INHIBITORY EFFECT OF CARDAMONIN ON TRANSFORMATION OF ARYL HYDROCARBON RECEPTOR

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1. Introduction

Dioxins invade the body mainly through the diet[1] and bind to an aryl hydrocarbon receptor (AhR)[2]. The AhR is a ligand-activated transcription factor, and certain polycyclic aromatic hydrocarbons and halogenated aromatic hydrocarbons such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and 3-methylcholanthrene (MC)[3] bind to this receptor, resulting in its transformation. The unliganded AhR exists in the cytosol forming a complex with two molecules of heat shock protein 90, hepatitis B virus X-associated protein 2 and p23[4]. When AhR binds to ligands, AhR complex translocates into the nucleus and hetero-dimerizes with the AhR nuclear trans-locator (ARNT). The AhR-ARNT complex binds to a specific DNA motif called dioxin responsive element (DRE), leading to transcriptional induction of downstream genes including cytochrome P450 1A1 (CYP1A1)[5]. Since AhR transformation is the initial step for dioxin toxicities, an inhibitor of AhR transformation is effective to reduce the toxicity. It is reported that certain natural compounds such as flavonoids suppressed AhR transformation *in vitro*[6] and *in vivo*[7]. Cardamonin, one of the chalcones isolated from *Alpina katsumadai* Hayata, has been reported to possess anti-inflammatory, anti-coagulative and anti-diabetic activities[8,9]. In this study, we investigated the effects of cardamonin on AhR transformation and its underlying molecular mechanism.

2. Materials and methods

2.1 Materials. TCDD was purchased from AccuStanda Inc. (New Haven, CT, USA). [^{3}H]-MC (1.9 Ci/mmol) was obtained from Moravek Bio-chemicals, Inc. (Brea, CA, USA). 2,3,7,8-Tetrachlorodibenzofuran (TCDF) was from Cambridge Isotope Laboratories (Andover, MA, USA). 4-Hydroxyderricin and xanthoangelol were isolated and purified from "Ashitaba Chalcone Powder" distributed commercially by Japan Bio Science Laboratory (Osaka, Japan). Cardamonin and alpinetin were isolated and purified from the seeds of *Alpina katsumadai*, which was cultivated in Hainan Island in Southern China and purchased from Mikuni & Co. (Osaka, Japan). DNA-grade hydroxyapatite (HAP) was purchased from Bio-Rad Laboratories (Brea, CA, USA). Chalcone was purchased from Tokyo Chemical Industry Co, Ltd. (Tokyo, Japan). 2'-Hydroxychalcone and 4'-methoxychalcone were from Extrasynthese Chemical (Z.I Lyon Nord, France). 4'-Hydroxychalcone, 2'-hydroxy-6'-methoxychalcone, 2',4'-dihydroxychalcone, 4',6'-dimethoxy-2'-hydroxychalcone (flavokawain B), 2',4',6'-trimethoxychalcone, 2',6'-dihydroxy-4,4'-dimethoxydihydrochalcone and 2',6'-dihydroxy-4,4'-dimethoxychalcone were from Indofine Chemical Company, Inc. (Hillsborough, NJ, USA). All reagents used were of the highest grade available from commercial sources.

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 (Permission #24-04-01), and carried out according to the Kobe University Animal Experimentation Regulation. 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 with HEDG buffer (25 mM Hepes of pH 7.4, 1.5 mM EDTA, 1.0 mM DTT, 10% glycerol) according to the previous report[10].

2.3 EMSA assay[10]. The cytosolic fraction (4 mg protein/mL) was incubated with 1 nM TCDD (final concentration) or dimethylsulfoxide alone as a vehicle control (final 0.5%) at 20°C for 2h in the dark. To evaluate the suppressive effect on AhR transformation, each compound at various concentrations was added to the cytosol fraction 20 min prior to addition of 1 nM TCDD. The prepared DRE double-strand oligonucleotide was 5′-end-labeled with T4 polynucleotide kinase (Takara Biochemicals, Otsu, Japan) and [γ-^{32}P]-ATP (Amersham Pharmacia Biotech, Buckinghamshire, England). The treated cytosolic fraction (10 μg protein) was incubated with 250 mg of poly[dI-dC] and the ^{32}P-labeled DRE (30 kcpm, 10 fmols) for 15 min. The mixture was loaded onto a 4% non-stacking polyacrylamidegel in TBE buffer (25 mM Tris, 22.5 mM borate and 0.25

mM EDTA). After electrophoresis, the AhR-DRE complex was determined by autoradiography, and the density of the specific AhR-DRE complex was analyzed by a Digital Imaging System Is-1000 (Alpha Innotech, San Lean dro, CA, USA).

2.4 *Ligand Binding Assay*[11]. The ability of cardamonin to compete with [^{3}H]-MC for binding to the AhR was assessed by incubating cytosol (2 mg protein/mL) with cardamonin (0.1, 0.2, 0.5, 1 μM) for 15 min, thereafter the reaction mixture was treated with 0.25 nM [^{3}H]-MC for 2h at room temperature. Non-specific binding was defined by the incubation of cytosol with 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 [^{3}H]-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 washing with 1 mL of HEDG buffer containing 0.5% Tween 80 for 5 times. Then the precipitation of HAP mixture was transferred to the scintillation vials with 2 mL of ethanol to measure the effect of cardamonin on the binding of [^{3}H]-MC to the AhR.

3. Results and discussion

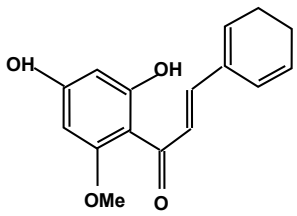

Figure.1 Chemical structure of cardamonin.

3.1 Inhibitory effect of cardamonin on TCDD-induced AhR transformation in cell-free system.

EMSA assay was used for estimating the effect of cardamonin on AhR transformation in cell-free system. The structure of cardamonin was shown in Figure 1. As shown in Figure 2, cardamonin suppressed TCDD-induced AhR transformation in a dose-dependent manner. We found that cardamonin at 20 and 50 μM significantly decreased AhR transformation by 26% and 23%, respectively. These results indicated that cardamonin suppressed TCDD-induced AhR transformation effectively. Results from the studies of interaction between the AhR and chemical compounds have revealed that polyphenols such as curcumin act as agonist or antagonists of the receptor[6]. Previous report demonstrated that curcumin itself induced AhR transformation and CYP1A1 expression, while it supressed dimethylbenzanthracene-induced AhR transformation and CYP1A1 expression in mouse hepatoma Hepa-1c1c7 cells by inhibiting the phosphorylation of AhR[12]. In this study, cardamonin itself slightly induced AhR transformation, whereas it suppressed TCDD-induced AhR transformation. These results indicate that cardamonin is a ligand of AhR and acts as mainly antagonist.

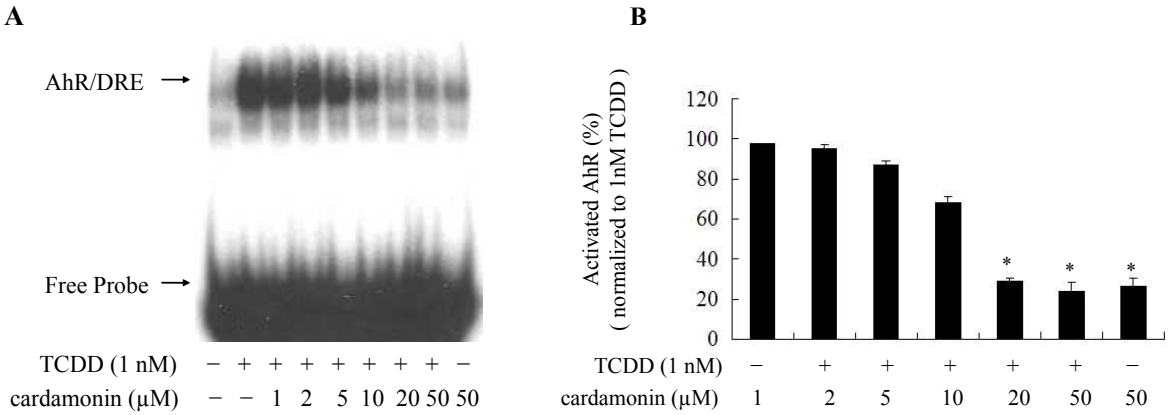

Figure.2 Effects of cardamonin on AhR transformation. (A) Representative EMSA result. Arrow indicates AhR/DRE complex. Cytosol was incubated with cardamonin at concentration of 1, 2, 5, 10, 20, 50 μM for 15 min. Subsequently, the reaction mixture was treated with 1 nM TCDD for 2h at room temperature. Then AhR/DRE complex was determined by EMSA assay. The densitiy of each band was quantified, and the value in positive control (1 nM TCDD treated cytosol) was set to 100%. The reasult is represented as the mean ±SE (n=3). *Indicates significant difference from the positive control by Dunnett's test ($p<0.05$).

3.2 Inhibitory effect of chalcones on TCDD-induced AhR transformation in cell-free system.

In this study, we found that cardamonin exerts antagonistic effects on AhR transformation (Figure 2). We, next, investigated that the effects of various chalcones on TCDD-induced AhR transformation (Figure 3). The result from EMSA assay showed that the synthetic chalcone, 2'-hydroxychalcone and 2',4'-dihydroxychalcone, significantly inhibited AhR transformation by 4% and 12%, respectively. Moreover, xanthoangeol and 4-hydroxyderricin, which were natural chalcones, also showed significant inhibitory effects on TCDD-induced AhR transformation. These results indicated that the structure of chalcones and their molecular size were important for inhibiting AhR transformation.

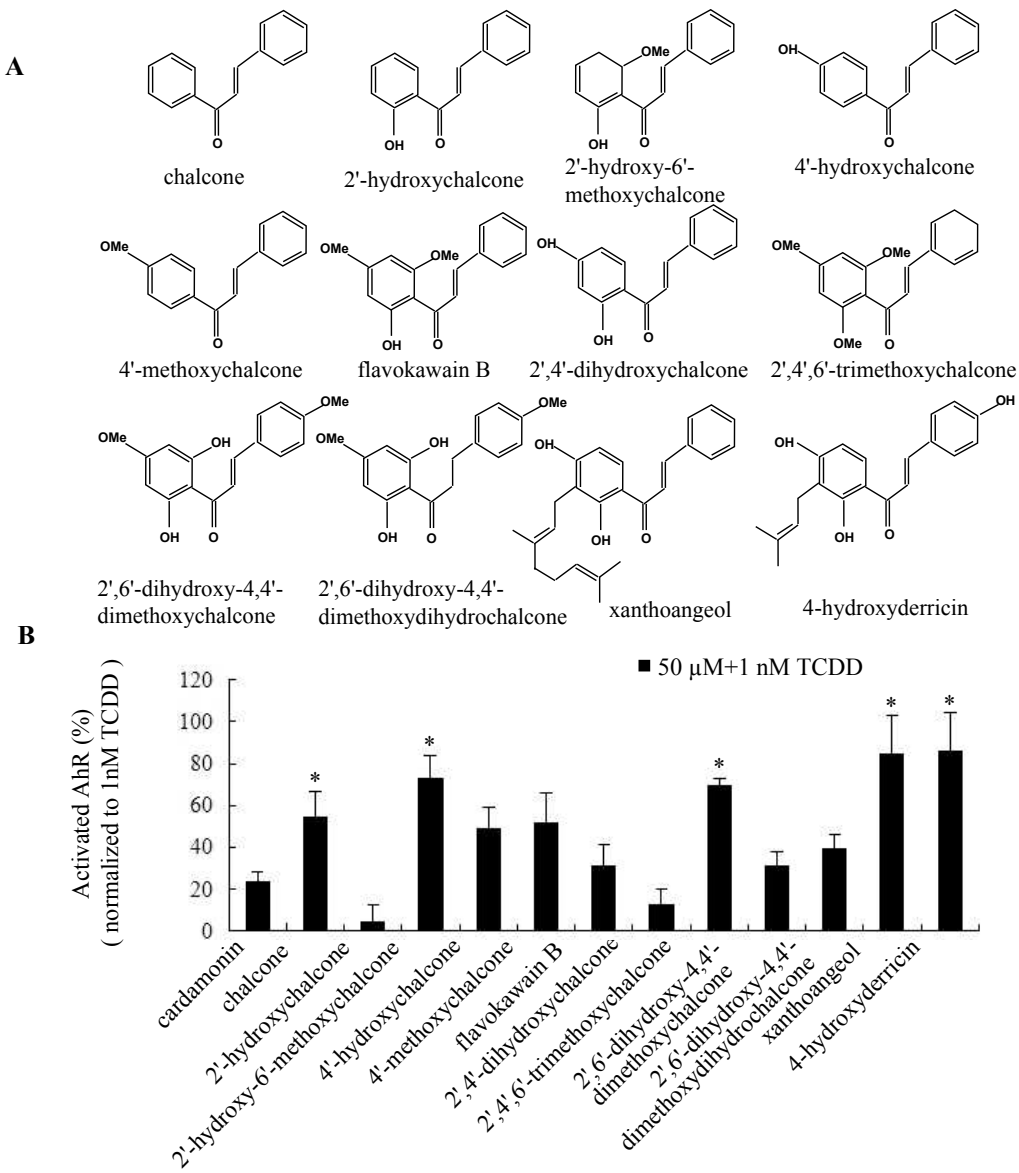

Figure.3 Effects of various chalcones on AhR transformation. (A) shows the chemical structures of uesd chalcones. (B) Rat cytosolic fraction was incubated with chalcones at 50 μM. Subsequently, the reaction mixture was treated with 1 nM TCDD for 2h at room temperature. AhR/DRE complex was determined by EMSA assay. The densitiy of each bond was quantified, and the value of (1 nM TCDD treated cytosol) was set to 100%. The reasult are represented as the mean ±SE (n=3). *Indicates significant difference from the 1 nM TCDD by Dunnett's test ($p<0.05$).

3.3 Cardamonin suppressed AhR transformation through competitively binding to the AhR.

To clarify the inhibitory mechanism of cardamonin, we examined whether cardamonin inhibit the binding of agonist to the AhR by ligand binding assay. In this assay, [^{3}H]-MC was used as an agonist to the AhR. When the inhibitory effects of cardamonin on the specific binding of [^{3}H]-MC to the AhR, cardamonin competitively inhibited the binding of [^{3}H]-MC to the AhR in a dose-dependent manner with a 50% inhibitory concentration of 0.48 μM. In a conclusion, we found cardamonin is a novel ligand of the AhR and it mainly acted as an antagonist.

References

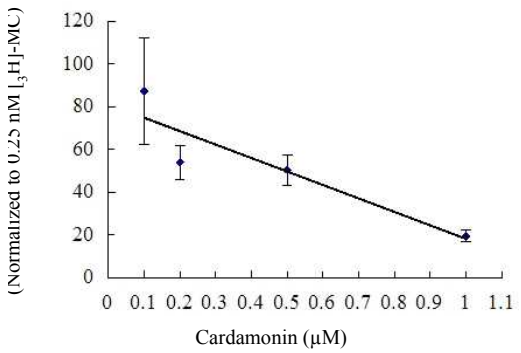

Figure.4 Effects of cardamonin on specific binding of [^{3}H]MC to the AhR in the cell-free system. Rat liver cytosolic fraction was pretreated with indicated concentrations of cardamonin, and the inhibitory effects of cardamonin on [^{3}H]MC binding to the AhR was measured as described in materials and methods. Non-specific binding was difined by adding excess amount if TCDF. The specific binding of [^{3}H]MC alone and AhR was set to 100% as positive control. Data are indicated as % of specific binding and represented as means ± SE (n=3) from triplicate independent experiments.

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