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The induction of human UDP-glucuronosyltransferase 1A1 mediated through a distal enhancer module by flavonoids and xenobiotics

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Abstract

We identified the UDP-glucuronosyltransferase (UGT) 1A1 5′-upstream region that confers UGT1A1 induction by various agents, including flavonoids, on a luciferase reporter gene and has the properties of a transcriptional enhancer. Chrysin- and rifampicin-response activities were traced to the same element as a 290-bp distal enhancer module (−3483/−3194), in which the reporter activities were enhanced by activators of nuclear receptors [constitutive androstane receptor (CAR) and pregnane X receptor (PXR)] and transcription factor [aryl hydrocarbon receptor (AhR)]. Utilizing transactivation experiments with the UGT1A1 290-bp reporter gene, we assessed UGT1A1 induction by various flavonoids. 5,7-Dihydroxyflavones with varying substituents in the B-ring and gallocatechin dimers increased the reporter activity in a time- and dose-dependent manner. The treatment of HepG2 cells with the flavonoids for 24 hr elevated the expression of mRNAs and proteins of UGT1A1 and CYP1A1, while the mRNA levels of CYP2B6, CYP3A4, CAR, PXR and AhR was not altered. Chrysin and rifampicin induced the activation of the wild-type reporter gene and T–3263G-mutated gene to a similar extent in HepG2 cells cotransfected with expression vectors of CAR and PXR. Mutation of the AhR core binding region most prominently suppressed the activation of the 290-bp reporter gene by chrysin and baicalein, while mutations of four putative nuclear receptor motifs (DR4 element, PXRE, CARE and DR3 element) partly decreased its activation. Taken together, the results indicate that UGT1A1 was induced in response to flavonoids and xenobiotics through the transactivation of the 290-bp reporter gene, that was a multi-component enhancer containing CAR, PXR and AhR motifs.

Keywords: Aryl hydrocarbon receptor (AhR); Constitutive androstane receptor (CAR); Pregnane X receptor (PXR); UDP-glucuronosyltransferase 1A1 (UGT1A1); Flavonoids; Xenobiotics

1. Introduction

UDP-glucuronosyltransferase, UGT1A1, plays a critical role in the detoxification of potentially neurotoxic bilirubin by conjugating it with glucuronic acid [1], and conjugates many drugs and other xenobiotics [2–4]. The requirements for the glucuronidation of bilirubin for excretion were first elucidated on the basis of the finding that the severe form of unconjugated hyperbilirubinemia in the absence of hemolysis or other liver disease, known as Crigler–Najjar type I syndrome, can be fatal [5]. Reduced UGT1A1 activity

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Abbreviations: AhR, aryl hydrocarbon receptor; AhRE, AhR response element; bp, base pair(s); CAR, constitutive androstane receptor; CARE, CAR response element; CYP, cytochrome P450; DMSO, dimethyl sulfoxide; DR, direct repeat; hAhR, human AhR; hCAR, human CAR; hPXR, human PXR; kb, kilobase pairs; PBREM, phenobarbital-responsive enhancer module; PCR, polymerase chain reaction; pGL3-tk, pGL3-tk-firefly luciferase vector; PXR, pregnane X receptor; PXRE, PXR response element; RXR, retinoid X receptor; tk, thymidine kinase promoter; UGT, UDP-glucuronosyltransferase.

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causes unconjugated hyperbilirubinemia (Crigler–Najjar syndrome and Gilbert's syndrome) [6] and decreases the glucuronidation of SN-38 (a pharmacologically active metabolite of the anticancer drug, irinotecan), leading to an increased risk for the development of severe irinotecan-associated toxicity [3,7]. Phenobarbital is used as a therapeutic drug for patients with Crigler–Najjar type II syndrome, because it increases the expression of bilirubin glucuronosyltransferase and markedly reduces the incidence of unconjugated hyperbilirubinemia [8]. The 51-bp phenobarbital-responsive enhancer module, which is conserved between species, has been identified in phenobarbital-inducible *CYP2B* genes and is regulated by the nuclear receptor known as constitutive androstane receptor (CAR; NR1I3) in response to phenobarbital induction [9]. Furthermore, we have identified the module in the phenobarbital-inducible human *UGT1A1* gene (UGT1A1 phenobarbital-responsive enhancer module, PBREM), and have identified CAR as a transcription factor regulating PBREM in response to phenobarbital induction [10]. PBREM is a composite 290-bp element consisting of four nuclear receptor motifs, named DR4 element, CAR response element (CARE) and DR3 element [10] and pregnane X receptor (PXR; NR1I2) response element (PXRE) [11], and aryl hydrocarbon receptor (AhR) response element (AhRE) [12]. The functional roles of DR4 and DR3 elements are still unknown, whereas CARE, PXRE and AhRE have been reported as the binding sites of CAR, PXR and AhR, respectively. Recently, a single nucleotide polymorphism changing T to G at nucleotide −3263 has been found in *UGT1A1* [13]. Since the T–3263G mutation in the direct-repeat spacer of the PBREM DR3 site significantly decreases the transcriptional activity (associated with increased plasma total bilirubin levels) [13], the DR3 site may play an important role in the regulation of UGT1A1 expression in human subjects.

The modulation of UGT1A1 expression by dietary factors is of considerable interest, because phenobarbital is a therapeutic drug for mild unconjugated hyperbilirubinemia but has the serious disadvantage of inducing sleep. Walle *et al.* have demonstrated that the flavonoid chrysin can induce UGT1A1 mRNA and protein in Caco-2 cells [14] and HepG2 cells [15,16]. As the mechanism by which chrysin induces UGT1A1 expression has not been addressed, we focused on the induction of UGT1A1 in response to dietary flavonoids. First, we clarified the location of the chrysin-responsive enhancer element in the *UGT1A1* gene using constructs in which various DNA fragments generated from a 11-kbp 5′-flanking region of *UGT1A1* were placed in front of the reporter luciferase gene and examined for enhancer activity in HepG2 cells. Surprisingly, we traced the chrysin-response activity as well as the rifampicin-response activity to the 290-bp distal enhancer module, which is activated by CAR, PXR and AhR [10–12]. In this study, we further investigated the effects of dietary flavonoids and xenobiotics on human UGT1A1 induction, utilizing a screening system for assessing UGT1A1 induction with the 290-bp distal enhancer module, and the characterization of UGT1A1 induction by flavonoids and xenobiotics.

2. Materials and methods

2.1. Drugs and materials

Chrysin, apigenin, luteolin, rutin hydrate, taxifolin, black tea polyphenol extract, methoxychlor, rifampicin, clotrimazole, 3-methylcholanthrene and *tert*-butylhydroquinone were purchased from Sigma-Aldrich Fine Chemicals. Quercetin dihydrate, kampferol, baicalein, naringenin, flavone, α-naphthoflavone, β-naphthoflavone and benzo[*a*]pyrene were from Wako Chemicals; 3-hydroxyflavone, 5-hydroxyflavone and 7-hydroxyflavone from Extrasynthese S.A.; metyrapone from Biomol Research Laboratories; green tea polyphenol extract from Kurita Co. Free theaflavin, theaflavin 3-*O*-gallate, theaflavin 3′-*O*-gallate, theaflavin 3,3′-di-*O*-gallate, (−)epicatechin, (−)epigallocatechin, (−)epicatechingallate, (−)epigallocatechingallate, theasinensin A, procyanidine B-2 3,3′-di-*O*-gallate and procyanidine B-5 3,3′-di-*O*-gallate were prepared as described previously [17].

2.2. Plasmids

The subfragments of the 11-kbp flanking region were obtained as follows: the UA fragment was generated from pCAT/2.9 kbp [18], the UB, UC, UD, UE and UF fragments were prepared from HepG2 genomic DNA (HepG2 cells came from the American Type Culture Collection, and from the Riken Cell Bank), and the U2K fragment and the 1650-, 462-, 290- and 190-bp subfragments of the U2K fragment were prepared from human genomic DNA and cloned into the pGL3-tk-firefly luciferase reporter plasmid as described by Sugatani *et al.* [10]. Since the T normally present at nucleotide –3263 in the distal enhancer region of *UGT1A1* is substituted with G in HepG2 genomic DNA with decreased reporter activity [13], we obtained wild-type U2K fragments having T at nucleotide −3263 from wild-type human genomic DNA by PCR amplification with the primers 5′-gtttccgctagcATCGCGTTTCTACGCGGT-3′ and 5′-gtttaactcgagCCTCTGCCTTGCTCTCAAAA, and cloned them into the *Nhe*I–*Xho*I sites in the pGL3-tk-firefly luciferase reporter plasmids as described by Sugatani *et al.* [10]. We used the wild-type 290 bp reporter gene unless stated otherwise. Bases in lower-case letters were added to digest the oligonucleotides with the restriction enzymes *Nhe*I and *Xho*I. The cDNA encoding the full-length human PXR was amplified by PCR with the primers 5′-gccggatccgcaaacATGGAGGTGAGACCCAAA-3′ and 5′-gcgctcgagTCAGCTACCTGTGATGCC-3′ and cloned into the *BamH*I and *Xho*I sites in the expression vector pCR3.1 (Invitrogen Corp.). The pCR3-human CAR expression

vector was a gift from Dr. M. Negishi (National Institute of Environmental Health Sciences). For deletion analysis of the 290-bp fragment, we amplified six subfragments located at nucleotide numbers −3483/−3434, −3433/−3384, −3392/−3333, −3343/−3284, −3293/−3234, and −3243/−3194 by using the following 12 primers, and cloned them into pGL3-tk-firefly luciferase reporter plasmid at the *Nhe*I and *Xho*I sites. The sequences of the primers were: (1) 5′-gtttccgctagcTACACTAGTAAAGGTCACT-3′, (2) 5′-gtttccgctagcACATTCTAACGGTTCATA-3′, (3) 5′-gtttccgctagcACATTCTAACGGTTCATA-3′, (4) 5′-gtttaactcgagGATAACACATCCTCATTA-3′, (5) 5′-gtttccgctagcTGTGTTATCTCACCAGAA-3′, (6) 5′-gtttaactcgagTTCAGGTTATGTAACTAG-3′, (7) 5′-gtttccgctagcCATAACCTGAAACCCGGA-3′, (8) 5′-gtttaactcgagAGCTTACTATGACTGTTC-3′, (9) 5′-gtttccgctagcATAGTAAGCTGGCCAAGG-3′, (10) 5′-gtttaactcgagCCTTTGATGTTCTCAAAT-3′, (11) 5′-gtttccgctagcACATCAAAGGAAGTTTGG-3′, (12) 5′-gtttaactcgagCCCTCTAGCCATTCTGGA-3′. Bases in lower-case letters were added to digest the oligonucleotides with the restriction enzymes *Nhe*I and *Xho*I. The mutations of the DR4 element, CARE and DR3 element [10], PXRE [11] and AhRE [12] in the 290 bp distal enhancer module were performed using a QuickChange site-directed mutagenesis kit according to the manufacturer's instructions. The primers used were; 5′-TACACTAGTAA*GGCGCC*CTCAATTCCAAGG-3′, 5′-AGAACAAACTTC*GGCGCC*TATATAACCTC-3′, 5′-TGGCCAAGGGTAGA*TTC*CAGTTTGAACAAAG-3′, 5′-ACATTCTAAC*TT*TTCATAAAGGGTATTAGG-3′, and 5′-CTTGGTAAG*AC*CGCAATGAAC-3′ and complements of these for DR4 element, CARE, DR3 element, PXRE and AhRE, respectively. Italicized characters show the bases for the mutation.

2.3. Transient transfection and luciferase assays

Transfection assays were performed as described by Sugatani *et al.* [10]. HepG2 cells (Riken Cell Bank) and LLC-PK1 cells (American Type Culture Collection) were plated at 1×10^5 cells/mL and 2.5×10^4 cells/mL in Dulbecco's modified Eagle's medium, respectively; 24 hr later they were transfected with UGT1A1-luciferase reporter constructs (0.2 μg) and pRL-SV40 plasmid (0.2 μg) using a calcium phosphate co-precipitation method (CellPhect Transfection Kit, Amersham Biosciences), and the medium was replaced with growth medium after 12 hr. When the effects of flavonoids and other xenobiotics were investigated, the cells were subsequently treated with dietary or synthetic flavonoids, methoxychlor, chlotrimazole, rifampicin, metyrapone, β-naphthoflavone, 3-methylcholanthrene, benzo[*a*]pyrene and *tert*-butylhydroquinone as 400× concentrated stocks in DMSO; controls received an equivalent volume of DMSO. The cells were harvested after an additional 24 hr of culture unless otherwise stated. Luciferase activity was measured simultaneously using the Dual–Luciferase reporter assay system (Promega).

2.4. mRNA levels

To measure the mRNA levels of UGT1A1, cytochrome P450 (CYP1A1, CYP2B6 and CYP3A4), AhR, CAR and PXR, cDNA prepared from total cellular RNA of HepG2 cells was subjected to quantitative real-time PCR using ABI GeneAmp 5700 (PE Applied Biosystems) as described previously [10,19]. The mRNA levels were normalized against β-actin mRNA determined by Pre-Developed TaqMan Assay Reagents for human β-actin (PE Applied Biosystem).

2.5. Western blot analysis

HepG2 cells cultured in three 75 T flasks and treated with chrysin (5 or 25 μM) or a vehicle (DMSO) were collected by centrifugation at 200 *g* for 2 min and homogenized with a motorized Teflon/glass homogenizer (10 strokes) in homogenized buffer, consisting of pH 7.4, 10 mM KH_2PO_4, 250 mM sucrose, 1 mM EDTA and 0.04 mg/mL PMSF. After 10-min low-speed (10,000 *g*) centrifugation at 4°, the supernatant was removed and the microsomes were pelleted by high-speed centrifugation (100,000 *g*, 60 min) as described by Chen *et al.* [20]. The protein concentration of microsomal protein was determined using a bicinchoninic acid protein assay kit (Pierce Chemicals), using bovine serum albumin as a standard. Microsomal proteins (20 μg) were resolved on a sodium dodecyl sulfate–12.5% polyacrylamide gel, electroblotted onto a polyvinylidene difluoride membrane, and incubated with anti-human UGT1A1 antibody (BD Biosciences or anti-rat CYP1A1 antibody (Daiichi Pure Chemical Co). After incubation with horseradish peroxidase-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories) for UGT1A1 detection or horseradish peroxidase-conjugated donkey anti-goat IgG for CYP1A1 detection (Jackson ImmunoResearch Laboratories), the resultant immunoproducts were visualized with an enhanced chemiluminescence system (Amersham Biosciences).

2.6. Statistical analysis

Data are expressed as the average ± SD. Statistical significance of differences between different groups was analyzed using ANOVA or unpaired *t*-test. Differences were considered significant at $P < 0.05$.

3. Results

3.1. Isolation and characterization of flavonoids- and xenobiotics-responsive enhancer elements in the UGT1A1 5′-flanking region

To test whether sequences upstream of the 5′-flanking region of *UGT1A1* may contribute to the activation process

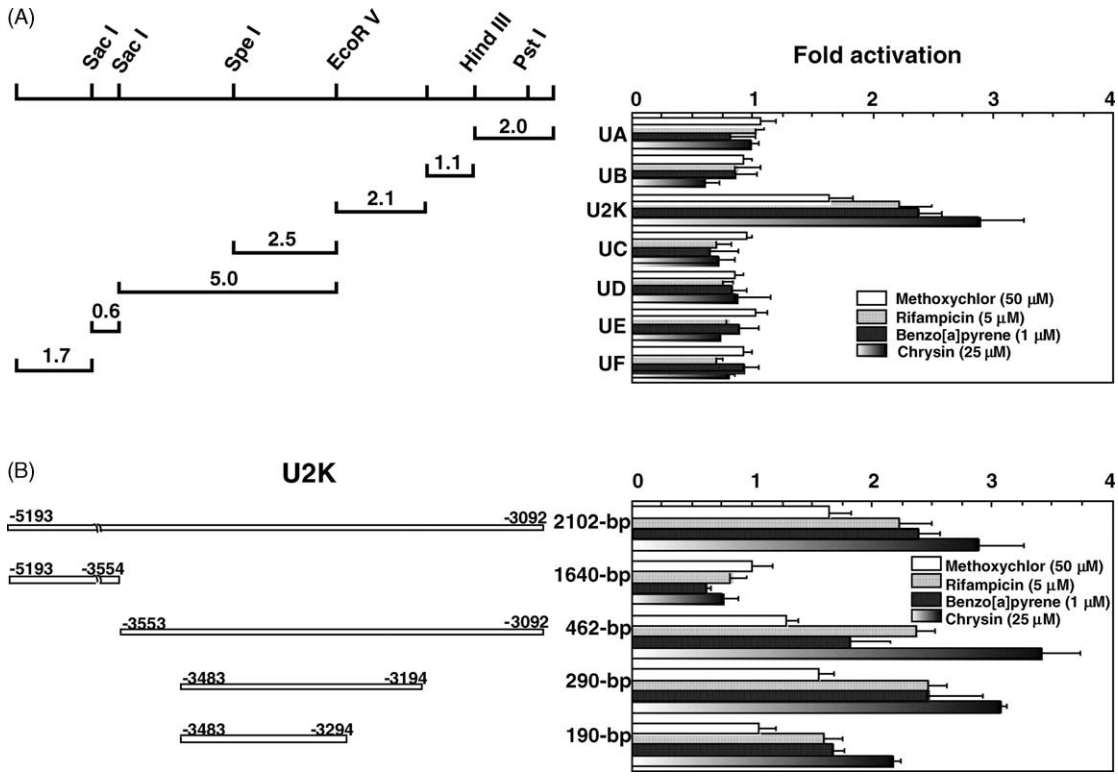

Fig. 1. Transcriptional activity of various 5′-flanking fragments in methoxychlor-, rifampicin-, benzo[*a*]pyrene- or chrysin-treated HepG2 cells. Various fragments were generated from the 11-kbp 5′-flanking DNA of the *UGT1A1* gene (A) and from the U2K DNA (B), constructed into the luciferase reporter gene plasmid, and co-transfected with pRL-SV40 (0.2 μg) for methoxychlor-, benzo[*a*]pyrene- or chrysin-activation or pRL-SV40 (0.2 μg) and hPXR-pCR3 expression vector (0.2 μg) for rifampicin-activation into HepG2 cells. Cells were then treated with the vehicle (DMSO) alone, methoxychlor (50 μM), rifampicin (5 μM), benzo[*a*]pyrene (1 μM) or chrysin (25 μM) for 24 hr, and the luciferase activity was determined as described in Section 2. Fold activation was calculated for each reporter construct by dividing the activity with the inducer by that without the inducer. Data presented are the average of three independent experiments ± SD.

by chrysin, we tested various DNA fragments generated from a 11-kbp 5′-flanking region in *UGT1A1* and placed in front of the reporter luciferase gene for their enhancer activities compared with those by methoxychlor (so-called phenobarbital-type inducer) [21], rifampicin (PXR activator) [22] and benzo[*a*]pyrene (AhR activator) [23] (Fig. 1). The U2K fragment (−5193/−3092) displayed the most prominent activation by 25 μM chrysin, 50 μM methoxychlor, 5 μM rifampicin and 1 μM benzo[*a*]pyrene (Fig. 1A). Further deletion assays were performed on the U2K fragment to delineate the minimum sequence that could be activated by chrysin, methoxychlor, rifampicin and benzo[*a*]pyrene (Fig. 1B). The 462-bpp 3′-fragment (−3553/−3092) produced full activation, while the 1640-bp 5′-fragment (−5193/−3554) produced no activation. The 290-bp fragment was fully activated. We used the 290-bp fragment including 3′ bases additional to the 190-bp fragment (−3483/−3194) for subsequent analysis, because the activation of the 190-bp fragment by 25 μM chrysin, 50 μM methoxychlor, 5 μM rifampicin and 1 μM benzo[*a*]pyrene was only 0.71×, 0.68×, 0.65× and 0.68× that of the 290-bp fragment, respectively. In addition, the 290-bp reporter gene displayed the most prominent activation by clotrimazole (10 μM), metyrapone (500 μM) and black tea polyphenol extract (100 μg/mL) (data not shown).

Furthermore, we investigated the effect of the compounds previously characterized as inducers of CYP2B/3A and CYP1A1 and chrysin on the activation of UGT1A1 290-bp reporter gene in HepG2 cells in the presence of exogenously expressed hCAR and hPXR. In the absence of exogenously expressed nuclear receptor, methoxychlor (50 μM), clotrimazole (10 μM), chrysin (5 μM) and benzo[*a*]pyrene (1 μM) elevated the reporter activities to 147, 210, 201 and 237%, respectively (Fig. 2). In the presence of exogenously expressed hCAR, the reporter activity was elevated in the absence of the inducers, and treatment of the cells with methoxychlor, clotrimazole, chrysin and benzo[*a*]pyrene resulted in further increases in the reporter activity, but phenobarbital and rifampicin gave no further increase (Fig. 2). On the other hand, treatment of hPXR-pCR3-transfected cells with methoxychlor, phenobarbital, rifampicin, clotrimazole, chrysin and benzo[*a*]pyrene elicited increases in the reporter activity (Fig. 2). These observations indicate that not only CAR but also PXR and AhR may regulate the transcription and drug induction of *UGT1A1* in manners analogous to those reported for human CYP2B/3A and CYP1A1.

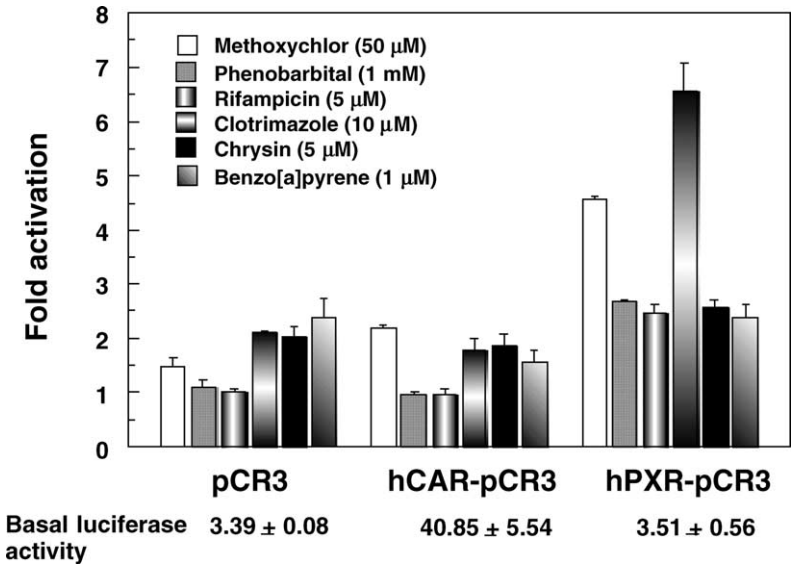

Fig. 2. Effects of various drugs on the transcriptional activation of UGT1A1 290-bp reporter gene by hCAR and hPXR. HepG2 cells were cotransfected with expression plasmid for hCAR or hPXR or control vector pCR3 and the 290-bp-tk-luciferase plasmid. The transfected cells were treated with vehicle (DMSO) alone, methoxychlor (50 μM), phenobarbital (1 mM), rifampicin (5 μM), clotrimazole (10 μM), chrysin (5 μM) or benzo[*a*]pyrene (1 μM) for 24 hr, harvested, and assayed for luciferase activity. Fold activation was calculated by dividing the activity with the inducer by that without the inducer. Basal luciferase activity, measured by pGL3-tk basic reporter gene in control vector-transfected and vehicle-treated HepG2 cells, is calculated as one. Data presented are the average of the three independent experiments ± SD.

3.2. Elevated reporter activity of the UGT1A1 290-bp distal enhancer module by various flavonoids

As the 290-bp distal enhancer module contains the DNA binding domains for CAR, PXR and AhR, the maximal time period for induction exposure using the activators of CAR, PXR and AhR was determined by establishing the time course of induction-mediated luciferase activity. In HepG2 cells without an exogenously expressed nuclear receptor, clotrimazole (10 μM), methoxychlor (50 μM), metyrapone (500 μM), β-naphthoflavone (2.5 μM), chrysin (25 μM) and *tert*-butylhydroquinone (80 μM) elevated the reporter activities, that reached near the plateau at 48 hr (248, 273, 226, 294, 476 and 226%, respectively), while the elevation in the reporter activities by 3-methylcholanthrene (1 μM) and benzo[*a*]pyrene (1 μM) reached the maximum at 12 hr (Fig. 3). Accordingly, in order to determine the flavonoid-structural requirements, we examined the effects of an additional 25 flavonoids, shown in Fig. 4, on UGT1A1 expression by measuring the reporter activity of the 290-bp distal enhancer module after 48 hr-incubation of human hepatoma cell line HepG2 cells and pig proximal tube-like cell line LLC-PK1 cells with those compounds. Flavone had the most potent induction; 5,7-dihydroxyflavones with varying substituents in the B-ring, such as luteolin, apigenin, chrysin and baicalein, 5-hydroxyflavone, 7-hydroxyflavone and α-naphthoflavone at a concentration of 25 μM elevated the reporter activities to 306–848% in HepG2 cells and 205–567% in LLC-PK1 cells (Fig. 5). The feature of the flavonoid structure associated with the induction of UGT1A1, obtained by using UGT1A1 reporter gene assays, was consistent with a previous observation using catalytic activity assays [16]. While black tea components such as theaflavin, theaflavin 3-*O*-gallate, theaflavin 3′-*O*-gallate and theaflavin 3,3′-di-*O*-gallate and green tea polyphenol components such as (−)-epicatechin, (−)-epigallocatechin, (−)-epicatechin gallate and (−)-epigallocatechin gallate at 2.5–25 μM

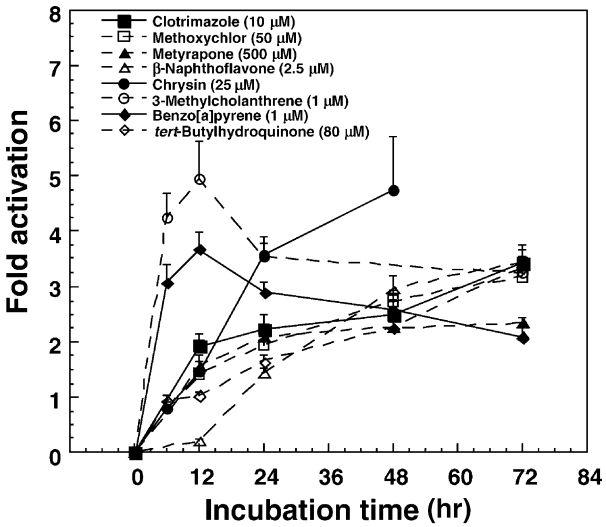

Fig. 3. Time dependence of inducer-mediated luciferase activity in HepG2 cells. The UGT1A1 290-bp enhancer module-tk-luciferase plasmid was co-transfected with pRLSV40 into HepG2 cells. The transfected cells were incubated with clotrimazole (10 μM), methoxychlor (50 μM), metyrapone (500 μM), β-naphthoflavone (2.5 μM), chrysin (25 μM), 3-methylcholanthrene (1 μM), benzo[*a*]pyrene (1 μM) or *tert*-butylhydroquinone (80 μM) for the indicated time periods, harvested, and assayed for luciferase activity. Fold activation was calculated by dividing the activity with the inducer by that without the inducer. Data presented are the average of three independent experiments ± SD.

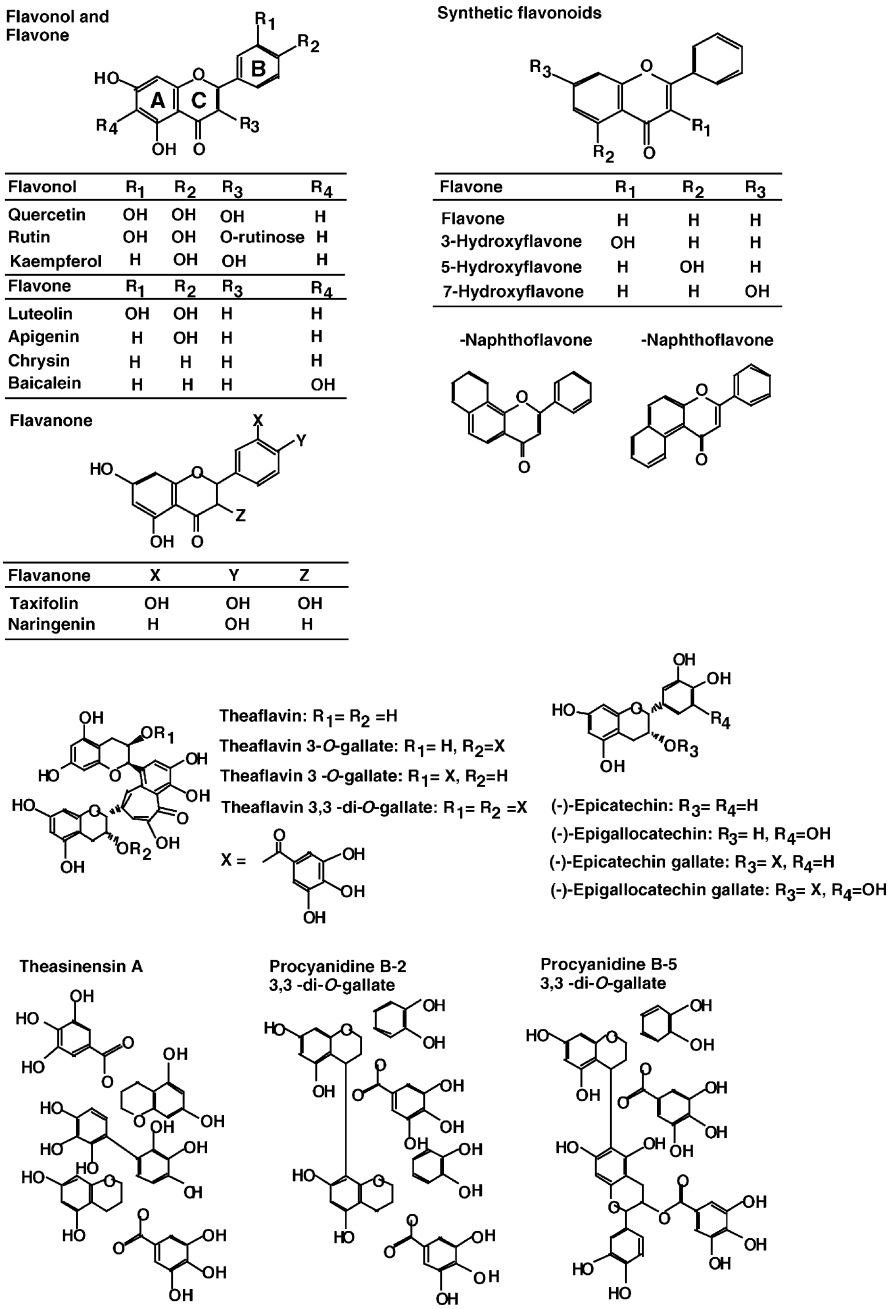

Fig. 4. The structures of dietary flavonoids, synthetic flavonoids, black tea polyphenols, green tea polyphenols, and gallocatechin dimers, used in this study.

had no effect on luciferase activity, procyanidine B-2 3,3′-di-*O*-gallate and procyanidine B-5 3,3′-di-*O*-gallate (gallocatechin dimers) at 25 μM potently enhanced the reporter activities in both HepG2 cells and LLC-PK1 cells (121–196% of the control activity in DMSO-treated cells) (Fig. 5). A dose-dependent effect was produced by these flavones and gallocatechin dimers, except apigenin, which had maximal activation at 2.5 μM (Fig. 6A). While maximum induction by apigenin and luteolin at 2.5 μM and procyanidine B-5 3,3′-di-*O*-gallate at 12.5 μM occurred at 24 hr, induction by chrysin at 5 μM and procyanidine B-2 3,3′-di-*O*-gallate at 12.5 μM reached near the plateau at 24 hr (Fig. 6B). These observations indicate that the reporter gene assay is quite sensitive within a short time period, compared with the catalytic activity assay [15,16].

3.3. Effects of treatment with chrysin, quercetin, procyanidine B-5 3,3′-di-O-gallate, benzo[a]pyrene and tert-butylhydroquinone on mRNA and protein levels of UGT1A1, CYP1A1, CYP2B6, CYP3A4, CAR, PXR and AhR in HepG2 cells

To examine the possible mechanisms mediating UGT1A1 induction, we compared the mRNA levels of UGT1A1 and CYP1A1 in chrysin- and procyanidine B-5 3,3′-di-*O*-gallate-treated HepG2 cells with those found in

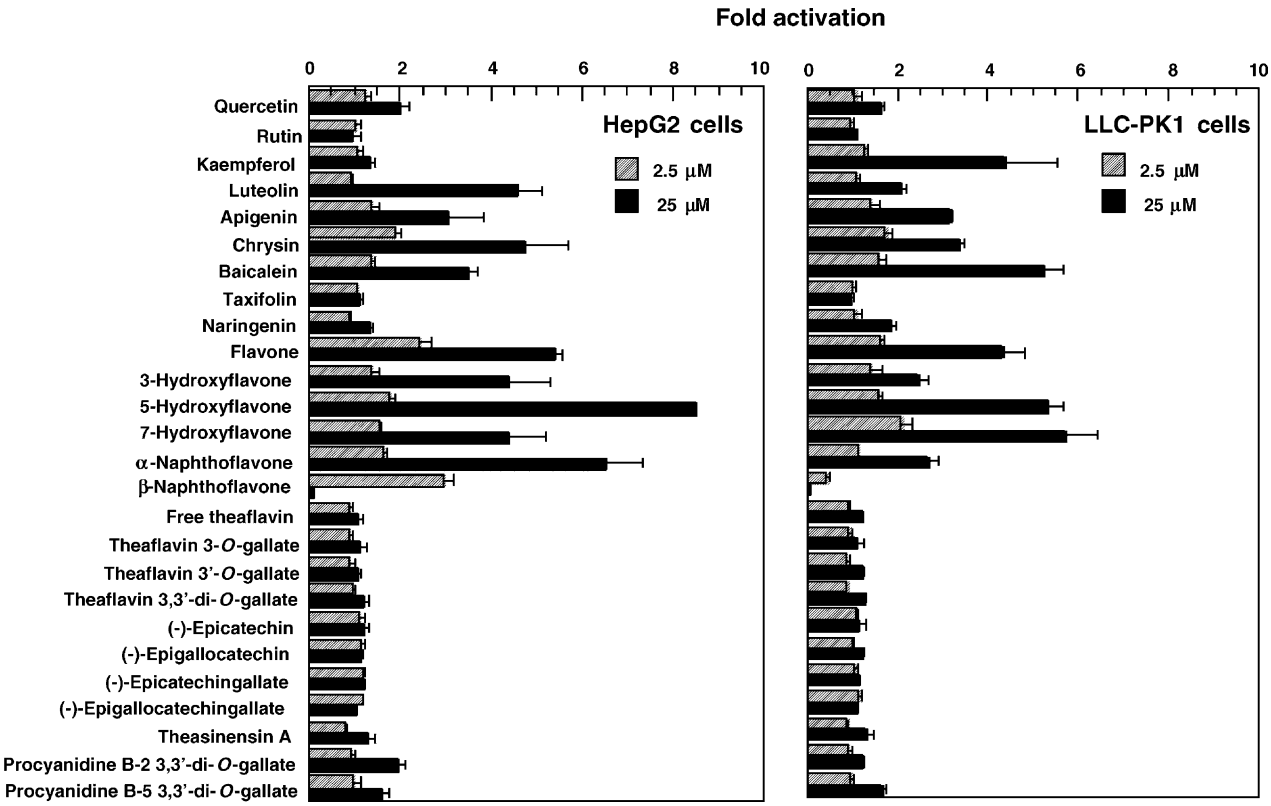

Fig. 5. Effects of various flavonoids on the transcriptional activation of UGT1A1 290 bp enhancer module in HepG2 cells and LLC-PK1 cells. The 290 bp-tk-luciferase plasmid was cotransfected with pRL-SV40 into the cells. The transfected cells were incubated with various flavonoids at concentrations of 2.5 and 25 μM for 48 hr, harvested, and assayed for luciferase activity. Fold activation was calculated by dividing the activity with the inducer by that without the inducer. Data presented are the average of three independent experiments ± SD.

cells treated with quercetin (AhR agonist [24]), benzo[*a*]-pyrene (AhR agonist) and *tert*-butylhydroquinone (antioxidant-type inducer [25]). Treatment with chrysin at 25 μM, procyanidine B-5 3,3′-di-*O*-gallate at 12.5 μM, quercetin at 25 μM and *tert*-butylhydroquinone at 80 μM for 24 hr produced a significant induction of UGT1A1 mRNA, 4.8, 0.72, 0.95 and 0.77 times that produced with benzo[*a*]pyrene treatment at 1 μM, while the increased induction of CYP1A1 mRNA was 0.3, 0.05, 0.13 and 0.09 times that of CYP1A1 mRNA in response to benzo[*a*]pyrene, respectively (Table 1). In the cells treated with chrysin at 25 μM for 48 hr, the increase in UGT1A1 mRNA induction was maintained at 15.77 ± 8.14 times that of the DMSO control, while the CYP1A1 mRNA level declined to

Table 1
Effects of flavonoids on levels of mRNAs specific for UGT1A1, CYP1A1, CYP2B6, CYP3A4, CAR, PXR and AhR in HepG2 cells

	Relative mRNA level				
	Chrysin (25 μM)	Procyanidine B-5 3,3′-di-*O*-gallate (12.5 μM)	Quercetin (25 μM)	Benzo[*a*]pyrene (1 μM)	*tert*-Butylhydroquinone (80 μM)
UGT1A1	12.13 ± 3.80**	1.83 ± 0.39*	2.41 ± 0.88*	2.55 ± 0.36**	1.97 ± 0.67*
CYP1A1	20.85 ± 6.63**	3.59 ± 2.03*	9.02 ± 5.42***	69.29 ± 10.72***	6.38 ± 0.94***
CYP2B6	3.04 ± 2.17	Not determined	Not determined	Not determined	Not determined
CYP3A4	1.82 ± 1.15	Not determined	Not determined	Not determined	Not determined
CAR	N.D.	Not determined	Not determined	Not determined	Not determined
PXR	1.49 ± 0.57	Not determined	Not determined	2.84 ± 1.10	2.00 ± 0.39*
AhR	1.99 ± 0.97	Not determined	Not determined	1.41 ± 0.18*	2.23 ± 0.31***

Total RNA was isolated from HepG2 cells treated for 24 hr with either the vehicle (DMSO), chrysin (25 μM), quercetin (25 μM), procyanidine B-5 3,3′-di-*O*-gallate (12.5 μM), benzo[*a*]pyrene (1 μM) or *tert*-butylhydroquinone (80 μM). Relative mRNA levels are expressed by taking the control values obtained from the vehicle-treated cells as one. Data presented are the average ± SD of normalized mRNA abundance (four samples per treatment group). N.D., not detected.

* $P < 0.05$ vs. vehicle.
** $P < 0.01$ vs. vehicle.
*** $P < 0.001$ vs. vehicle.

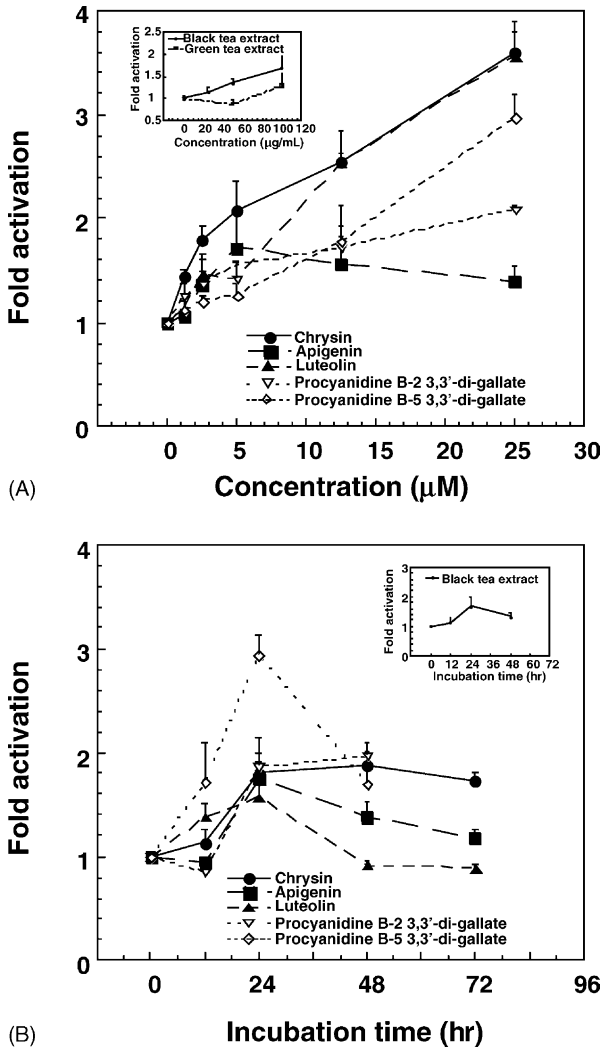

Fig. 6. Dose (A)- and time (B)-dependent transcriptional activation of UGT1A1 290 bp enhancer module by various flavonoids and tea polyphenol extracts in HepG2 cells. The 290 bp-tk-luciferase plasmid was co-transfected with pRL-SV40 into HepG2 cells. (A) Doses ranged from 1 to 25 μM of each flavonoid and from 25 to 100 μg/mL of tea polyphenol extracts with 24 hr of exposure. (B) The transfected cells were incubated with various flavones at 2.5 μM, gallocatechin dimers at 25 μM or black tea polyphenol extract at a concentration of 100 μg/mL for 12 to 48 hr, harvested, and assayed for luciferase activity. Fold activation was calculated by dividing the activity with the inducer by that without the inducer. Data presented are the average of three independent experiments ± SD.

4.88 ± 2.26 times that of the DMSO control (data not shown). Treatment with chrysin at 5 μM for 24 hr produced a significant increase in the UGT1A1 mRNA level to 2.19 ± 0.49 times that of the DMSO control ($P < 0.05$) (data not shown), whereas mRNA levels of CYP2B6 and CYP3A4 did not significantly change in response to chrysin at 25 μM for 24 hr (Table 1). In fact, treatment with chrysin at 5 and 25 μM for 24 hr induced the expression of microsomal UGT1A1 protein (2.5 and 8.2 times that of the DMSO control) and microsomal CYP1A1 protein (15.1 and 31.3 times that of the DMSO control), respectively (Fig. 7). Furthermore, we considered whether the expression of CAR, PXR and AhR could be increased by chrysin. CAR mRNA was not detectable in either untreated HepG2 cells or cells treated with chrysin (Table 1). The levels of PXR and AhR mRNAs did not significantly change in response to chrysin (Table 1).

3.4. Characterization of the UGT1A1 290-bp distal enhancer module by directed mutagenesis

First, we investigated the effect of T–3263G mutation in the DR3 motif of the 290-bp distal enhancer module on CAR- and PXR-mediated UGT1A1 induction and enhancement by chrysin. In the presence of exogenously expressed CAR, the reporter gene expression was elevated in the absence of the inducers, and treatment of the cells with chrysin (5 μM) resulted in further increases in luciferase activity [1.84 times that of the DMSO control (Fig. 8)]. On the other hand, treatment of hPXR-pCR3-transfected cells with chrysin (5 μM) elicited increases in the luciferase activity 1.32 times that in the cells transfected with the control vector pCR3 and treated with chrysin (5 μM) (data not shown). The activation extent of the mutated 290-bp reporter gene by 5 μM chrysin (2.17 and 2.99 times that of the DMSO control) was greater than that of the wild-type 290-bp reporter gene (1.84 and 1.57 times that of the DMSO control) in the exogenously hCAR- and hPXR-expressed cells, respectively. Similarly, rifampicin (5 μM) increased the PXR-mediated reporter gene activities of the wild-type and mutated genes (2.47 and 3.66 times that of the DMSO control, respectively), indicating that flavones and xenobiotics are effective in the

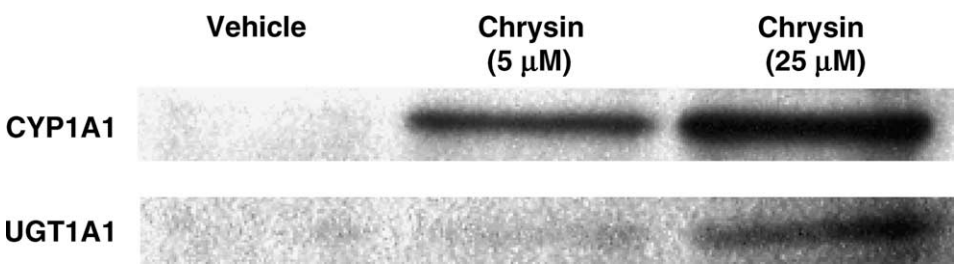

Fig. 7. Induction of UGT1A1 and CYP1A1 in HepG2 cells treated with chrysin. The microsome proteins were prepared from cells treated with chrysin (5 or 25 μM) or the vehicle (DMSO) alone for 24 hr, and subjected to Western blot analysis using an anti-human UGT1A1 antibody or anti-rat CYP1A1 antibody. The signal intensities were determined with a Fujix BAS-2000 bioimage analyzer (Fuji Photo Film).

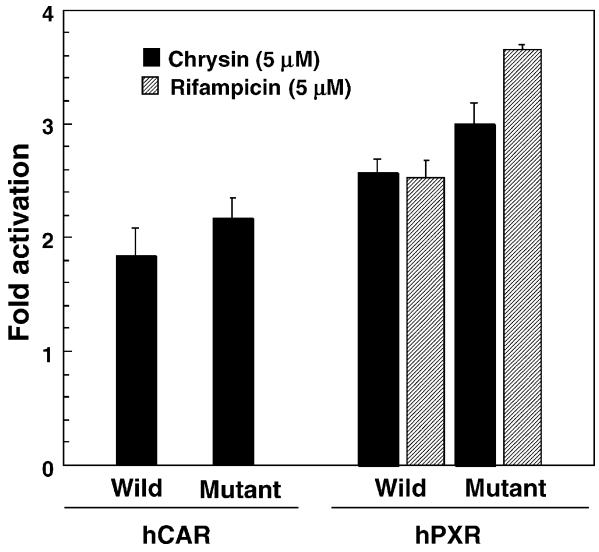

Fig. 8. Effects of chrysin and rifampicin on the transcriptional activation of the T–3263G-mutated UGT1A1 290-bp reporter gene. The wild-type UGT1A1 290-bp reporter gene- or the T–3263G-mutated reporter gene-tk-luciferase plasmid (0.2 μg) was co-transfected with hCAR-pCR3 or hPXR-pCR3 expression vector (0.2 μg) and pRL-SV40 plasmid (0.2 μg) into HepG2 cells as described in Section 2. The cells were treated for 24 hr, with the vehicle (DMSO) alone, chrysin (5 μM) or rifampicin (5 μM). Fold activation was calculated by dividing the activity with the inducer by that without the inducer. Data are the average of three independent experiments ± SD.

transactivation of not only the wild-type reporter gene but also the mutant gene.

When we tested the enhancer activities of six constructs from the 290-bp enhancer module to identify the elements through which the hCAR-, hPXR- or hAhR-dependent induction was acting, none of the six constructs was responsible for CAR-activator methoxychlor (50 μM)-, PXR-activator rifampicin (5 μM)- and AhR-activator benzo[*a*]pyrene (1 μM)-dependent induction in HepG2 cells (data not shown). In addition, the activation of the reporter gene by chrysin was abolished in all six fragments in the absence and presence of exogenously expressed nuclear receptor hCAR or hPXR (Fig. 9). Accordingly, in order to evaluate the role of four potential nuclear receptor motifs (DR4 element, PXRE, CARE and DR3 element) and AhRE in the transactivation of the UGT1A1 reporter gene by chrysin and baicalein, the 5′-hexamers of four nuclear receptor motifs (DR4 element, PXRE, CARE and DR3 element) and the AhR core binding region were mutated individually by directed mutagenesis using pGL3-tk-290-bp reporter gene as a template (Fig. 10A). The DNA was subjected to transient transfection assays in HepG2 cells without an exogenously expressed nuclear receptor. Mutations of DR4 element, PXRE, CARE and DR3 resulted in the decreased activation of the 290-bp reporter gene in response to 25 μM chrysin and 25 μM baicalein (Fig. 10B). On the other hand, AhRE mutation most strongly suppressed the activation in response to 25 μM chrysin and 25 μM baicalein (56.1 ± 7.9% and 44.3 ± 3.9% of the activation of the wild-type reporter gene, respectively) (Fig. 10B). Also, in HepG2 cells co-transfected with CAR and PXR expression vectors (0.2 μg), AhRE mutation displayed the most prominent decrease in its enhancer activities after treatment with chrysin at 5 μM for 24 hr (57.0 ± 6.7% and 38.0 ± 4.2% of the activation of the wild-type reporter gene, respectively) (data not shown). These results indicate that AhRE may play a prominent role in the activation of the 290-bp distal enhancer module in response to chrysin and baicalein.

4. Discussion

The glucuronidation of endogenous and exogenous lipophilic chemicals has profound effects on their biological activities and their excretion in urine or bile [2–4,26].

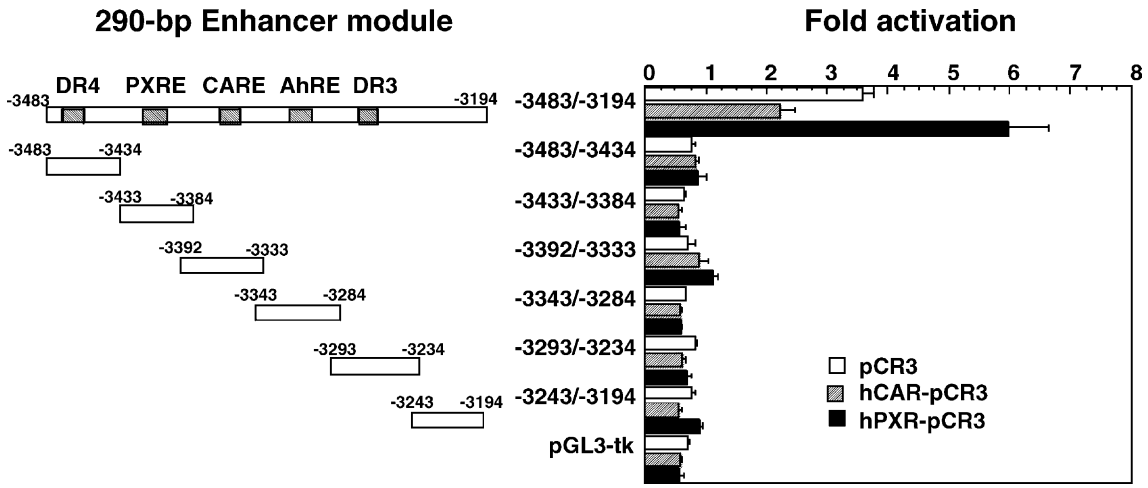

Fig. 9. Deletion analysis of the UGT1A1 290-bp reporter gene. A series of luciferase constructs was prepared with deletions in the 290-bp reporter gene and was tested by transient cotransfection with the expression plasmid for hCAR or hPXR or with the control vector pCR3 in HepG2 cells as described in Section 2. The cells were treated for 24 hr with vehicle (DMSO) alone or chrysin (25 μM). Fold activation was calculated by dividing the activity with the inducer by that without the inducer. Data are the average of three independent experiments ± SD.

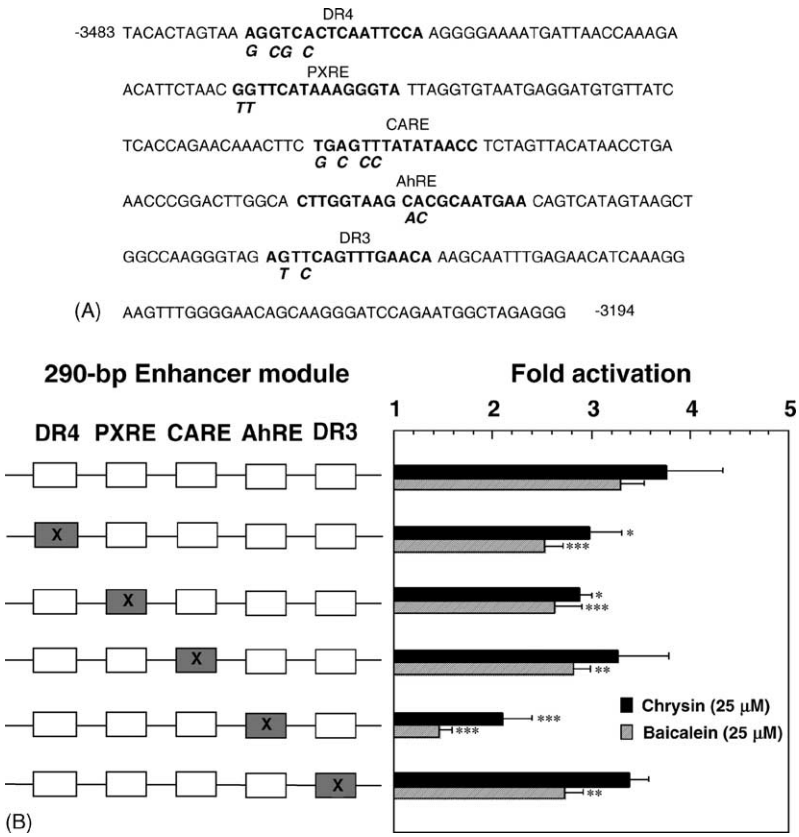

Fig. 10. Roles of nuclear receptor/transcription factor binding elements in the transcriptional activation of UGT1A1 290 bp reporter gene by flavonoids. (A) Mutations of nuclear receptor/transcription factor binding elements, which are underlined, were introduced into the UGT1A1 290-bp reporter gene as described in Section 2. Italicized characters show the bases for the mutation. (B) Each mutated DNA (indicated by the closed boxes with X) was co-transfected into HepG2 cells with pRL-SV40. The transfected cells were incubated with the vehicle (DMSO) alone, chrysin (25 μM) or baicalein (25 μM) for 24 hr, harvested, and assayed for luciferase activity. Fold activation was calculated by dividing the activity with the inducer by that without the inducer. Data are the average of three independent experiments ± SD.

Many investigators have demonstrated that reduced bilirubin glucuronosyltransferase activity (UGT1A1 activity) is associated with unconjugated hyperbilirubinemia (Crigler–Najjar syndrome and Gilbert's syndrome) [6] and predisposes patients to SN-38-initiated toxicity [7,27]. Phenobarbital, a UGT1A1 inducer, is an effective therapeutic drug for treating patients with Crigler–Najjar type II syndrome, but has the serious disadvantage of inducing sleep. UGT1A1 inducers can prevent and treat unconjugated hyperbilirubinemia and enhance the glucuronosyltransferase activity against xenobiotics and their metabolites, such as SN-38, to facilitate the biotransformation to more water-soluble compounds. UGT1A1 induction by chrysin and other flavonoids in HepG2 cells and Caco-2 cells and by rifampicin in primary rat hepatocytes has been demonstrated by using catalytic activity assays and Western and Northern blotting [14–16] and by using the bilirubin conjugation activity assay and Western blotting [28]. However, the mechanisms underlying UGT1A1 induction remain to be resolved.

In this study, we investigated the mechanism by which UGT1A1 induction in response to chrysin and other flavonoids is regulated in HepG2 cells. By using transient transfection studies, we characterized an upstream enhancer element that responds to chrysin and other xenochemicals in the *UGT1A1* 5′-flanking region. We identified and characterized a distal enhancer module at −3483/−3194 that directs the transactivation of the *UGT1A1* gene induced by chrysin, methoxychlor, rifampicin and benzo[*a*]pyrene (Figs. 1 and 2). Strikingly, this element is the same as that identified as PBREM, which directs the CAR-mediated transactivation of the *UGT1A1* gene [10]. The 290-bp distal enhancer module is a complex array of transcription-factor-binding sites that includes two *DR4* sites (direct repeat with a four-nucleotide spacer), designated DR4 element and CARE, two DR3 sites (direct repeat with a three-nucleotide spacer), designated PXRE and DR3 element [10,11], and the AhR core binding region [12]. CAR and PXR, members of the steroid/retinoid/thyroid hormone receptor superfamily, can bind to and transactivate the *CYP2B6*-DR4 site in PBREM located at –1733/–1683 upstream of the *CYP2B6* promoter site [9,29,30], the *CYP2C9*-DR4 site located at −1803/−1818 of *CYP2C9* [31], the *CYP3A4*-ER-6 site in prPXRE at −172/−148 and the *CYP3A4*-DR3 site in XREM at −7836/−7208 of *CYP3A4* [32], the *CYP2B1*-DR4 site in PBRE at −2301/−2142 of *CYP2B1*, and the *CYP3A1*-DR3 site in PXRE of *CYP3A1* [33,34]. The finding that rifampicin induces

the PXR-mediated transactivation of the *UGT1A1* 290-bp reporter gene (Fig. 1) supports the fact that rifampicin is a known inducer of *UGT1A1* both *in vitro* [28] and *in vivo* [35]. In *UGT1A1* induction, not only PXRE and CARE, but also AhRE have been reported to contribute to the transactivation of the UGT1A1 reporter gene mediated by the nuclear receptor/transcription factor. Multiple binding sites of nuclear receptors and receptor-type transcription factor are close to each other in the UGT1A1 290-bp enhancer module: the five nuclear receptor/transcription factor binding sites (DR4 element, PXRE, CARE, AhRE and DR3 element) are separated by 33, 42, 32 and 27 bases, respectively, in the 290-bp enhancer module [9–12]. In our previous study, we found that the plasma total bilirubin levels of heterozygotes for T–3263G were significantly higher than those of normal subjects and that the T–3263G mutation resulted in reduced transcriptional activity, leading to elevated plasma total bilirubin levels [13]. While the DR3 site may contribute to the constitutive transcriptional regulation of UGT1A1 expression *in vivo*, dietary flavones such as chrysin and baicalein may be useful for treatment and prevention of hyperbilirubinemia in subjects with reduced UGT1A1 activity due to the T–3263G mutation (Fig. 8). The regulation of the UGT1A1 290-bp reporter gene by multiple nuclear receptors/transcription factors may be effective in defending the body against a broad array of potentially harmful xenobiotics.

Reactive oxygen species (H_2O_2) and catalase inhibitor 3-amino-1,2,4-triazole represse phenobarbital-dependent CYP2B1 mRNA induction on the transcriptional level and glutathione precursor *N*-acetylcysteine enhances CYP2B1 mRNA induction by phenobarbital [36]. On the other hand, tea polyphenols have been reported to be potent inhibitors of the liver microsomal UGT1A1-dependent glucuronidation of estradiol and estrone at concentrations of 10–50 μM [37,38]. Here, we investigated whether tea polyphenols (catechin and their gallates in green tea and theaflavins in black tea), which are powerful chemopreventers of reactive oxygen species, can affect *UGT1A1* gene transcription. Tea polyphenols did not affect *UGT1A1* induction at concentrations of 2.5–25 μM. Furthermore, quercetin has hydroxyl substituents in the B-ring that are essential for antioxidant activity but is a modest inducer of *UGT1A1* (Table 1 and Fig. 5). Accordingly, *UGT1A1* induction by chrysin without hydroxyl substituents in the B-ring appears not to depend on antioxidant activity (Table 1 and Figs. 5 and 6).

The following findings demonstrate that flavonoids are likely to induce UGT1A1 mainly via the AhR-mediated transactivation of the 290-bp reporter gene: (1) that chrysin is an activator for the 290-bp reporter gene in the UGT1A1 5′-flanking region (Fig. 1), (2) that chrysin induces the expression of not only UGT1A1 mRNA and protein, but also CYP1A1 mRNA and protein in HepG2 cells (Table 1 and Fig. 7), and (3) that mutation of the functional nuclear receptor binding site AhRE in the 290-bp reporter gene most strongly abrogates reporter activity in HepG2 cells treated with chrysin and baicalein (Fig. 10). However, bilirubin (a ligand of the aryl hydrocarbon receptor [39]) slightly increased the reporter activity of UGT1A1 290-bp distal enhancer module (unpublished data). In addition, the observation that the chrysin- and baicalein-induced activation of the 290-bp reporter gene mutated at each of four nuclear receptor motifs (DR4 element, PXRE, CARE and DR3 element) was slightly but significantly decreased in HepG2 cells suggests that these motifs may contribute to the overall UGT1A1 response to the flavonoids (Fig. 10). We are currently investigating the mechanism regulating the functional cross-talk between the 290-bp reporter gene and receptor-type transcription factors and the recruitment of co-activators and co-repressors.

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