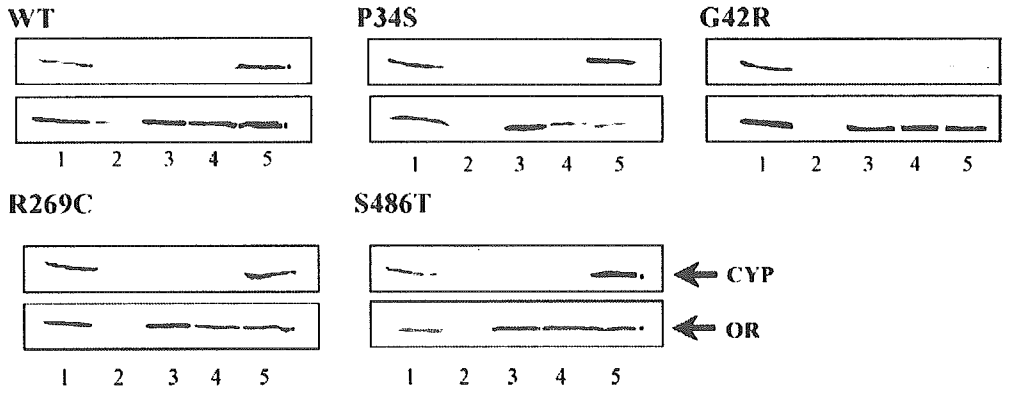

Fig. 2. Western blot analysis of CYP2D6 and OR proteins in HepG2/OR cells which were transfected with cDNAs encoding CYP2D6-WT or its variants (P34S, G42R, R269C and S486T). Upper and lower PVDF membrane strips of each panel show CYP2D6 and OR protein bands, respectively. Lane 1, human liver microsomes (50 μg); lane 2, control HepG2 (3×10^5 cells); lane 3, HepG2/OR (3×10^5 cells); lane 4, HepG2/OR/mock (3×10^5 cells); lane 5, HepG2/OR/CYP2D6-WT or -variant (3×10^5 cells). The arrows show bands corresponding to CYP2D6 and OR proteins.

3.2. Establishment of HepG2 transfectants stably expressing OR and CYP2D6 or its variants

Fig. 2 shows the results of Western blot analysis of whole-cell lysate fractions from geneticin-resistant HepG2 transfectants. Each panel has two PVDF membrane strips, and the upper and lower strips are for CYP2D6 enzymes and OR, respectively. Human liver microsomes (lane 1) exhibited protein bands corresponding to CYP2D6 (upper strip) and OR (lower strip). All of the HepG2/OR/CYP2D6-WT and its variants (lane 5 of each panel) yielded protein bands corresponding to CYP2D6 and OR, whereas other HepG2 cells such as control HepG2 cells (lane 2), HepG2/OR (lane 3) and HepG2/OR/mock (lane 4) showed an OR protein band only. In lane 2, there appeared a weakly stained band, corresponding to the endogenous OR protein in control HepG2 cells.

3.3. Assay of cytochrome c reductase and BF 1″-hydroxylase activities

As described above, the cytochrome *c* reductase activities of the control HepG2 and HepG2/OR cells were 27 and 116 units/mg protein, respectively (Fig. 3A). Cytochrome *c* reductase activities similar to those of HepG2/OR cells were also seen in HepG2/OR/2D6-WT and its variants except for P34S. The transfectant expressing P34S had about two-fold higher cytochrome *c* reductase activity than the control HepG2 cells.

BF 1″-hydroxylase activities are shown in Fig. 3B. The activities of the control HepG2 and HepG2/OR

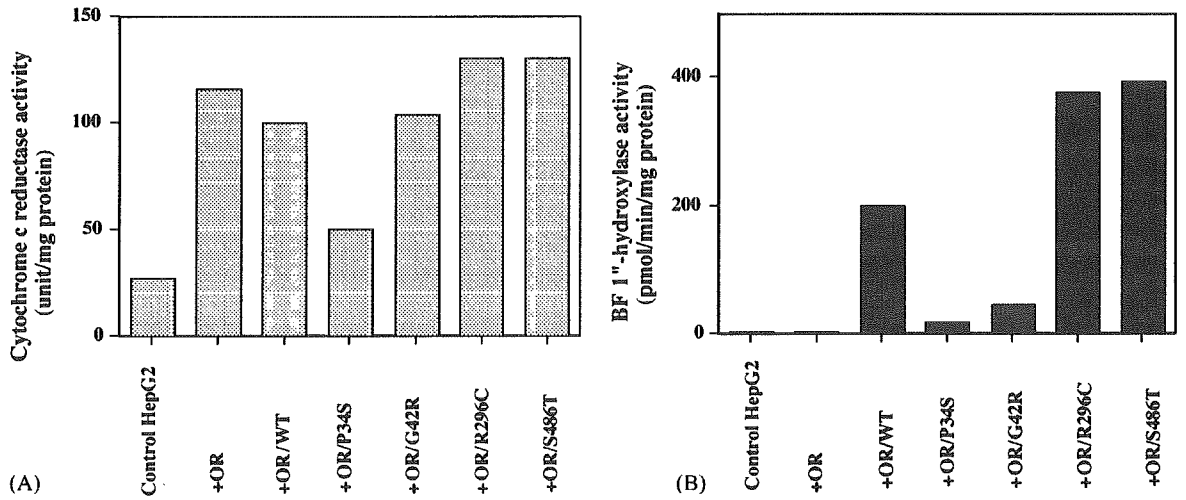

Fig. 3. Comparison of OR activity (A) and CYP2D6 activity (B) of microsomal fractions from HepG2/OR cells which were transfected with cDNAs encoding CYP2D6-WT or its variants. (A) OR activity was expressed as cytochrome *c* reduction. (B) CYP2D6 activity was expressed as BF 1″-hydroxylation. Each value represents the mean of two determinations.

cells were 3.0 and 4.9 pmol/min/mg protein, respectively. In contrast, much higher activities were seen for HepG2/OR/2D6-WT (197 pmol/min/mg protein) and its variants, except for P34S and G42R. The activities of HepG2/OR/2D6-P34S and -G42R were 10 and 23% of that of HepG2/OR/2D6-WT. These results are in accord with our previous observations using the yeast cell expression system [5,10].

3.4. Effects of DMSO and hemin on function and protein level of CYP2D6

HepG2 cells (HepG2/OR/2D6 and HepG2/2D6) were cultivated in the medium containing DMSO (0.1% of a final concentration) and/or hemin (5 μg/ml of a final concentration) for 24 h, and then microsomal fractions were prepared. CYP2D6 protein levels and BF 1″-hydroxylase activities were measured by Western blot analysis and HPLC, respectively. Western blot analysis revealed that the cultivation of HepG2/2D6 and HepG2/OR/2D6 with DMSO and/or hemin did not change the level of CYP2D6 protein in the microsomal fraction (Fig. 4A and B, upper panels).

As shown in the lower panels of Fig. 4A for HepG2/OR/2D6, the addition of DMSO to the culture medium increased BF 1″-hydroxylase activity up to 4.2-fold that of the control HepG2 cells cultivated in the medium without DMSO. In contrast, the treatment of HepG2/OR/2D6 with hemin did not cause such a remarkable increase of BF 1″-hydroxylase activity. Similar effects were also observed in HepG2/2D6, though the BF oxidation activities were much lower, i.e., one-third to one-fifth those of HepG2/OR/2D6 (Fig. 4B, lower panel). The addition of DMSO markedly elevated the BF 1″-hydroxylase activities by four- to six-fold compared to those of the control, whereas the addition of hemin did not affect the activity.

3.5. Kinetic analysis of BF 1″-hydroxylation by HepG2/OR/2D6 and HepG2/2D6 cultivated with or without DMSO

Fig. 5 shows Lineweaver–Burk plots for BF 1″-hydroxylation by microsomal fractions from HepG2/OR/2D6 and HepG2/2D6 cultivated with or without DMSO. Kinetic parameters were calculated as follows: apparent K_m values were 3.5, 3.4, 9.8 and 7.0 μM for HepG2/OR/2D6 without DMSO (Fig. 5A), and with DMSO (Fig. 5B), HepG2/2D6 without DMSO (Fig. 5C) and with DMSO (Fig. 5D), respectively; while the V_{max} values were 197, 978, 92 and 291 pmol/min/mg, respectively.

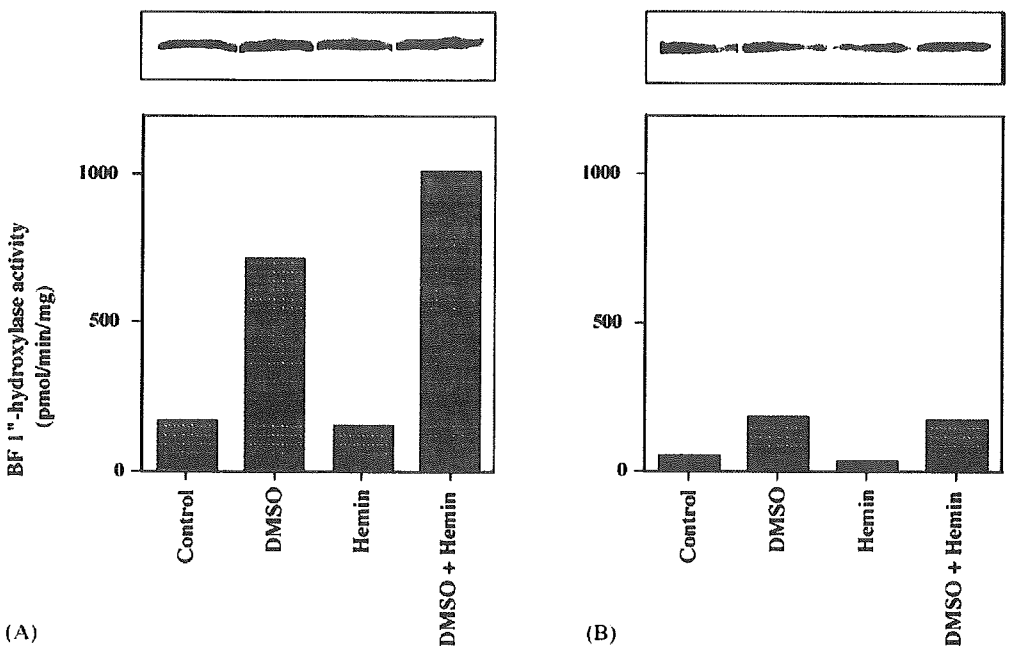

Fig. 4. Effects of the addition of DMSO or hemin to the culture medium on the contents of CYP2D6 protein (upper panels) and on the CYP2D6 activity (lower panels) in HepG2/OR/2D6 (A) and HepG2/2D6 (B). HepG2/OR/2D6 or HepG2/2D6 cells were cultured in the medium containing DMSO (0.1%) and/or hemin (5 μg/ml) for 24 h prior to the preparation of microsomal fractions. In Western blot analysis, each lane contained 100 μg of microsomal protein. CYP2D6 activity was expressed as BF 1″-hydroxylation. Each value represents the mean of two determinations.

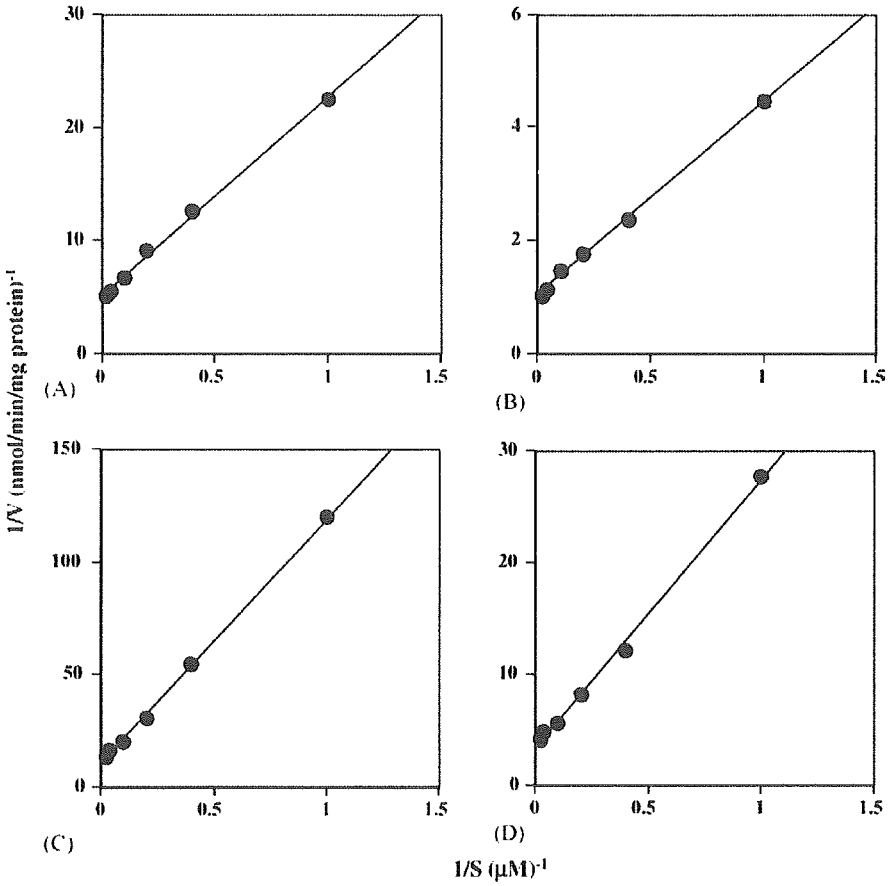

Fig. 5. Lineweaver–Burk plots for BF 1″-hydroxylation by microsomal fractions from HepG2/OR/2D6 ((A) and (B)) and HepG2/2D6 ((C) and (D)). HepG2/OR/2D6 or HepG2/2D6 cells were cultivated for 24 h with or without DMSO (0.1%), and microsomal fractions were prepared as described. BF 1″-hydroxylase activities were measured using final substrate concentrations ranging from 1 to 50 μM as described in Section 2. (A) and (C) without DMSO; (B) and (D) with DMSO. Each value represents the mean of two determinations.

3.6. Spectral analysis of microsomal fractions from HepG2/OR/2D6 and HepG2/2D6 cultivated with or without DMSO

The amounts of functional CYP2D6 in microsomal fractions from HepG2 cells were measured spectrophotometrically. Fig. 6 summarizes the reduced CO-difference spectra. Compared to the spectra obtained from HepG2/OR/2D6 (Fig. 6A) and HepG2/2D6 (Fig. 6C) that were cultivated without DMSO, the spectra from HepG2/OR/2D6 (Fig. 6B) and HepG2/2D6 (Fig. 6D) cultivated with DMSO showed increased peak heights at 450 nm. The estimated functional P450 contents were as follows: HepG2/OR/2D6 without DMSO, 6.0 pmol/mg protein; HepG2/OR/2D6 with DMSO, 18.9 pmol/mg protein; HepG2/2D6 without DMSO, 0.9 pmol/mg protein; HepG2/2D6 with DMSO, 7.4 pmol/mg protein.

4. Discussion

The CYP2D6 gene has numerous kinds of single nucleotide polymorphism, resulting in stop codons (forming short proteins), frame shifts, splicing defects at the gene level and substitutions of amino acid residues at the protein level [6]. In particular, the substitution of amino acid residues in CYP2D6 protein may cause not only quantitative but also qualitative changes in the enzymatic function. Namely, there is a possibility that CYP2D6 variants would produce novel reactive metabolites from substrates that are not produced by CYP2D6-WT, resulting in as-yet-unidentified biological activities that may cause unknown toxicities including side-effects of medicines. Thus, the aim of the present study using the HepG2 cell line was to establish an appropriate screening system for cytotoxicities caused by unknown reactive

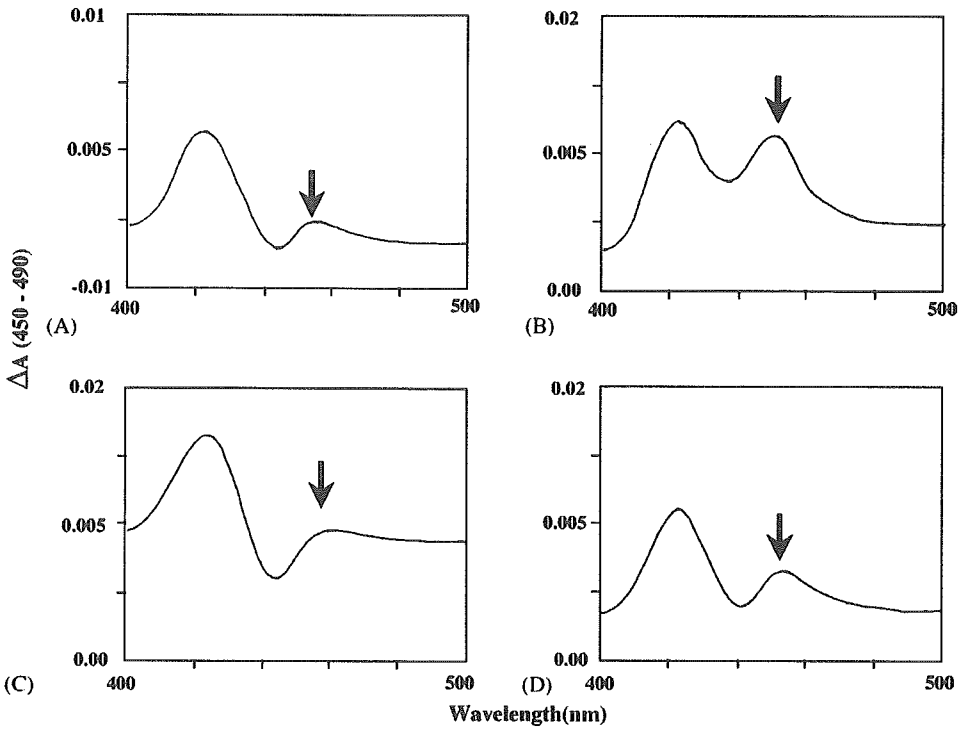

Fig. 6. Spectral analysis of microsomal fractions from HepG2/OR/2D6 ((A) and (B)) and HepG2/2D6 ((C) and (D)). HepG2/OR/2D6 or HepG2/2D6 cells were cultivated for 24 h with or without DMSO (0.1%), and microsomal fractions were prepared as described. Reduced CO-difference spectra were measured. (A) and (C) without DMSO; (B) and (D) with DMSO. The arrow indicates the P450 peak.

metabolites formed from substrates by various CYP2D6 variants.

As a recent typical example, Flanagan et al. [14] examined a CYP2D6 mutant having alanine instead of phenylalanine-120 (which has not yet been identified as a CYP2D6 variant). The mutant yields a novel metabolite (7-hydroxydextromethorphan) from dextromethorphan [14]. In our experiments independently perfomed during the same time period, the CYP2D6-Phe120Ala was found to cause a reverse in substrate enantioselectivity in 1″-hydroxylation of BF enantiomers [7] and a remarkable increase in V_{max} (about 100-fold) for bunitrolol 4-hydroxylation as compared with the profiles of CYP2D6-WT [8].

After the present project employing the HepG2 cell line was started in 2000, Yoshitomi et al. [17] reported the establishment of HepG2 cell lines stably expressing various CYP enzymes, including CYP2D6. For the transfection of HepG2 cells, they employed a plasmid into which a cDNA encoding each CYP enzyme, but not one encoding OR, was introduced. In this study, we therefore attempted to construct HepG2 cell lines stably expressing both OR and CYP2D6-WT or each of the CYP2D6 variants (P34S, G42R, R296C and S486T).

As shown in Figs. 1 and 2, we successfully obtained HepG2 cells stably expressing OR and CYP2D6 or its variants. The enzymatic function as BF 1″-hydroxylase of HepG2/OR/2D6-WT was much higher than that reported by Yoshitomi et al. [15] (Table 2). Therefore, the HepG2 cell line stably expressing OR and CYP2D6-WT or its various variants established in the present study is a promising tool for screening cell toxicities of unknown reactive metabolites of various substrates formed by CYP2D6 variants.

In BF 1″-hydroxylation, HepG2/OR/2D6-P34S and -G42R showed much lower activities compared to HepG2/OR/2D6-WT. However, the profile of BF 1″-hydroxylation is similar to that obtained in the yeast cell expression system reported previously from this laboratory. In contrast, the activities of HepG2/OR/2D6-R296C and -S486T tended to be higher than that of the wild-type. In our previous studies using yeast cell expression system, the two variants (R296C and S486T) exhibited BF 1″-hydroxylase activities at a similar level to that of the wild-type [5]. Consequently, it seems likely that the variants, R296C and S486T, do not remarkably affect BF 1″-hydroxylation. We recently reported that these variants, especially R296C, in the yeast cell expression system showed lower progesterone 21-hydroxylase activities compared to those of the wild-type [16]. Further studies will be necessary to understand whole properties of these variants.

Table 2
Comparison of BF 1″-hydroxylase activities in three cell lines expressing CYP2D6

Cell line	Transfectant	K_m (μM)	V_{max} (pmol/min/mg protein)	Reference
HepG2	HepG2/2D6WT	9.8	92	
	HepG2/OR/2D6-WT	3.5	197	This study
	HepG2/OR/2D6-WT (with DMSO)	3.4	987	
HepG2	Hepc/2D6.39[a]	17	14	[15]
CHO	DUKX/2D6[b]		147 ± 12	
	DUKX/2D6/CPR[c]		388 ± 52	[17]
	DUKX/2D6/CPR-3[d]		439 ± 33	

[a] HepG2 cell line expressing exogenous CYP2D6 but not exogenous OR.
[b] CHO cell lines expressing exogenous CYP2D6 but not exogenous OR.
[c] CHO cell lines expressing exogenous CYP2D6 and exogenous OR (45 pmol/mg protein).
[d] CHO cell lines expressing exogenous CYP2D6 and exogenous OR (75 pmol/mg protein). For CHO cell lines, BF 1″-hydroxylase activities were measured at a racemic BF concentration of 30 μM, which was sufficient to obtain the maximum velocity for BF 1″-hydroxylation by CYP2D6-WT. The values of this study are the mean of two determinations.

While the present project was underway, Ding et al. [17] published the interesting finding that the addition of DMSO to the culture medium increased the content of functional CYP2D6 expressed in Chinese hamster ovary (CHO) cells (Table 2). Although the BF 1″-hydroxylase activity of our HepG2 cell line was considerably higher than that of the cell line developed by Yoshitomi et al. [15], much higher activity would be required for our purpose of screening cytotoxicities caused by unknown reactive metabolites. Therefore, the effects of the addition of DMSO to the culture medium were examined in our HepG2 cell expression system. The results showed that the addition of DMSO (final concentration of 0.1%) to the culture medium increased the BF 1″-hydroxylase activities of the HepG2/2D6 and HepG2/OR/2D6 cell lines three- to four-fold compared to those of the respective control cells without DMSO, though the level of CYP2D6 activity of HepG2/2D6 cells was one-fifth that of HepG2/OR/2D6 cells.

Kinetic analysis using microsomal fractions from HepG2/2D6 or HepG2/OR/2D6 cells revealed that the addition of DMSO did not change the apparent K_m values but increased the V_{max} values. This means that the treatment with DMSO might increase the functional CYP2D6 contents without changing the affinity of CYP2D6 for the substrate. Spectral analysis of the enzyme supported this notion. That is, the addition of DMSO to the culture medium markedly increased the functional P450 content as shown by the reduced CO-difference spectra of microsomal fractions from HepG2/2D6 or HepG2/OR/2D6 cells. Furthermore, Western blot analysis showed that the total content of immunoreactive CYP2D6 protein (functional and non-functional) was similar between DMSO-treated and non-treated HepG2 cells regardless of the expression of OR.

In Table 2, the BF 1″-hydroxylase activity of HepG2/OR/2D6 cells pretreated with DMSO was the highest among the values of Yoshitomi et al. [15], Ding et al. [17] and our study. Unfortunately, Ding et al. [17] showed the effects of DMSO on the contents of functional P450 spectrophotometrically, but reported no data for BF 1″-hydroxylase activity. Therefore, although direct comparison cannot be performed, the HepG2/OR/2D6 cells developed in the present study may have the advantage of high levels of enzymatic activity and easy handling with respect to cultivation.

Furthermore, Ding et al. [17] observed that the addition of DMSO increased spectrally active CYP2D6 contents in CHO cells expressing both CYP2D6 and OR but did not cause a change in those expressing CYP2D6 only. Moreover, the same group reported that the addition of exogenous hemin (5 μg/ml) also increased CYP2D6 contents in CHO cells expressing CYP2D6 and OR. In contrast, the addition of hemin (5 μg/ml) did not change either the CYP2D6 contents or activities of HepG2 cells expressing CYP under the conditions used in the present study.

The levels of endogenous OR are very similar between HepG2 cells (27 units/mg protein as cytochrome *c* reductase activity) that we used in the present study and CHO cells (22 units/mg protein) that Ding et al. [17] employed. Therefore, the different effects of the addition of hemin seen in HepG2 and CHO cells might be due to differences of the levels of endogenous heme between the cell lines used. That is, the HepG2 cell line derived from hepatic cells may have a biosynthetic pathway for heme which is more efficient than that of the

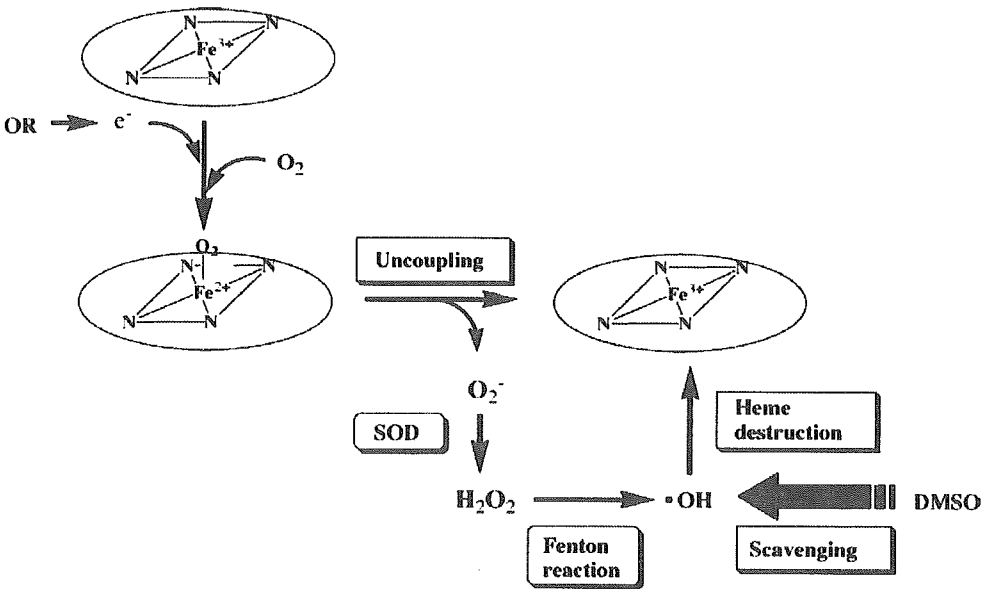

Fig. 7. Hypothetical mechanism for the increase in CYP2D6 activity caused by cultivating HepG2 cells in medium containing DMSO.

CHO cell line, resulting in a higher level of endogenous heme in HepG2 than in CHO cells. The lower level of endogenous heme might account for the clear increase of functional CYP2D6 content by exogenously added hemin in the CHO cell line.

It is known that CYP can activate oxygen molecules to reactive oxygen species, including superoxide anions, by the so-called uncoupling reaction using the electron-transfer system via OR, and the resulting superoxide anions might be converted to hydrogen peroxides by superoxide dismutase [18]. In the presence of iron, hydrogen peroxides are converted to hydroxyl radicals, which cause the destruction of the heme moiety of holo CYP2D6, resulting in the conversion of holo CYP2D6 to its apoprotein (Fig. 7). Reactive oxygen species, including hydroxyl radicals, could also cause the destruction of CYP proteins. However, the present study indicated that the levels of immunochemically stained protein band corresponding to CYP2D6 were very similar between DMSO-treated and non-treated HepG2 cells in Western blot analysis. Therefore, it is reasonable to think that no remarkable destruction of CYP2D6 proteins by reactive oxygen species occurred under the conditions employed.

DMSO is a detoxifier of reactive oxygen species, with an especially high affinity for the hydroxy radical [19]. Thus, it is thought that the DMSO added to the culture medium in the present study scavenged hydroxyl radicals and increased the contents of functional CYP enzymes expressed in HepG2 cells, thereby causing an increase in substrate oxidation activities. This effect was observed in HepG2 cells expressing CYP2D6-WT regardless of the expression of OR, which is different from the reported results [17] in the CHO system, where the effect of DMSO was seen only in the cells expressing both CYP2D6 and OR.

In summary, HepG2 cells stably expressing OR and/or CYP2D6-WT or its variants were established in the present study. The cultivation of HepG2/2D6 or HepG2/OR/2D6 in the medium containing DMSO (0.1% final concentration) remarkably increased BF 1″-hydroxylase activities compared with those of control cells that were cultivated in the medium without DMSO. The addition of hemin instead of DMSO to the culture medium did not cause such an increase. Western blotting and spectrophotometric analyses indicated that the radical-scavenging effects of DMSO were responsible for the increased activity. These results indicate that HepG2 cell lines stably expressing CYP isoenzymes and OR are good tools for screening cytotoxicities of chemical compounds which undergo oxidation by CYP isoenzymes. However, it should be noted that there is a possibility that DMSO as a scavenger of reactive oxygen species masks cell toxicities in which reactive oxygen species formed in the reaction play a major role. In such a case, careful comparison should be performed for results obtained from experiments in which HepG2/OR/2D6 cells are cultivated with and without DMSO.

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Functional analysis of four naturally occurring variants of human constitutive androstane receptor

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Abstract

The human constitutive androstane receptor (CAR, NR1I3) is a member of the orphan nuclear receptor superfamily that plays an important role in the control of drug metabolism and disposition. In this study, we sequenced all the coding exons of the *NR1I3* gene for 334 Japanese subjects. We identified three novel single nucleotide polymorphisms (SNPs) that induce non-synonymous alterations of amino acids (His246Arg, Leu308Pro, and Asn323Ser) residing in the ligand-binding domain of CAR, in addition to the Val133Gly variant, which was another CAR variant identified in our previous study. We performed functional analysis of these four naturally occurring CAR variants in COS-7 cells using a *CYP3A4* promoter/enhancer reporter gene that includes the CAR responsive elements. The His246Arg variant caused marked reductions in both transactivation of the reporter gene and in the response to 6-(4-chlorophenyl)imidazo[2,1-*b*][1,3]thiazole-5-carbaldehyde *O*-(3,4-dichlorobenzyl)oxime (CITCO), which is a human CAR-specific agonist. The transactivation ability of the Leu308Pro variant was also significantly decreased, but its responsiveness to CITCO was not abrogated. The transactivation ability and CITCO response of the Val133Gly and Asn323Ser variants did not change as compared to the wild-type

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CAR. These data suggest that the His246Arg and Leu308Pro variants, especially His246Arg, may influence the expression of drug-metabolizing enzymes and transporters that are transactivated by CAR.

Keywords: Nuclear receptor; NR1I3; CAR; Constitutive androstane receptor; SNP; Amino acid alteration

Introduction

The constitutive androstane receptor (CAR) encoded by *NR1I3* is expressed predominantly in the liver [1] and it belongs to the nuclear receptor subfamily 1I. This subfamily also includes the pregnane X receptor (PXR) and the vitamin D receptor. CAR regulates transcription of the genes encoding drug/steroid-metabolizing enzymes and transporters, as well as other physiologically important enzymes [2–4]. CAR also regulates thyroid hormone and bilirubin metabolism [5,6]. In humans, CAR transactivates several major hepatic drug-metabolizing enzymes, such as the cytochrome P450s (CYPs) and transferase, including CYP2B6 [7], CYP3A4 [7,8], CYP2C9 [9,10], CYP2C19 [11], and UGT1A1 [12]. CAR forms a heterodimer with the retinoid X receptor (RXRα) and this binds to DNA motifs such as DR3, DR4, DR5, or ER6, of the target genes. It is noteworthy that, unlike most nuclear receptors, CAR is constitutively active in the absence of any added ligand [1,13]. However, ligand binding modulates the transcriptional activity of CAR. For example, 6-(4-chlorophenyl)imidazo[2,1-*b*][1,3] thiazole-5-carbaldehyde *O*-(3,4-dichlorobenzyl)oxime (CITCO) is an agonistic ligand of human CAR and it also triggers nuclear translocation [14].

NR1I3 is located on chromosome 1 and consists of nine exons. The DNA binding domain is encoded by exons 2, 3, and 4. A small hinge domain is encoded by a portion of exon 4, while the ligand-binding domain corresponds to exons 4–8 and a 5′ portion of exon 9 [15,16]. Recently, we sequenced all the coding exons of the *NR1I3* gene for 253 Japanese subjects and identified novel SNPs in the *NR1I3* gene [17]. These SNPs included one non-synonymous amino acid change that was localized to the ligand-binding domain of CAR. It is thought that SNPs may induce changes in the function or expression of the *NR1I3* gene, and this may explain variations in drug metabolism among humans. In this study, we analyzed the *NR1I3* sequence in an additional set of 334 Japanese subjects and found three novel, non-synonymous SNPs. We performed functional analysis of these four CAR variants using a reporter gene assay carried out with COS-7 cells.

Materials and methods

Human genomic DNA samples

The 334 subjects used in this study were Japanese cancer patients who were administered irinotecan or paclitaxel. Genomic DNA was extracted from blood leukocytes and was used as a template for the polymerase chain reaction (PCR). This study was approved by all the Ethnic Committees of the National Cancer Center, the National Cardiovascular Center, and the National Institute of Health Sciences. Written informed consent was obtained from all participants.

Conditions for PCR and DNA sequencing

The conditions and primers for PCR and DNA sequencing in these experiments were essentially the same as described previously [17]. Briefly, sequencing templates from the *NR1I3* gene were prepared by two rounds of PCR as described previously [17]. First, the entire region of the *NR1I3* gene (exon 1 to exon 9) was amplified by Z-Taq (Takara, Bio., Shiga, Japan) using each individual genomic DNA as a template (1st round PCR). Next, each exon was amplified by Ex-Taq (Takara, Bio) using the appropriate set of *NR1I3*-specific primers in the introns, and using the 1st round PCR product as a template. The PCR products were purified, and both strands were directly sequenced using the ABI BigDye Terminator Cycle Sequencing Kit Version 3.1 (Applied Biosystems, Foster City, CA, USA) and an ABI Prism 3730 DNA Analyzer (Applied Biosystems). The sequences were confirmed independently at least twice. Electropherograms were analyzed by two researchers.

Plasmids

Variant CAR (V133G, H246R, L308P, and N323S) expression plasmids were constructed by site-directed mutagenesis of the wild-type CAR (WT) expression plasmid [18] using the PCR-based QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA). All the CAR constructs were introduced to the pcDNA6.2/nLumio-DEST mammalian Gateway vector to generate a six amino acids-Tag (Lumio tag) at the N-terminus, according to the manufacturer's protocol (Invitrogen, Carlsbad, CA, USA) to facilitate in-gel detection of Lumio-tagged CAR proteins. All the coding regions of wild-type and variant CARs were confirmed by DNA sequencing. The luciferase reporter plasmid, pCYP3A4XREM 362/53Luc [19] that contains the XREM (xenobiotic-responsive enhancer module) and proximal promoter region of the *CYP3A4*

5′-flanking region (−7835 to −7208 and −362 to +53) including CAR responsive elements [8], was used for reporter gene assays.

Luciferase assay

COS-7 cells (African green monkey kidney cell line) were maintained in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) supplemented with 10% fetal bovine serum (FBS, Hyclone Laboratories) under 5% CO_2 atmosphere at 37 °C. One day before transfection, the cells were plated onto 24-well plates at a density of 6×10^4 cells/well. The cells were transiently transfected with 0.175 μg each of the CAR expression plasmids, including the wild-type and the variants, together with 0.175 μg of the pCYP3 A4XREM 362/53Luc and 0.05 μg phRL-TK plasmid (encoding *Renilla* luciferase under control of the TK promoter; Promega, Madison, WI, USA) as the internal control. Mock transfections were carried out in parallel, using the pDNA6.2/nLumio-DEST without any inserts. All transfections were performed in 24-well plates using LipofectAmine2000 reagent (Invitrogen) according to the manufacturer's instructions. The total DNA was mixed in 50 μl of serum-free Opti-MEM I (Invitrogen). LipofectAmine2000 in 50 μl Opti-MEM I was added to the mixture and incubated for an additional 20 min. Cells were rinsed with growth medium without antibiotics, and incubated with 0.5 ml of the DNA/liposome mixture at 37 °C. Six hours after transfection, the medium was replaced with DMEM containing 10% charcoal-dextrane-treated FBS, and the cells were incubated at 37 °C overnight. Twenty-four hours after transfection, the cells were treated with the vehicle (dimethyl sulfoxide) or with various concentrations of the CITCO (0.1–10 μM) and then cultured for an additional 24 h. Cell lysates were prepared using the Dual-Glo Luciferase Assay System (Promega). Luciferase activity was then measured using an ARVO SX Multilabel Counter (Perkin-Elmer Wallac, Turku, Finland). All transfection efficiencies were normalized to the *Renilla* luciferase activity.

Detection and quantification of CAR variant proteins

Forty-eight hours after transfection with CAR expression plasmids, the cells were harvested and Lumio-tagged CAR proteins were detected using the Lumio Green Detection Kit (Invitrogen) as recommended by the manufacturer. Samples with 40 μg of total cellular protein were subjected to electrophoresis on NuPAGE Novex 4–20% Bis-Tris gel with MES buffer (Invitrogen). The fluorescent bands were then visualized and quantified using a fluorescent image analyzer FLA-3000 (Fuji film, Tokyo, Japan).

Results

Four non-synonymous SNPs in CAR

Previously, we identified the variant 398T > G in the *CAR* gene after screening 253 Japanese patients. This mutation leads to the amino acid alteration Val133Gly [17]. In this study, we surveyed an additional set of 334 Japanese subjects and identified novel non-synonymous SNPs 737A > G (His246Arg), 923T > C (Leu308Pro), and 968A > G (Asn323Ser), found in two, one, and one subjects, respectively. The electropherograms of the three novel SNPs are shown in Fig. 1. All four SNPs were found as heterozygotes and all mapped to the ligand-binding domain (LBD), which is known to be the most important structural domain of nuclear receptors. We next looked for functional alterations resulting from these four amino acid substitutions using a luciferase reporter gene.

Expression of variant CAR proteins in COS-7 cells

COS-7 cells were transiently transfected with the wild-type and four variant CAR expression plasmids. The expression levels of the CAR proteins were determined by detection of the Lumio tag using the Lumio Green detection In Gel System. As shown in Fig. 2, the relative expression levels of all four variants were

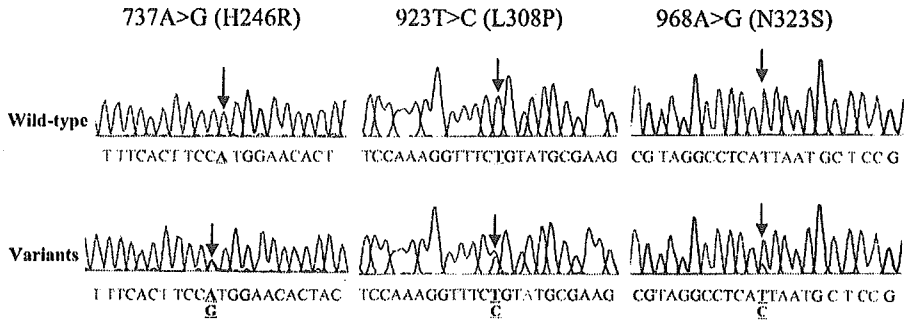

Fig. 1. Electropherograms (sense strands for 737A > G and 923T > C; antisense strand for 968A > G) of three novel non-synonymous variants of NR1I3. The first A of the translational initiation codon ATG in exon 2 is defined as position 1. Arrows indicate the nucleotide positions of variations.

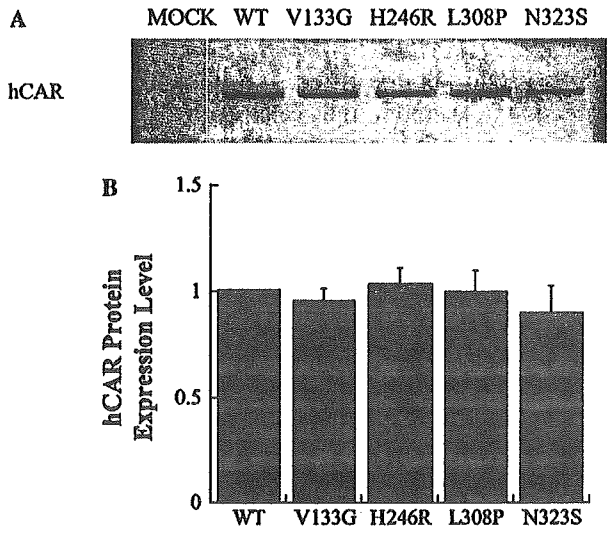

Fig. 2. Expression of the wild-type and variant CARs in COS-7 cells. (A) Aliquots (40 μg) of the pooled whole cell lysates from three independent preparations were loaded in each lane and Lumio Green detection was performed as described in Materials and methods. Whole cell lysates were prepared from COS-7 cells transfected with the empty expression vector (MOCK), the CAR wild-type plasmid (WT), or the variant expression plasmids as indicated. (B) Expression levels of CAR proteins are shown relative to that of the wild-type protein. Results are indicated as the means ± SD from three independent preparations.

equivalent to that of the wild-type protein, indicating that these substitutions do not affect protein stability and translational efficiency.

Reduced transactivation of the CYP3A4 promoter/enhancer by CAR variants

To evaluate the functional characteristics of the CAR variants with respect to transactivation, reporter gene assays were carried out. COS-7 cells were transiently transfected with a *CYP3A4* promoter/enhancer luciferase reporter plasmid that includes the CAR responsive elements [8], together with an expression plasmid for each CAR variant. As shown in Fig. 3, the His246Arg variant showed substantially less transactivation activity than that of the wild-type protein. The Leu308Pro variant also showed reduced activity. However, statistically significant differences in the transactivation levels of the Val133Gly and Asn323Ser variants versus the wild-type CAR were not observed. As the expression levels of the CAR proteins were similar (Fig. 2), this suggests that the reduced transactivation by these two CAR variants was due to a functional retardation associated with the amino acid alterations. Next, we investigated the effect of CITCO, which is a human CAR-specific agonist, on the activity of all the CAR variants. As shown in Fig. 4, all the variants except for His246Arg showed enhanced transactivations of the *CYP3A4* promoter/enhancer activity in the

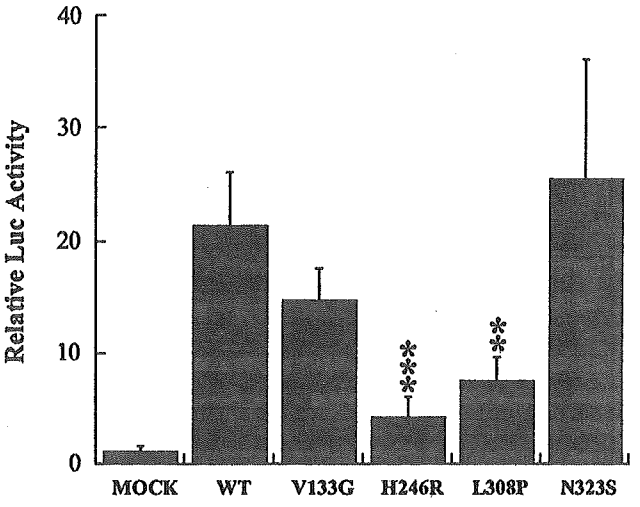

Fig. 3. Effect of CAR variants on the *CYP3A4* promoter/enhancer reporter activity. COS-7 cells were transfected with the *CYP3A4* luciferase reporter plasmid together with the empty expression vector (MOCK), the CAR wild-type plasmid (WT), or the variant expression plasmids as indicated. Data are shown as the means ± SD from four independent transfections. Statistically significant differences compared with the wild-type CAR are indicated by asterisks (**$p < 0.005$; ***$p < 0.0005$; by the one-way ANOVA and Dunnet's test).

presence of 0.1–10 μM of CITCO in COS-7 cells. In contrast, the CITCO-induced enhancement was completely abrogated in His246Arg.

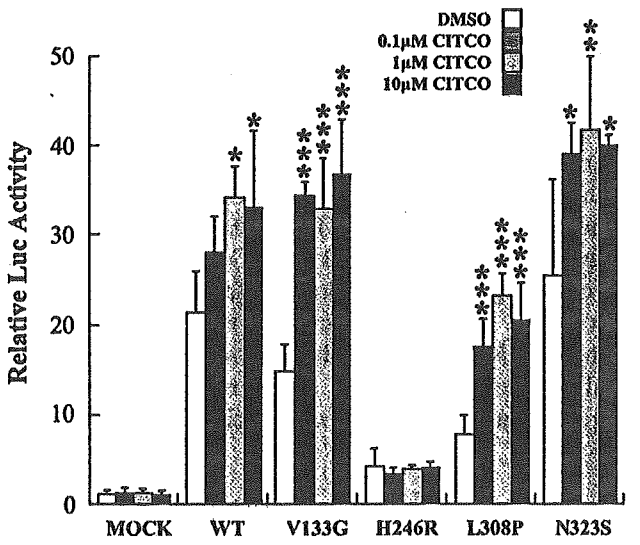

Fig. 4. Effect of CAR variants on the enhanced *CYP3A4* promoter/enhancer reporter activity in response to CITCO. COS-7 cells were transfected with the *CYP3A4* promoter/enhancer luciferase reporter plasmid together with the empty expression vector (MOCK), the CAR wild-type plasmid (WT), or the variant expression plasmids as indicated. After 24 h, the cells were further treated with CITCO (0.1–10 μM) for another 24 h. As a control, the cells were treated with vehicle (dimethyl sulfoxide, 0.4% v/v) alone (DMSO). Data are shown as the means ± SD from four independent transfections. Statistically significant differences compared with the activity of the DMSO-treated cells in each CAR are indicated by asterisks (*$p < 0.05$; **$p < 0.005$; ***$p < 0.0005$; by the one-way ANOVA and Dunnet's test).

Discussion

CAR, as well as PXR, regulates the expression of genes involved in the metabolism and transport of xenobiotics and endobiotics [20–22]. It is possible that functional alterations resulting from genetic polymorphisms may influence drug metabolism and therapeutic outcomes. To date, several non-synonymous SNPs that affect the transactivation ability of human PXR have been reported [19,23,24]. These PXR variants might cause variations between individuals in their responses to drugs. CAR is a close relative of PXR, with which it shares several overlapping properties. However, little is known about SNPs in *CAR*. Recently, we found one non-synonymous SNP in exon 4 that induces a Val133Gly substitution in the Japanese population [17]. In this study, we sequenced the *CAR* gene in an additional set of Japanese subjects.

In the coding region for CAR, we found a total of four SNPs with changes that would lead to amino acid alterations (Val133Gly, His246Arg, Leu308Pro, and Asn323Ser). These variants, along with wild-type CAR, were transiently expressed in COS-7 cells, and their transactivation abilities were determined by utilizing the *CYP3A4* promoter/enhancer luciferase reporter gene system in the presence or absence of an agonistic ligand, CITCO. The His246Arg variant showed a dramatically decreased constitutive activity and was no longer activated by CITCO. The Leu308Pro variant also had a reduced constitutive transactivation ability but retained its responsiveness to the ligand. The other two variants, Val133Gly and Asn323Ser, showed similar levels of constitutive activity to that of the wild-type CAR (Fig. 3). The responsiveness to CITCO of the two variants were also retained (Fig. 4). As shown in Fig. 4 and also in our recent study [18], the increasing effect of CITCO is relatively small (at most 2-fold even at 10 μM) in the reporter gene assay using transfected cell lines, which makes it difficult to determine the reliable EC50 or affinity for CITCO. To improve accuracy, a different approach, e.g., a usage of primary hepatocyte cultures, might be warranted.

All four of these amino acid variations were located in the LBD. The LBD of most nuclear receptors is a multifunctional domain that mediates ligand binding, dimerization with RXR, interactions with co-activator proteins, nuclear localization, and most importantly, transactivation functions via the C-terminal transactivation domain AF2. The X-ray crystal structure of CAR shows that the LBD is composed of 11 α-helices, two 3_{10} helixes, and three β-strands, while Val133, His246, Leu308, and Asn323 map to helices H2, H7, H10, and H10, respectively [25–27]. H7 contributes to the ligand binding pocket and participates in interactions with the ligand [27]. Recently, it has been reported that H7 is involved in the selectivity for CAR ligands [28]. This finding might explain the observation that the His246Arg variant lacks responsiveness to CITCO. Activation of nuclear receptors requires that the AF2 helix be precisely positioned. In CAR, H10 interacts with the AF2 helix to maintain the active conformation of the protein [25]. The reduced transactivation activities of the His246Arg and Leu308Pro variants might be due to conformational changes of AF2 resulting from the amino acid alterations. Thus, it is possible that His246 and Leu308 play an important role in stabilizing the active conformation of the receptor.

CAR regulates a number of genes encoding enzymes involved in xenobiotic/endobiotic metabolism, conjugation, and transport of small hydrophobic substrates. Moreover, recent studies have indicated that CAR participates in bilirubin clearance [5], bile acid detoxification [29], and adaptive responses to nutrition stress [6]. The physiological importance of CAR is becoming increasingly apparent. Thus, variations of CAR resulting in functional alterations may have serious impacts on pharmacological and physiological responses of the target genes.

In conclusion, we functionally characterized four naturally occurring CAR variants. The variant His246Arg showed a marked reduction of constitutive activity and failed to respond to CITCO. Although the Leu308Pro variant retained normal responsiveness to CITCO, its constitutive transactivation ability was significantly reduced.

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