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Activation of Pregnane X Receptor and Induction of MDR1 by Dietary Phytochemicals

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The pregnane X receptor (PXR) is understood to be the key regulator for gene expression of such drug-metabolizing enzymes and transporters as multidrug-resistant protein 1 (MDR1) and the cytochrome *P*450 (CYP) family. We examined the effect of dietary phytochemicals on the PXR-dependent transcriptional activity in human intestinal LS180 cells by using a reporter assay. Among ~40 kinds of phytochemicals, tangeretin and ginkgolides A and B markedly induced the PXR-dependent transcriptional activity and also the activity of the human MDR1 promoter. The expression levels of MDR1 mRNA as well as of CYP3A4 mRNA, another gene regulated by PXR, were significantly increased by these phytochemicals. Furthermore, an increase was observed of the MDR1 protein and its functional activity by tangeretin and by ginkgolides A and B. These findings strongly suggest that tangeretin and ginkgolides A and B activated PXR, thereby regulating detoxification enzymes and transporters in the intestines.

KEYWORDS: Pregnane X receptor; MDR1; CYP3A4; tangeretin; ginkgolide

INTRODUCTION

The intestinal epithelium lining the gut has such diverse functions as nutrient absorption and as a barrier for body defense. The intestinal defense system comprises both physical and biological barriers. The physical barrier consists of such molecules as the tight junction, which plays an important role in restricting intestinal permeability only for nonharmful low-molecular weight compounds (*1*). On the other hand, the biological barrier undertakes detoxification and excretion of xenobiotics, that is, chemical xenobiotics including drugs are metabolized by drug-metabolizing enzymes and excreted to the luminal side from intestinal epithelial cells by drug transporters (*2*). Such enzymes as the cytochrome *P*450 family (CYP), UDP glucuronosyltransferases (UGTs), and glutathione *S*-transferases (GSTs) are involved as drug-metabolizing enzymes, and multidrug-resistant protein 1 (MDR1) and multidrug-resistant associated proteins (MRPs) are known to act as drug transporters.

It is understood that the gene expression of many of these drug-metabolizing enzymes and transporters is regulated by the pregnane X receptor (PXR), one of the ligand-regulated orphan nuclear receptors (*3–5*). As PXR is mainly expressed in the small intestine, liver, and colon, PXR is a key regulator of biological barriers, regulating the expression of these enzymes and transporters in the enterocyte, especially intestinal epithelial cells. PXR is known to be activated by such diverse natural and synthetic compounds as rifampicin, phenobarbital, SR12813, hyperforin, ritonavir, and bile acids (*6–8*). The increased gene transcription by PXR is initiated by ligand binding to the receptor and subsequent cytoplasmic−nuclear translocation. A nuclear localization signal also was identified in PXR (*9, 10*), although the precise molecular mechanism remains to be determined. Moreover, the promiscuity of PXR toward xenobiotics of diverse chemical character indicates a prominent role for this receptor in xenosensing and environmental adaptation. The X-ray crystal structure of the PXR ligand-binding domain in its apo- and drug-bound forms demonstrates a large, flexible, hydrophobic ligand-binding cavity that can accommodate a variety of chemical structures in multiple binding modes (*11, 12*). It is thus more likely that unknown chemical substances having a ligand or ligand-like activity toward PXR are present in natural products.

The regulation of PXR by dietary factors is of considerable importance and interest because PXR regulates the drug-metabolizing enzymes as already described, and so it is likely that pharmacokinetics could be modulated by dietary factors via PXR. Furthermore, as intestinal epithelial cells are basically exposed to dietary factors at high concentrations, it is plausible that these dietary factors could modulate the intestinal biological barrier or pharmacokinetics by regulating the PXR activity.

Activated PXR increases the expression of MDR1, which belongs to the ATP-binding cassette (ABC) transporter superfamily. Several studies have reported the regulation or modulation of MDR1 by food-derived compounds (*13–15*). However, there is no report on food-derived substances in terms of their transcriptional regulation of MDR1 expression via activated

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PXR, except in the case of hyperforin in St. John's wort as a ligand of PXR (*16, 17*).

The aim of this present study was to search for PXR activity-modulating chemical compounds among food-derived natural products, using the reporter assay that can estimate the PXR-dependent transcriptional activity. We also examined the regulation of MDR1, one of the typical genes targeted by PXR, by those compounds, using human intestinal epithelial-like LS180 cells that are frequently utilized for studies on PXR (*18*). Among the various dietary factors, we particularly focused on phytochemicals, including flavonoids, carotenoids, and terpenoids, since these chemical compounds are good candidates for agonists or antagonists regulating the drug receptors or nuclear receptors due to their chemical structure.

MATERIALS AND METHODS

Materials. The LS180 cell line (derived from human colonic cancer tissue) was obtained from the American-type Culture Collection (Rockville, MD). Dulbecco's modified Eagle's medium (DMEM) and Hanks' balanced salt solution (HBSS) were purchased from Sigma (St. Louis, MO), and fetal calf serum was purchased from Asahi Technoglass (Chiba, Japan). The lipofectamine reagent was purchased from Invitrogen (Carlsbad, CA), and the dual-luciferase reporter assay system was purchased from Promega (Madison, WI). QuantiTect SYBR Green for real-time PCR was from Qiagen (Hilden, Germany), and rhodamine-123 was purchased from Kanto Chemicals (Tokyo, Japan). The protease inhibitor cocktail (complete, EDTA-free) was from Roche (Basel, Switzerland). All other chemicals used were of reagent grade.

Phytochemicals. Myricetin, morin, puerarin, fisetin, taxifolin, polyphenon, daidzein, 3-epigallocatechin gallate, (+)-catechin, 3-catechingallate, 3-epicatechin, 3-epigallocatechin, tearubigin, theaflavin, diosmin, coumaric acid, hesperetin, genistein, and rifampicin were obtained from Sigma (St. Louis, MO). Kaempherol, luteolin, eriodictol, and tamarixetin were from Extrasynthese (Genay, France), and galangin was obtained from Sigma-Aldrich (Tokyo, Japan). Chlorogenic acid, tangeretein, and rutin were from Wako Pure Chemicals (Tokyo, Japan), and quercitrin was from Tokyo Kasei Kogyo (Tokyo, Japan). Gallocatechin gallate and gallocatechin were purchased from Kurita Kogyo (Tokyo, Japan), and biochanin A was from Mitsui Norin (Tokyo, Japan). Genistin was from Fujicco (Kobe, Japan), and ginkgolides A−C and J were from Tama Biochemical (Tokyo, Japan). The purity of these phytochemicals was >95%. Each phytochemical was diluted in dimethyl sulfoxide (DMSO) before use.

Cell Culture. LS180 cells were cultured at 37 °C in a humidified atmosphere of 5% CO_2 in air with a culture medium consisting of DMEM, 10% fetal bovine serum, 1% nonessential amino acids, 200 U/mL penicillin and 200 μg/mL streptomycin. Each phytochemical was dissolved in DMSO and added to LS180 cells at a DMSO concentration in the medium never exceeding 0.1% (v/v).

Construction of Reporter Vector. The reporter gene plasmid containing the PXR-binding site, a DR4 nuclear response element, was constructed as described previously (*19*) by ligating the annealed oligonucleotides, which contained four tandemly arranged DR4s, into the *Mlu*II/*Bgl*II-digested pGL3 promoter vector; four DR4s sense, 5′-cgcgttgaactaacttgacctaaattttgaactaacttgaccttctagatgaactaacttgacctaaattttctaacttgaccta-3′; and four DR4s antisense, 5′-gatctaggtcaagttagttcaaaatttaggtcaagttagttcatctagaaggtcaagttagttcaaaatttaggtcaagttagttcaa-3′. The MDR1 promoter fragment (-7975 to -7013) containing the cluster of nuclear receptor response elements was, as described previously (*19*), amplified by PCR from human genomic DNA with primers 5′-TCT GCT AGC AGT GTT TCT TGT-3′, containing a natural *Nhe*I site, and 5′-AAT AGA TCT CAT ATA AGG CAA CTG TTT TGT T-3′, introducing a *Bgl*II site. The *Nhe*I/*Bgl*II-digested PCR fragment was ligated between the *Nhe*I/*Bgl*II sites of a pGL3 basic vector and was sequenced. The open reading frame of human PXR was amplified from LS180 cells by using the primer 5′-TCG AAT TCC ACC ATG GAG GTG AGA CCC AAA GAA AGC-3′, which introduced an *Eco*RI site and an optimized Kozak consensus sequence, and the primer 5′-CGT CTA GAT CAG CTA CCT GTG ATG CCG AAC A-3′, which introduced an *Xba*I site. The *Eco*RI/*Xba*I-digested PCR fragment was ligated into appropriately digested pcDNA3.1A (Invitrogen), creating pcDNA-hPXR, and then was sequenced.

Table 1. Primers Used for Real-Time PCR Analysis

gene	primer	sequence (5′−3′)
MDR1 (NM_000927)	forward	TGG TTT GAT GTG CAC GAT GT
	reverse	GGC CAA AAT CAC AAG GGT TA
CYP3A4 (NM_017460)	forward	ACC GTA AGT GGA GCC TGA AT
	reverse	CCC CGG TTA TTT ATG CAG TC
β-actin (NM_001101)	forward	GCGAGAAGATGACCCAGATCATGTT
	reverse	GCTTCTCCTTAATGTCACGCACGAT

Reporter Assay. The reporter assay was carried out as previously described (*20*). The cells were seeded at 1.5×10^5 cells/well in a 12-well plate that had been precoated with 0.2 mg/mL collagen (Nitta Geratin, Osaka, Japan) for 30 min. After 1 day of culture, LS180 cells were transfected with the reporter plasmid by a lipofection technique with lipofectamine and the PLUS reagent (Invitrogen). One day before transfection, 1.5×10^5 cells/well were plated in 12-well plates. The medium in the dishes was replaced with a serum-free medium 1 h before this transfection. Plasmid DNA (0.2 μg of pcDNA-hPXR or pcDNA3.1A, 1 μg of pGL3-DR4, and 0.05 μg of pRL-CMV) and the PLUS reagent (4 μL) were diluted in 250 μL of the serum-free medium in each well and then incubated at room temperature for 15 min. Lipofectamine (6 μL) was diluted in 250 μL of the serum-free medium. The plasmid and lipid dilutions were combined, gently mixed, and then incubated at room temperature for 15 min. Meanwhile, the medium in the wells was replaced with 1 μL of serum-free medium. A 200 μL portion of the DNA/lipid suspension was added to each well and gently mixed, before the medium was replaced with a growth medium containing each phytochemical. After this transfection, the medium was replaced with a medium containing each phytochemical, and the LS180 cells were cultured for 24 h. The luciferase activity was determined by a dual-luciferase reporter assay (Promega, Tokyo, Japan) with an LB9507 Lumet luminometer (Berthold Technologies, Bad Wildbad, Germany).

Isolation of Total RNA and Real-Time PCR. After incubating the LS180 cells in 12-well plates at 2.5×10^5 cells/well for 6 days, the total RNA was extracted from the cells by using Isogen as previously described (*20*). cDNA was prepared from 1 μg of the total RNA. Real-time PCR was performed with SYBR Green I also as previously described (*20*). After denaturing at 95 °C for 15 min, PCR was performed for 40 cycles, each of which consisted of denaturing at 95 °C for 15 s, annealing at 61 °C in MDR1 and at 60 °C in CYP3A4 for 15 s, and extension at 72 °C for 10 s. The PCR primers used for MDR1, CYP3A4, and β-actin are shown in **Table 1**. The effect of the phytochemical treatment on β-actin mRNA expression was not significant at any stage, indicating that β-actin could be used as a stable housekeeping gene throughout the experiment.

Western Blot Analysis. LS180 cells cultured in 6-well plates at 5×10^5 cells/well with each phytochemical for several days were washed twice with ice-cold PBS. To prepare the membrane fraction of LS180 cells for the Western blot analysis of MDR1, the cells were scraped off and then suspended in 1 mL of an ice-cold suspension buffer (10 mM Tris-HCl, 1 mM EDTA, 250 mM sucrose, 0.1 mM PMSF, and 0.1% of an inhibitor cocktail at pH 7.5). The precipitate obtained by centrifugation at 1000 rpm for 5 min at 4 °C was homogenized with 0.5 mL of a hypotonic buffer (10 mM Tris-HCl, 1 mM EDTA, 0.1 mM PMSF, and 0.1% of an inhibitor cocktail at pH 7.5) and left at 4 °C for 20 min. The homogenate was centrifuged at 3000 rpm for 10 min at 4 °C, and the resulting supernatant also was centrifuged at 14 000 rpm for 30 min at 4 °C. The precipitate was homogenized in 1 mL of the ice-cold suspension buffer, and the membrane fraction was obtained. This fraction was dissolved in 5× Ling's buffer (50 mM Tris-HCl, 5 mM EDTA, 1.5 M sucrose, 350 mM SDS, 200 mM DTT, and 0.165 mM Pyronin Y) at 55 °C for 5 min, and the medium immediately was refreshed. A 2× Urea buffer (20% 5× Ling's buffer and 10 M Urea) was then added to the solution, and the obtained sample was used for Western blotting as described previously (*20*). Each protein in the cell lysate was fractionated by SDS−PAGE (12.5% polyacrylamide gel)

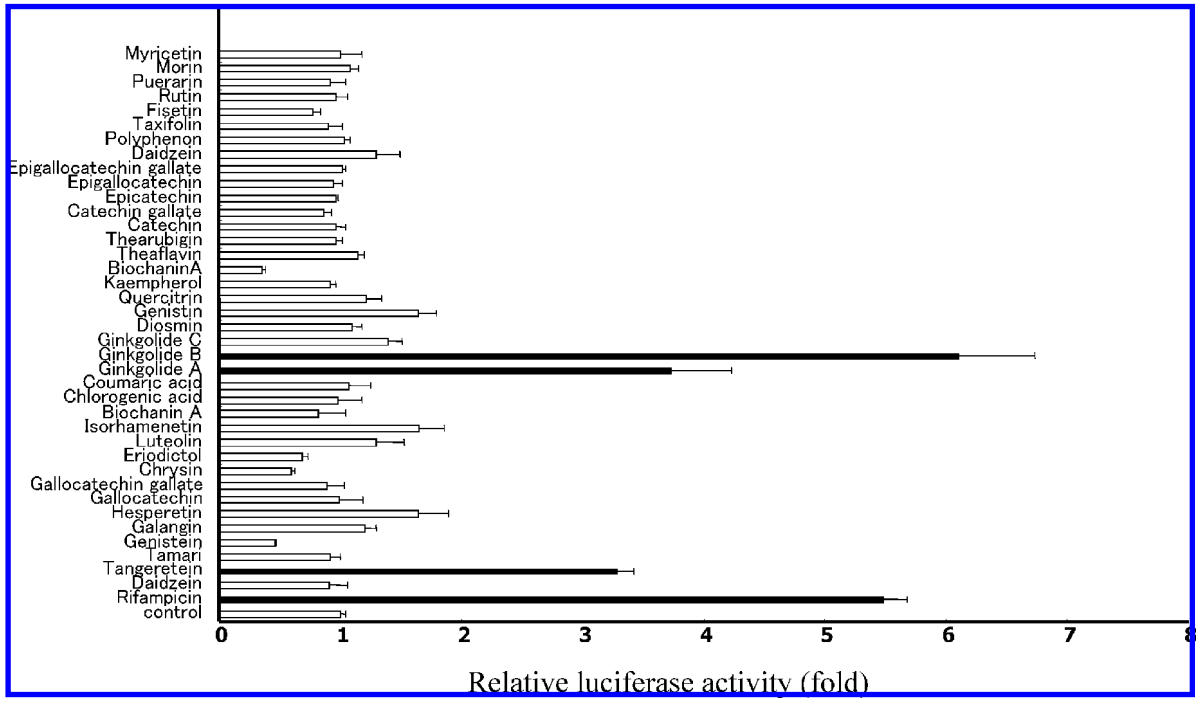

Figure 1. Effect of phytochemicals on PXR-dependent transcriptional activity in LS180 cells. Transfected cells were incubated with each phytochemical at 100 μM for 24 h, and the reporter assay was performed as described in the Materials and Methods. The relative luciferase activity measured for the vehicle (dimethyl sulfoxide)-treated LS180 cells was taken as 1. Each value is the mean ± SD (n = 3).

and transferred to a PVDF membrane (Millipore). The membrane was blocked overnight at 4 °C by PBS-T containing 5% BSA. The membrane was then incubated for 1 h with antihuman MDR1 antibody (sc-8313: Santa Cruz Biotechnology) (1:1000 dilution) or monoclonal anti-β-actin antibody (AC-15: Sigma) (1:2500 dilution). The blot was washed in PBS-T and then incubated for 1 h with antigoat IgG-HRP (1:1000 dilution). A chemiluminescent substrate (ECL; Amersham Biosciences) was used for detection.

Accumulation Experiment of Rhodamine 123. LS180 cells were seeded into 24-well plates at 2.5 × 10^5 cells/well. After 1 day of culture, the medium was exchanged for a fresh culture medium containing one of the phytochemicals, and the cells were incubated with serum for 6 days more. The cells were then washed twice with PBS and equilibrated with HBSS at 37 °C for 10 min. An accumulation experiment was started by adding 300 μL of fresh HBSS containing 15 μM rhodamine 123, and the mixture was incubated for 2 h at 37 °C. The reaction was stopped by aspirating HBSS from the wells, before washing twice with ice-cold PBS. The fluorescence intensity of rhodamine 123 then was measured at an excitation wavelength of 485 nm and an emission wavelength of 544 nm by using a Fluoroskan Ascent CF instrument (Labsystems).

Statistical Analysis. Each value is expressed as the mean ± SD. Differences between control and test groups were assessed by the Student's t test, a p value of <0.05 indicating a significant difference. The results shown are from a representative run of two individual experiments performed in triplicate.

RESULTS

Effect of Each Phytochemical on PXR-Dependent Transcriptional Activity in LS180 Cells. Approximately 40 kinds of phytochemicals were examined by the reporter assay to identify as to whether or not they could increase the PXR-dependent transcriptional activity. As a result of the reporter analysis, the three phytochemicals, tangeretin and ginkgolides A and B, substantially induced the luciferase activity (**Figures 1** and **2**), while the other phytochemicals had no such inducing effect.

To confirm the results shown in **Figure 1**, we further examined the dose dependence of the PXR-dependent reporter activity by tangeretin and ginkgolides A and B. Tangeretin, ginkgolide A, and ginkgolide B at concentrations of 10, 50, and 100 μM were each added to the culture medium and subjected to the reporter analysis. Tangeretin induced a significant increase of PXR-dependent reporter activity (**Figure 3A**). **Figures 3B**,**C** clearly shows that ginkgolides A and B significantly induced the PXR-dependent reporter activity in a dose-dependent manner. These findings support the results that tangeretin and ginkgolides A and B induced the PXR-dependent transcriptional activity in LS180 cells.

Effect of Tangeretin and Ginkgolides on Transcriptional Activity of Human MDR1 Promoter. It was reported that the transcription of the human MDR1 gene was regulated by PXR. The presence of a cis element in the gene, which involves a PXR-responsive element such as DR4, also was reported (*19*). We therefore examined as to whether ginkgolides A and B and tangeretin could regulate the promoter activity of the human MDR1 gene. We constructed a promoter vector containing the 5′ upstream region of the human MDR1

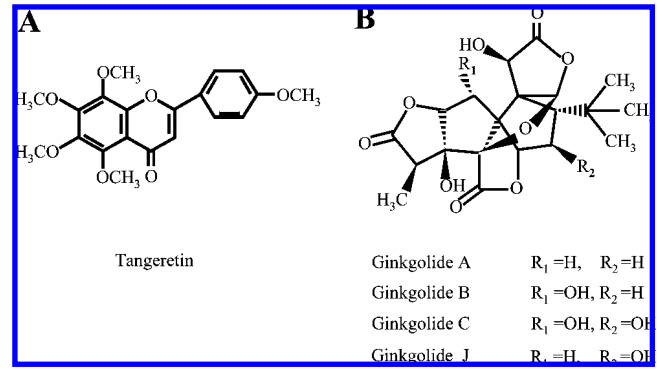

Figure 2. Chemical structures of tangeretin and ginkgolides: (**A**) tangeretin and (**B**) ginkgolides (A: R_1 = H, R_2 = H; B: R_1 = OH, R_2 = H; C: R_1 = OH, R_2 = OH; and J: R_1 = H, R_2 = OH).

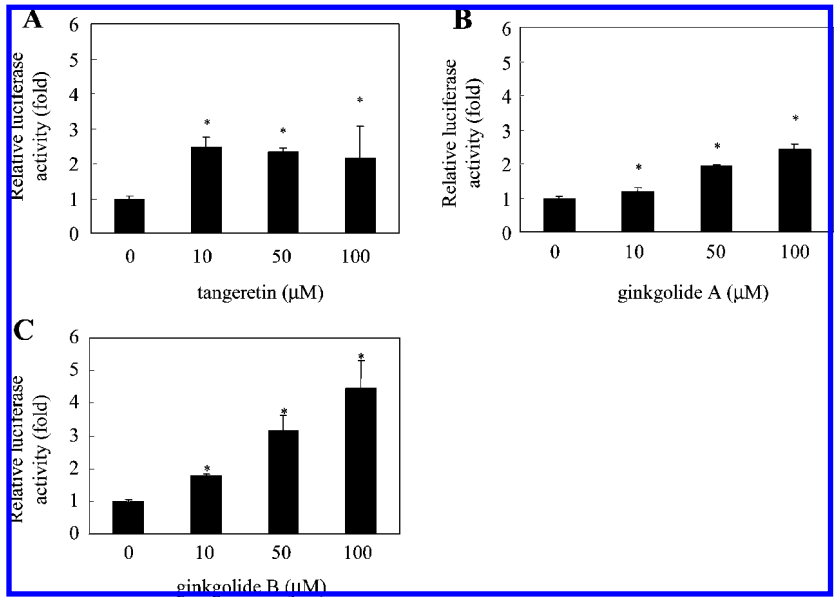

Figure 3. Dose dependence of PXR-dependent induction of reporter activity by phytochemicals in LS180 cells. Transfected cells were incubated with tangeretin (**A**), ginkgolide A (**B**), and ginkgolide B (**C**) at 10, 50, and 100 μM for 24 h, and the reporter assay was performed as described in the Materials and Methods. The relative luciferase activity measured for the vehicle (dimethyl sulfoxide)-treated LS180 cells was taken as 1. Each value is the mean ± SD ($n = 3$). *: Significantly different from the control value ($p < 0.05$).

promoter from -7975 to -7013, as described in the Materials and Methods, for use with the reporter assay. **Figure 4** shows that the reporter activity was significantly increased by treating the PXR-overexpressing cells with tangeretin (**Figure 4A**), ginkgolide A (**Figure 4B**), and ginkgolide B (**Figure 4C**). These phytochemicals induced luciferase activity less significantly than with pcDNA-hPXR in the cells transfected with a mock vector. On the other hand, ginkgolides C and J did not or only slightly increased the reporter activity (**Figure 4D**,**E**). These results strengthen our findings that these phytochemicals could regulate the transcription of human MDR1 via PXR.

Effect of Tangeretin and Ginkgolides on Expression Level of Human MDR1 mRNA. To reveal as to whether tangeretin and ginkgolides A and B could actually regulate the expression level of MDR1 mRNA, the amount of MDR1 mRNA in LS180

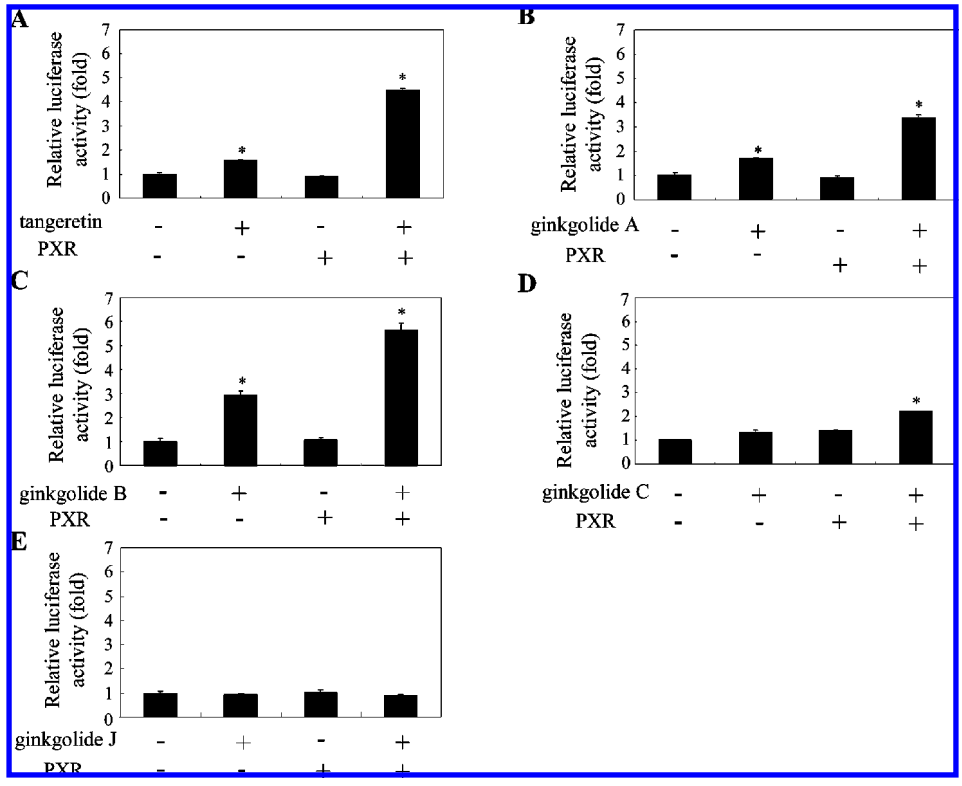

Figure 4. Effect of phytochemicals on transcriptional activity of the human MDR1 promoter in LS180 cells. Transfected cells were incubated for 24 h with tangeretin (**A**), ginkgolide A (**B**), ginkgolide B (**C**), ginkgolide C (**D**), and ginkgolide J (**E**) at 100 μM, and the reporter assay was performed as described in the Materials and Methods. The relative luciferase activity measured for the vehicle (dimethyl sulfoxide)-treated LS180 cells was taken as 1. Each value is the mean ± SD ($n = 3$). *: Significantly different from the control value ($p < 0.05$).

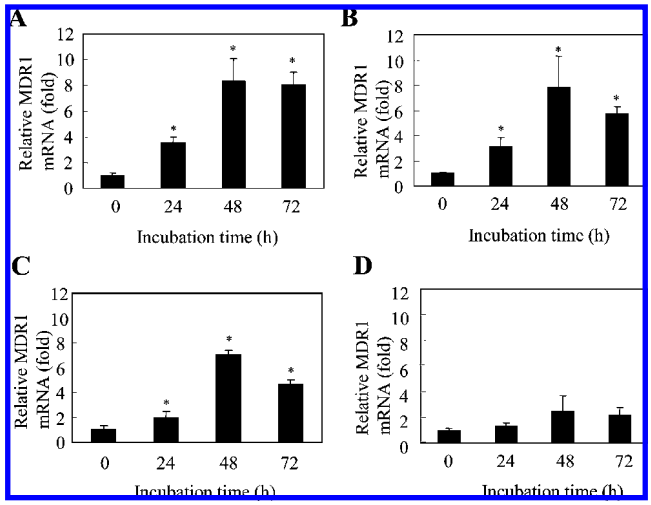

Figure 5. Effect of tangeretin and ginkgolides on the expression level of human MDR1 mRNA. LS180 cells were incubated in the presence of 100 μM tangeretin (**A**), ginkgolide A (**B**), ginkgolide B (**C**), and ginkgolide C (**D**). Total RNA was extracted from the cells after incubating with each phytochemical for 0, 24, 48, or 72 h. The mRNA expression of MDR1 was detected by real-time PCR as described in the Materials and Methods. Each value is the mean ± SD ($n = 3$). *: Significantly different from the control value ($p < 0.05$).

cells was measured by real-time PCR. The exposure of LS180 cells to tangeretin significantly increased the expression level of MDR1 mRNA in a time-dependent manner (**Figure 5A**). Both ginkgolides A and B also increased the expression level of MDR1 mRNA, the highest induction being observed after a 48 h treatment (**Figure 5B**,**C**). On the other hand, ginkgolide C did not significantly increase the expression level of MDR1 mRNA (**Figure 5D**), in accordance with the result of the reporter assay (**Figure 1**).

Effect of Tangeretin and Ginkgolides on Expression Level of Human CYP3A4 mRNA. We also examined the expression level of CYP3A4 mRNA, another target gene of PXR. As the culture time of LS180 cells with tangeretin was increased, the

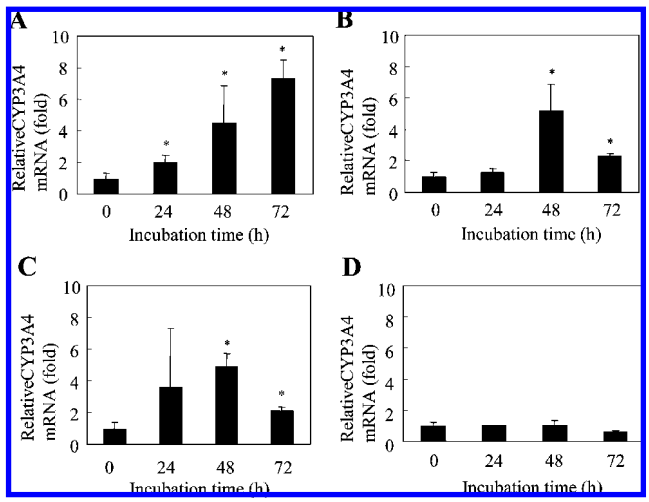

Figure 6. Effect of tangeretin and ginkgolides on expression level of human CYP3A4 mRNA. LS180 cells were incubated in the presence of 100 μM tangeretin (**A**), ginkgolide A (**B**), ginkgolide B (**C**), and ginkgolide C (**D**). Total RNA was extracted from the cells after incubating with each phytochemical for 0, 24, 48, or 72 h. The mRNA expression of CYP3A4 was detected by real-time PCR as described in the Materials and Methods. Each value is the mean ± SD ($n = 3$). *: Significantly different from the control value ($p < 0.05$).

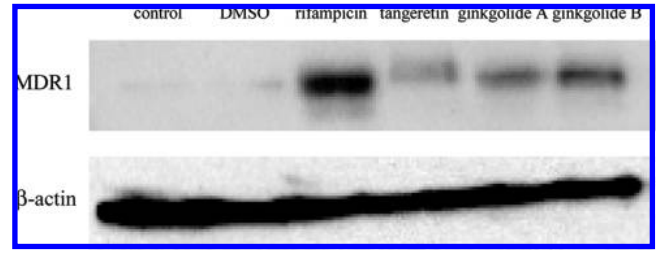

Figure 7. Effect of tangeretin and ginkgolides on expression level of the MDR1 protein in LS180 cells. LS180 cells were incubated with 100 μM rifampicin, tangeretin, ginkgolides A and B, and DMSO for 4 days, and membrane fractions were prepared from each cell. A Western blot analysis was then performed as described in the Materials and Methods.

CYP3A4 mRNA level also gradually and significantly was increased (**Figure 6A**). Ginkgolides A and B had a similar effect as shown in **Figure 6B**,**C**, but ginkgolide C did not (**Figure 6D**). These results strongly suggest that PXR was activated by tangeretin and ginkgolides A and B in LS180 cells.

Effect of Tangeretin and Ginkgolides on Expression Level of *P*-Glycoprotein (P-gp). A Western blot analysis was performed to confirm as to whether the change in MDR1 mRNA was associated with the increased expression of P-gp, a gene product of MDR1. **Figure 7** shows that the expression level of P-gp was markedly increased by tangeretin. Furthermore, the expression of P-gp was clearly up-regulated by exposure to ginkgolides A and B. These results suggest that tangeretin and ginkgolides A and B up-regulated the MDR1 expression, not only at the mRNA level but also at the protein level.

Effect of Tangeretin and Ginkgolides on Activity of P-gp. The effect of tangeretin and ginkgolides on the functional activity of P-gp was measured by using rhodamine 123, one of the typical substrates for human MDR1. LS180 cells were cultured with tangeretin or a ginkgolide at concentrations of 0, 10, 50, and 100 μM for 6 days, and the MDR1 activity was measured. As shown in **Figure 8B**, the accumulation of rhodamine 123 was significantly decreased by treating the cells with tangeretin, its effect being similar to that of rifampicin (**Figure 8A**). Ginkgolides A and B slightly but significantly decreased the accumulation of rhodamine 123 in LS180 cells (**Figure 8C**,**D**), reflecting the increase of P-gp activity. These results demonstrate that tangeretin and ginkgolides A and B increased not only the expression levels of MDR1 mRNA and the glycoprotein but also its functional activity.

DISCUSSION

We examined in the present study the effect of phytochemicals on PXR-dependent transcriptional activity by a reporter analysis and revealed that tangeretin and ginkgolides A and B induced PXR-dependent transcriptional activity. Increased mRNA and protein expression, together with an increased functional activity of MDR1, by these compounds also were demonstrated.

It has been reported that several food-derived compounds, especially flavonoids, have antagonistic and/or agonistic activity toward drug receptors and nuclear receptors. For example, flavones and flavonols have been reported to inhibit the transformation of the aryl hydrocarbon receptor (AhR) and the CYP1A1 expression induced by dioxins (*21–23*). Such flavonoids as apigenin, chrysin, and kaempferol also were reported to show ligand-like activity toward the peroxisome proliferator-activated receptor gamma (PPARγ) that suppresses the expression of such inflammation-related genes as cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) in mouse

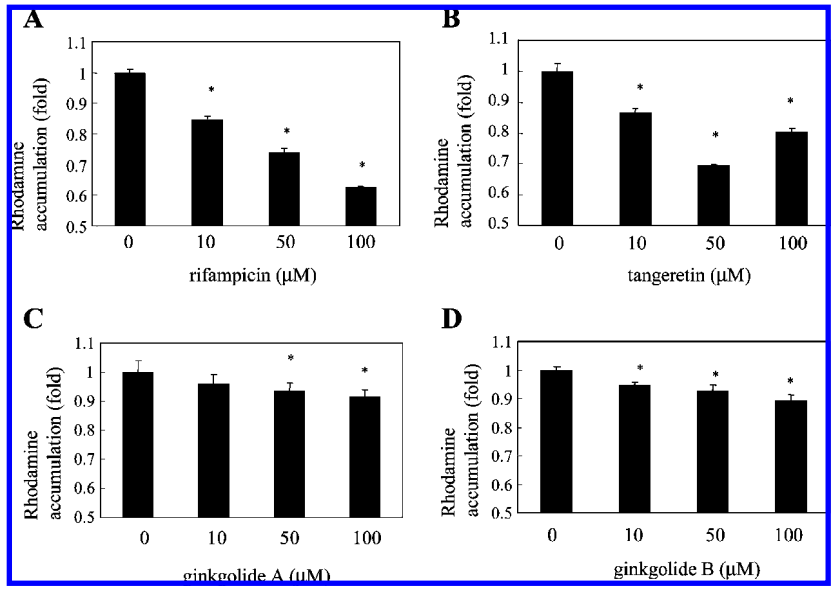

Figure 8. Effect of tangeretin and ginkgolides on accumulation of rhodamine 123 in LS180 cells. LS180 cells were pretreated with rifampicin (**A**), tangeretin (**B**), ginkgolide A (**C**), and ginkgolide B (**D**) at 100 μM for 6 days, and then the rhodamine 123 accumulation was measured. The relative value measured for the vehicle (dimethyl sulfoxide)-treated LS180 cells was taken as 1. Each value is the mean ± SD ($n = 3$). *: Significantly different from the control value ($p < 0.05$).

macrophages (*24*). It is therefore likely that phytochemicals, including flavonoids, could regulate or modulate the activity of drug receptors and nuclear receptors. Several studies have been reported concerning the relationship between PXR and dietary products (*25, 26*). In the present study, we also focused on such phytochemicals as flavonoids and terpenoids and revealed for the first time that tangeretin and ginkgolides A and B activated PXR.

Tangeretin (4′,5,6,7,8-pentamethoxyflavone) is a natural compound and is contained in citrus fruits, with high concentrations in tangerine juice (~10 μM) and tangerine peel oil (~5000 μM) having been reported (*27*). Previous studies have demonstrated that tangeretin inhibited a number of cell activities such as the invasion of tumor cells in vitro (*28*) and also cytolysis by natural killer (NK) cells in vivo (*29*). Furthermore, Slambrouck et al. (*30*) reported that tangeretin inhibited the phosphorylation of extracellular signal-regulated kinases 1/2 (ERK1/2) in human T47D mammary cancer cells. In terms of interaction with drug receptors, we recently found that tangeretin significantly inhibited the TCDD-induced transformation of AhR as well as the expression of CYP1A1 in human hepatoma HepG2 cells (*20*). Our present finding that tangeretin could be involved in the regulation of such nuclear receptors as PXR is quite interesting, providing new insight into the physiological functions of this polyphenol.

Ginkgolides, the other PXR activators found in our study, are understood to be the main active ingredients in a *Ginkgo biloba* extract (*31*). Mauri et al. (*32*) reported that a *G. biloba* extract contains 1.49% ginkgolide A, 0.581% ginkgolide B, 2.567% ginlgolide C, and 0.728% ginkgolide J. The use of medicinal plants or their crude extracts in the prevention and/or treatment of several chronic diseases traditionally has been practiced in different societies worldwide. Extracts of *G. biloba* have been used for centuries in traditional Chinese medicine. The physiological effects of ginkgo extract have been widely reported (i.e., on memory disorders, obstructive arteriosclerosis, Alzheimer's disease, ischemic heart disease, cerebral infarction, aging, and age-related macular degeneration *33–35*). Ginkgo extracts also have been shown to exert a wide range of biological activities at the cellular level, including antiangiogenic and antioxidative properties: Park et al. (*36*) reported the preventive effect of ginkgo extract on the lipopolysaccharide-induced expression of iNOS and COX-2 in the macrophage cell line RAW 264.7. Furthermore, rat studies indicated that an orally administered ginkgo extract induced hepatic CYPs, including CYP3A4 (*37*), although the molecular mechanism has not yet been revealed. Considering that these drug-metabolizing enzymes in hepatocytes are thought to be mainly regulated by PXR, and that ginkgolides A and B are the main active constituents in ginkgo extracts, our present study would successfully explain the cellular regulatory mechanism for this phenomenon. Among these three phytochemicals, the inducing effect of ginkgolide B on the expression level of MDR1 protein is higher than that of tangeretin (**Figure 7**), although the MDR1 activity was higher with tageretin than with ginkgolide B (**Figure 8**). This difference may be due to the incubation time with tangeretin and ginkgolides A and B, that is, the expression level of the MDR1 protein was measured after 4 days of incubation with them, while the MDR1 activity was measured after 6 days of incubation.

The PXR-dependent transcriptional activity reached a plateau at lower concentrations of tangeretin (**Figure 3**). This is because tangeretin may have had a greater affinity for PXR and/or higher permeability to the cell membrane of LS180 cells than the other two active ginkgolides. Furthermore, it is of particular interest that ginkgolides A and B showed a pronounced PXR-dependent agonistic activity, whereas ginkgolides C and J did not (**Figures 1**, **4**, **5**, and **6**). These results for the structure−activity relationship suggest that the proton residue in the R2 region was essential for the activation of PXR, whereas the R1 region was not necessary (**Figure 2**). Moreover, we examined as to whether or not ginkgolides C and J were antagonists of PXR. No antagonistic activity of these compounds was apparent (data not shown). This result suggests that ginkgolides C and J did not bind PXR, while ginkgolides A and B may have directly or indirectly interacted with PXR. This result may be useful in elucidating a more concise regulatory mechanism.

In addition to the well-known PXR function of regulating xenobiotic metabolism, the physiological significance of PXR extensively was increased by recent studies. Tabb et al. (*38*)

reported that the improvement of osteoporosis by vitamin K2 was mediated by PXR. The mutual involvement between inflammation and xenobiotic metabolism was observed (*39, 40*). Zhou et al. (*41*) recently reported that the activation of PXR inhibited the activity of nuclear factor-kappa B (NF-κB), a key regulator of inflammation and of immune response, and, conversely, the suppression of PXR activity by NF-κB. PXR activators such as tangeretin and ginkgolides A and B may therefore function as effective therapeutic or preventive agents for the management of osteoporosis and inflammatory diseases.

We focused in the present study on MDR1 (P-gp) as a target gene product of PXR. P-gp is highly expressed in the cell membrane of tumor cells and excretes hydrophobic drugs from the cells in an ATP-dependent manner. Furthermore, the broad specificity of P-gp causes multidrug resistance (*42*). P-gp is expressed in many normal tissues, especially in the brain, liver, intestine, kidney, and placenta that have barrier functions (*43*). It has been reported that P-gp in normal tissues affected the pharmacokinetics and efficacy of several drugs (*43*). P-gp is expressed on the apical surface of intestinal epithelial cells and excretes drugs and xenobiotic compounds ingested with foods to the apical side. P-gp thus regulates the intestinal absorption of xenobiotic compounds.

Several studies reported the regulation or modulation of MDR1 by food-derived compounds (*13–15*). Ohnishi et al. (*13*) reported that an MDR1 inhibitory substance was present in an extract of grapefruit that was identified as 6′,7′-dihydroxybergamottin. We also found the inhibitory effect of a bitter melon extract on the MDR1 activity in Caco-2 cells and identified 1-monopalmitin as one of the active substances in this extract (*14*). With respect to the regulation of MDR1 at the expression level, Vilaboa et al. (*44*) reported that sodium butyrate significantly induced the transcriptional activity of MDR1 via the activation of heat-shock transcription factor 1. In our previous study, tributyl-tin (TBT) significantly increased the activity of MDR1, this being associated with increased expression levels of MDR1 mRNA and protein (*45*). However, there have been few reports on food-derived substances that could regulate MDR1 expression via PXR activation.

We revealed in this paper for the first time that such phytochemicals as tangeretin and ginkgolides A and B could induce PXR-dependent transcriptional activity. Activation of the MDR1 promoter by these compounds also was observed. These phytochemicals increased the expression level of CYP3A4 mRNA as well as that of MDR1 mRNA. Furthermore, the protein expression and functional activity of MDR1 were both up-regulated by these three compounds in human intestinal LS180 cells. These findings on the regulation of drug-metabolizing enzymes by food-derived compounds will lead to a better understanding of drug−food interactions in the intestinal epithelium.

ABBREVIATIONS USED

PXR, pregnane X receptor; MDR1, multidrug-resistant protein 1; CYP, cytochrome P450; UGT, UDP-glucuronosyltransferase; GST, glutathione *S*-transferase; MRP, multidrug resistant-associated protein; PCR, polymerase chain reaction; P-gp, P-glycoprotein; AhR, aryl hydrocarbon receptor; PPARγ, peroxisome proliferator-activated receptor gamma; COX-2, cyclooxygenase-2; iNOS, inducible nitric oxide synthase; LC-MS/MS, high-performance liquid chromatography-tandem mass spectrometry; NF-κB, nuclear factor-kappa B.

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