Inhibition of P-Glycoprotein Enhances the Suppressive Effect of Kaempferol on Transformation of the Aryl Hydrocarbon Receptor

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Dioxins enter the body mainly through the diet, bind to the aryl hydrocarbon receptor (AhR), and cause various toxicological effects. In this study, we found that oral administration of kaempferol or ginkgo biloba extract (EGb) containing 24% flavonol at 100 mg/kg body weight suppressed AhR transformation induced by 3-methylcholanthrene at 10 mg/kg body weight in the liver of mice. The suppressive effect of kaempferol was enhanced by verapamil, an inhibitor of P-glycoprotein (P-gp), in ex vivo experiments using a hepatic cytosolic fraction and 2,3,7,8-tetrachlorodibenzo-p-dioxin. Enhancement of the suppressive effect by verapamil was also observed in mouse hepatoma Hepa-1c1c7 cells, accompanied by an increase in the uptake of kaempferol into the cells. In conclusion, inhibition of P-gp enhanced the suppressive effect of kaempferol on AhR transformation through an increase in the intracellular kaempferol concentration.

Key words: aryl hydrocarbon receptor; kaempferol; 3-methylcholanthrene; P-glycoprotein; 2,3,7,8-tetrachlorodibenzo-p-dioxin

Dioxins cause various toxicological effects through transformation of the aryl hydrocarbon receptor (AhR), a ligand-dependent transcription factor. After binding to a ligand, the AhR dissociates its partner proteins, translocates into the nucleus, and forms a heterodimer with the aryl hydrocarbon receptor nuclear translocator (Arnt). This AhR/Arnt heterodimer binds to a DNA sequence called the dioxin responsive element (DRE), and induces expression of a battery of genes, including those for drug-metabolizing enzymes. A series of these sequential actions is called AhR transformation. Several drug-metabolizing enzymes, including cytochrome P450 1A1 (CYP1A1), are induced by transformed AhR, and CYP1A1 is involved in the activation of latent endogenous and exogenous carcinogens. In addition, AhR transformation disrupts intracellular signal transduction by changing the phosphorylation state of several regulatory proteins. Since AhR-knockout mice are resistant to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), the AhR plays an important role in the development of the toxicological effects of dioxins. Thus suppression of the AhR transformation is able to reduce the toxicological and biological effects of dioxins.

Flavonols such as kaempferol and galangin have been reported to suppress transformation of the AhR in vitro. Recently it was reported that these flavonols suppressed transcriptional activity of the AhR after permuting human colon adenocarcinoma Caco-2 cells, a model for enterocytes. It was also reported that kaempferol is a substrate for the ATP-dependent efflux transporter P-glycoprotein (P-gp). P-gp is a 170-kDa plasma membrane protein encoded by the human MDR1 and MDR3 genes, the murine mdr1a, mdr1b, and mdr2 genes, and the rat ppgp1, ppgp2, and ppgp3 genes. P-gp is highly expressed in many tumor cell lines and is found in many normal tissues and cells, including the intestinal epithelium, brain capillary endothelial cells, hepatocytes, and renal tubular cells, indicating that it plays an important role in the absorption, excretion, and tissue distribution of drugs. The bioavailability of glabridin, a prenyl flavonoid, in rats was increased by orally injected verapamil, a specific inhibitor of P-gp. Thus inhibition of P-gp affects the suppressive effect of flavonoids on AhR transformation. In this study, we investigated the suppressive effects of kaempferol and ginkgo biloba extract (EGb) containing 24% flavonol on the transformation of the AhR in vitro and examined to determine whether inhibition of P-gp would enhance the effect of kaempferol ex vivo and in vitro.

Materials and Methods

Materials. Corn oil and 3-methylcholanthrene (MC) were purchased from Nacalai Tesque (Kyoto, Japan). TCDD was from AccuStandard (New Haven, CT) and was dissolved in dimethylsulfoxide (DMSO). [3H]-Kaempferol was from Moravek Biochemicals (Brea, CA). Kaempferol and propylene glycol were from Wako Pure Chemical Industries (Osaka, Japan). EGb, which consists of 24% flavonoids, including kaempferol, quercetin, and isorhamnetin, and 6% terpenes, was from Asahi Food and Healthcare (Tokyo, Japan). Rhodamine-123 was from Sigma (St. Louis, MO).

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Abbreviations: AhR, aryl hydrocarbon receptor; Arnt, AhR nuclear translocator; DMSO, dimethylsulfoxide; DRE, dioxin responsive element; DTT, dithiothreitol; EGb, extract from ginkgo biloba; HAH, halogenated aromatic hydrocarbon; hsp90, 90-kDa heat shock protein; MC, 3-methylcholanthrene; PAS, Per-Arnt-Sim; P-gp, P-glycoprotein; PMSF, phenylmethylsulfonyl fluoride; SW-ELISA, southwestern ELISA; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin
Animal treatments. Animal treatments in this study conformed to the “Guidelines for the Care and Use of Experimental Animals on the Rokkodai Campus of Kobe University.” We carried out 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 three groups of six each. After diet was withheld for 18 h, the mice in the first and second groups were given kaempferol and EGb orally at 100 mg/kg of body weight respectively. The mice in the third group received propylene glycol alone at 10 ml/kg of 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 with MC at 10 mg/kg of body weight 2 h after the first administration, whereas the other was injected with corn oil (5 ml/kg of body weight) alone as a vehicle control. These mice were killed 2 h after MC injection. The liver was removed and used in the measurement of AhR transformation by SW-ELISA.

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

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

SW-ELISA. To evaluate AhR transformation, the DNA-binding activity of AhR was measured by SW-ELISA. For the in vivo and cultured cell experiments, the reaction mixture consisted of 12.5 μl of nuclear extract (12.5 mg of protein/assay) from the liver of mice or Hepa-1c1c7 cells 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 mM KCl and 40 μl of the cytosolic fraction prepared from the liver of mice. The reaction mixture was plated into a 96-well microtiter plate (Maxisorp, Nalge Nunc International, Tokyo) coated with anti-FITC antibody (DakoCytomation, Kyoto, Japan), as capturing antibody and FITC-labeled DELTAP (=GAT CCT CGG TCT CCG CAC CTC CG-3’) (prepared from Holkaido System Science, Sapporo, Japan) for 2 h at 20°C. After incubation, the plate was washed 3 times with 0.05% tween20 containing PBS (PBST), and then specific antibody against AhR (anti-AhR, MA1-514, Affinity BioReagents, Golden, CO) was used as the primary antibody. After the plate was washed 3 times with PBST, a biotinylated secondary antibody was added to each well and incubated for 1 h at 20°C. After the plate was washed 3 times again with PBST and then specific antibody against Arnt (anti-Arnt C-19, Santa Cruz Biotechnology, Santa Cruz, CA) for nuclear protein from cells was added to each well for 1 h at 20°C. For animal samples, a specific antibody against AhR (Anti-AhR, MA1-514, Affinity BioReagents, Golden, CO) was used as the primary antibody. After the plate was washed 3 times with PBST, a biotinylated secondary antibody was added to each well and incubated for 1 h at 20°C. The plate was washed 3 times again with PBST, and peroxidase-conjugated streptavidin (DakoCytomation) was added to each well and incubated for 30 min at 20°C. After the plate was washed 3 times with PBST and once with PBS, bound-peroxidase activity was visualized with tetramethylbenzidine (DakoCytomation), and color development was stopped by adding 2 M sulfuric acid. Transformed AhR was quantified by measuring the absorbance at 450 nm using a Wallac ARVO sx multilabel counter (Perkin-Elmer Life Sciences), whereas the other was injected with corn oil (5 ml/kg of body weight) alone as a vehicle control. These mice were killed 2 h after MC injection. The liver was removed and a cytosolic fraction was prepared for the ex vivo experiment.

Statistical analysis. Data were expressed as the mean ± SE of at least three independent determinations for each experiments. Data from the in vivo and ex vivo experiment were analyzed by two-way ANOVA with the Tukey multiple comparison test. Data from the cultured cell experiment were analyzed by one-way ANOVA with the Tukey multiple comparison test.

Results

Kaempferol and EGb suppressed MC-induced AhR transformation in the liver of mice

We investigated the effects of kaempferol and EGb on AhR transformation in vivo (Fig. 1). After the data were analyzed by two-way ANOVA, both group and treatment effects were observed. MC at 10 mg/kg of body weight significantly promoted the transformation in the liver of mice. Kaempferol and EGb at 100 mg/kg of body weight completely suppressed the MC-induced transformation to the control level, whereas they had no effect in the liver of control animals. These results suggest that kaempferol was incorporated into the liver of mice and suppressed the transformation.

Verapamil enhanced the suppressive effect of kaempferol on AhR transformation ex vivo

To determine whether verapamil, an inhibitor of P-gp, can enhance the suppressive effect of kaempferol, an ex vivo experiment was carried out. As shown in Fig. 2, both group and treatment effects were observed by two-way ANOVA. TCDD at 1.0 nM induced AhR transformation in the liver of vehicle-dosed mice. The liver of kaempferol-dosed mice tended to show less transformation than that of the vehicle-dosed control mice. It is noteworthy that in the livers of both the verapamil and the kaempferol-dosed mice, TCDD-induced AhR transformation was significantly suppressed as compared with that in the livers of the control mice. Thus pre-administration of verapamil enhanced the suppressive effect of kaempferol on AhR transformation in the livers of the mice.
[58x426]Fig. 1. EGB and Kaempferol Suppressed AhR Transformation Induced by MC in the Livers of Mice.

Mice were orally administered kaempferol or EGB at 100 mg/kg of body weight, then given an intraperitoneal injection of MC at 10 mg/kg of body weight. Hepatic nuclear protein was prepared and subjected to measurements of AhR transformation by SW-ELISA as described in “Materials and Methods.” Data are represented as the mean ± SE (n = 3). Group and treatment effects were analyzed by two-way fractional ANOVA. Different letters indicate statistically significant differences as evaluated by the Tukey multiple comparison test.

N.S., Not significant.

Table 1. Uptake of Kaempferol in Hepa-1c1c7 Cells

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<td>Percent uptake (pmols/4 × 10^6 cells)</td>
<td>21.4 ± 1.8^a</td>
<td>227 ± 12.5^b</td>
<td>427 ± 33.1^c</td>
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Each value represents the means ± SE from at least three independent experiments. The kaempferol effect was analyzed by one-way ANOVA. Different letters indicate statistically significant differences as evaluated by the Tukey multiple comparison test.

Fig. 2. Verapamil Enhanced the Suppressive Effect of Kaempferol on AhR Transformation ex Vivo.

Mice were orally administered verapamil at 100 mg/kg of body weight, then given an oral administration of kaempferol at 100 mg/kg of body weight. A hepatic cytosolic fraction prepared from the mice was subjected to measurements of AhR transformation by SW-ELISA. Data are represented as the mean ± SE (n = 3). Group and treatment effects were analyzed by two-way fractional ANOVA. Different letters indicate statistically significant differences as evaluated by the Tukey multiple comparison test.

accompanied by an increase in the uptake of this flavonol by hepatocytes without cytotoxicity.

**Discussion**

The toxicological effects of dioxins are induced mainly by transformation of the AhR,9,10 and the liver is one of the major target organs of dioxins. Kaempferol has been reported to suppress AhR transformation in vitro.10,11 In the present study, we found that kaempferol and EGB suppressed MC-induced AhR transformation in vivo (Fig. 1), and that verapamil enhanced the suppressive effect of kaempferol ex vivo (Fig. 2) and in cultured cells (Fig. 4), and increased the amount of kaempferol in Hepa-1c1c7 cells (Fig. 3). These findings indicate that verapamil increased the amount of kaempferol in the liver through inhibition of P-gp and enhanced the suppressive effect on AhR transformation. In a previous study, it was reported that kaempferol inhibited the binding of agonist to the AhR.18 Therefore, the suppressive effect depends on the amount of kaempferol in the cells. This is the first report that a flavonol suppressed AhR transformation in vivo and that inhibition of P-gp enhanced this suppressive effect.

The flavonol aglycone but not its glycoside had a suppressive effect on AhR transformation in vitro.10 In
In this study, we used kaempferol aglycone to estimate the suppressive effect on AhR transformation in vivo. It is known that flavonols undergo metabolism, i.e., glucuronidation and/or sulfation in the body. A conjugation position-specific effect of quercetin metabolites on AhR transformation has been reported. Thus not only kaempferol aglycone but its metabolites might suppress AhR transformation in vivo. Further study is needed to investigate the effects of the metabolites on AhR transformation. As for EGb, it contains flavonols such as kaempferol, quercetin, and isorhamnetin. 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, were detected in the urine and plasma. A previous report indicated that certain phenolic acids also suppressed AhR transformation in a cell-free system. Therefore, these metabolites, in addition to kaempferol, might be present in the liver after the administration of EGb and might have contributed to the suppressive effect in vivo.

P-gp is expressed in many organs, including the small intestine, liver, and brain, and it has an important role in the absorption, elimination, and distribution of drugs. Verapamil is able to inhibit the P-gp-mediated efflux of its substrate competitively, and kaempferol is reported to be a substrate for P-gp. From these reports, it is hypothesized that the inhibition of P-gp reduced the excretion of kaempferol from the target cells and enhanced the biological activity in vivo. In the present study, treatment with verapamil enhanced the suppressive effect of kaempferol on AhR transformation in Hepa-1c1c7 cells (Fig. 4). This is the first report that the inhibition of P-gp enhanced the suppressive effect of a flavonoid on AhR transformation. It has been reported that the bioavailability of glabridin, a prenyl flavonoid,
was increased by oral intake of verapamil through inhibition of P-gp in vivo.\textsuperscript{14,25} The uptake of quinine, a drug for malaria and excreted by P-gp, into the brain was increased by verapamil.\textsuperscript{26} These reports suggest that verapamil inhibited P-gp both in the small intestine and in the livers of mice, resulting in an increased amount of kaempferol, which in turn enhanced the suppressive effect on AhR transformation in vivo. It is necessary to investigate the effect of verapamil on the bioavailability of kaempferol in the future. Since certain flavonoids reported to be substrates or inhibitors for P-gp,\textsuperscript{27} there is a possibility that P-gp is one of the target molecules for estimation of the functions of flavonoids. It is, therefore, worth investigating the suppressive effect of combinations of flavonoids.

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### References