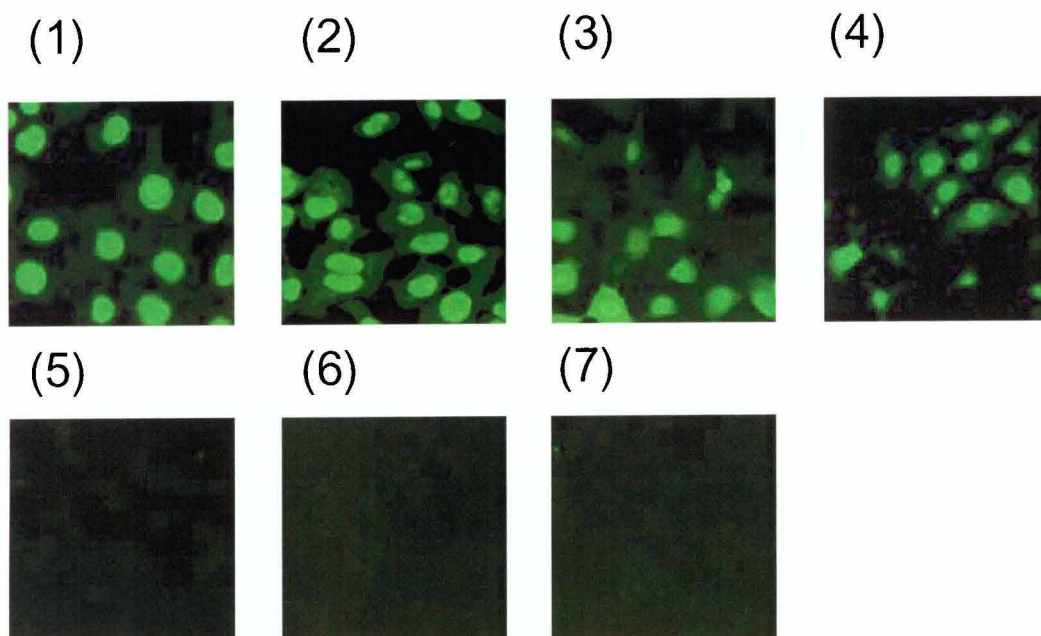


**Fig. 4 Cellular uptake of various flavonoids visualized by fluorescence microscopy.**
Hepa-1c1c7 cells were seeded onto glass bottom dish and treated with (1) galangin, (2) isorhamnetin, (3) morin, (4) quercetin, (5) kaempferol-3-glucoside, (6) kaempferol-3-rutioside, or (7) rutin at 50 μM for 70 min. Green fluorescence was measured under a confocal microscope.

### 3-3 Accumulation of kaempferol and galangin in the nucleus

To determine the subcellular localization of flavonols in intact mouse hepatoma Hepa-1c1c7 cells, we stained the nucleus with PI and found red fluorescence in the nuclear compartment (Fig. 5). This red fluorescence overlapped with the green fluorescence from flavonol because yellow fluorescence was observed in merged images. Therefore, kaempferol and galangin were incorporated into Hepa-1c1c7 cells, and accumulated in the nucleus.
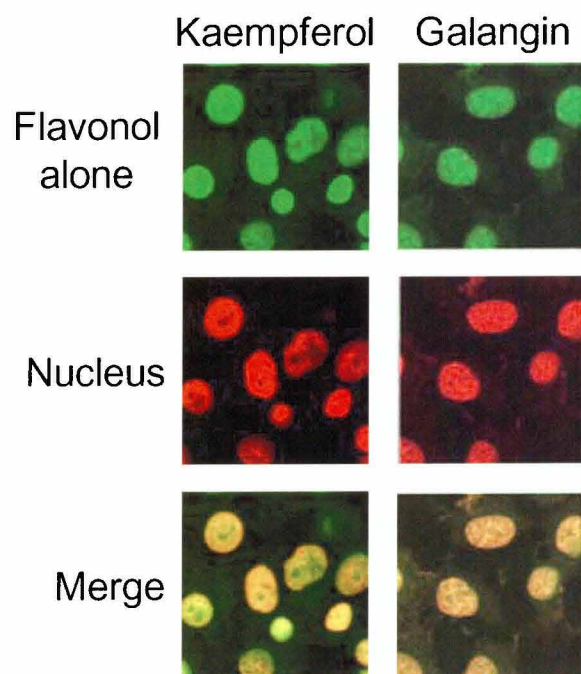
**Fig. 5 Subcellular localization of kaempferol and galangin in Hepa-1c1c7.** The cells were seeded onto glass bottom dish and treated with kaempferol (left) or galangin (right) at 50 µM for 70min and then stained with propidium iodide. Green fluorescence (Ex. 488 nm- Em. 515-535 nm) from flavonol (top) and red fluorescence (Ex. 543 nm-Em. 560-615) from PI (middle) were monitored by the confocal laser fluorescent microscope, and then these images were merged (bottom).

## 4 Discussion

In the present study, we demonstrated that flavonol aglycones specifically accumulated into the nucleus of Hepa-1c1c7 cells using a confocal laser scanning fluorescent microscope (Figs 2-4). It is likely that flavonol aglycones easily cross cellular and nuclear membranes through the passive diffusion because of their hydrophobicity. Previously, it was demonstrated that quercetin but not its conjugated form emitted fluorescence in Caco-2 cells by a fluorescence microscope but did not clarify the subcellular localization (180). Thus, this is the first report demonstrating the nuclear accumulation of flavonol aglycones detected with a fluorescence microscope. This method is superior to HPLC and LC/MS because it does not require intracellular fractions and extraction of the compound from the cells, although fluorescence microscopy has a limitation to detect the compound belonging to flavonol subclass and it can not quantify the compound.

As to the relationship between chemical structure and autofluorescence, a hydroxyl group at the C3-position in flavonol skeleton is important because no fluorescence was observed in the cells treated with compounds belonging to the flavone, flavanone and catechin subclasses. Even flavonol lost its autofluorescence when a sugar moiety was bound to the C3-position. Further study is needed to clarify the structure-fluorescence relationship.

Flavonoids have been detected *in vivo* not only in the intestine but also in various tissues and organs including the liver by HPLC and LC/MS (131,183,184). The liver plays an important

role the metabolism of flavonoids because aglycone and its metabolites are detected in this organ (150,183). In the present study, we quantified the uptake of flavonoid into hepatocytes using [$^3$H]-kaempferol, and found that the amount of radioactivity in the cells increased dose-dependently with a constant absorption ratio. This result indicates that a certain amount of flavonoid incorporated into the cells. In previous reports, the absorption of flavonoids was increased by lipids (185) and their excretion was reduced by an inhibitor of P-glycoprotein (180,186), indicating that these chemicals increase the bioavailability of flavonoids. It is worth investigating the subcellular localization of flavonols using fluorescence microscopy after pretreatment with these chemicals.

Flavonoids are reported to interact with isolated DNA *in vitro*, and to protect it from damage induced by oxidative stress (187,188). It was also reported that flavonoids induced the oxidative breakage of cellular DNA in the presence of copper ions in cancer cells as a preventive effect on cancer promotion (189). Taken together, these results and the findings of our study suggest that flavonol aglycones accumulate in the nucleus of hepatocytes and act to prevent the DNA damage caused by oxidative stress and chemical carcinogens.

## 5 Summary

Flavonoids are widely distributed in the plant kingdom and show various biological activities. The bioavailability of flavonoids in biological samples has conventionally been quantified by HPLC and MS, but with these analytical techniques it is difficult to estimate the subcellular localization of flavonoids in intact cells. In this study, we attempted to examine the localization of flavonoids in cultured cells using a confocal laser scanning fluorescence microscope and mouse hepatoma Hepa-1c1c7 cells. Five flavonol aglycones showed autofluorescence in the cells under the conditions (Ex. 488 nm- Em. 515-535 nm), whereas three flavonol glycosides and eight compounds belonging to other flavonoid subclasses, i.e., flavones, flavanones, and catechins, did not. The autofluorescence of galangin and kaempferol appeared stronger in the nucleus than cytoplasm, suggesting that they are incorporated into the cells and accumulated in the nucleus. The proposed method provided evidence that flavonol aglycones are incorporated into, and accumulated in the nucleus of, hepatocytes.

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# Acknowledgements

## List of Publications

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## B. Miscellaneous Papers

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