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Differences in Cytochrome P450 and Nuclear Receptor mRNA Levels in Liver and Small Intestines between SD and DA Rats

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Summary: This study aimed to clarify the differences in mRNA levels of cytochrome P450 (CYP) isoforms and nuclear receptors between Dark Agouti (DA) and Sprague-Dawley (SD) rats which are animal models for poor metabolizers and extensive metabolizers for CYP2D6, respectively. Using liver and small intestine tissues of both rat strains, we investigated the mRNA levels of CYP1A, 2A, 2B, 2C, 2D, 2E, and 3A subfamilies and nuclear receptors which regulate the transcription of CYP isoforms. In the liver, male DA rats showed a low CYP2D2 mRNA level but high mRNA levels of CYP3A1, 3A2, and 1A1 compared to SD rats. No significant difference was noted in other CYP isoforms. The mRNA levels of CAR were higher in DA rats than those in SD rats. In small intestine, the mRNA levels of CYP isoforms and nuclear receptors exhibited no significant strain differences. In addition, the activity of CYP3A in small intestinal microsome did not differ between SD and DA rats. Female DA rats exhibited higher mRNA levels of CYP3A1, 3A2, and 2B1 in the liver than female SD rats. In conclusion, the mRNA levels of CYP3A1 and 3A2 isoforms and CAR in the liver but not in the small intestines were different between DA and SD rats in both sexes.

Keywords: strain difference; cytochrome P450; nuclear receptor; SD rat; DA rat

Cytochrome P450 (CYP) plays an important role in first-pass metabolism in the liver. Recently, it has been shown that the small intestines function as a xenobiotic-metabolizing organ while being the absorption site of nutrients, water, and xenobiotics.1–4) CYP isoforms in the intestines in addition to those in the liver are also involved in first-pass elimination in various species including rats. Both phase I and phase II metabolic enzymes are expressed, together with associated transporters in the small intestines.5,6) Zhang et al. reported that in the human small intestines, CYP1A1, 1B1, 2C, 2D6, 2E1, 3A4, and 3A5 mRNAs were present but there was no sign of CYP1A2, 2A6, 2A7, 2B6, 2F1, 3A7, and 4B1 mRNAs.7) In the rat small intestines, several CYPs are expressed including CYP1A1, 2B, 2C, 2D, and 3A but this range is smaller than that expressed heptatically.8,9)

It has been reported that there are strain differences in CYP-mediated drug metabolism in rats. Dark Agouti (DA) rats exhibit quite low mRNA levels of CYP2D2,10) and low metabolic activities for the biotransformation of some typical CYP2D6 substrates (debrisoquine, bunitrolol, alpranolol, and metoprolol),11–14) compared to Sprague-Dawley (SD) or Wistar rats. Therefore, DA rats have been regarded as an animal model for human poor metabolizers of CYP2D6. Some attempts have been made to clarify CYP-mediated drug metabolizing activities, and metabolic activities of major CYP isoforms including that of CYP2D have been demonstrated in liver microsomes of DA and Wistar rats.15–18) In contrast to a low activity of CYP2D, CYP1A and CYP3A activities are higher in DA rats than those in Wistar rats.14) However, the strain differences in hepatic mRNA levels of CYP isoforms remain unclear.

Nuclear receptors including constitutive androstane receptor (CAR, NR1I3) and pregnane X receptor (PXR, NR1I2) are involved in the primary response to xenobiotics and endogenous toxins. Specifically, these receptors respond to ligands by activating the expressions of genes encoding enzymes involved in phase I (functionalization reactions) and phase II (conjugation reactions) metabolism and transporters.19–21) CAR is normally sequestered in the cytoplasm of untreated liver cells, and becomes translocated into the nucleus after exposure to phenobarbital or phenobarbital-like chemicals. Cytoplasmic CAR retention protein (CCRIP) has been shown to maintain the cytoplasmic localization of CAR by forming a complex with CAR and...
Strain Difference of CYP and Nuclear Receptor Levels in Rats

hsp90.\textsuperscript{22,23} Retinoid X receptor (RXR) plays a central role in regulating the activity of other nuclear receptors by acting as a partner for heterodimers.\textsuperscript{24} The peroxisome proliferator-activated receptor (PPAR\textalpha) also functions as a heterodimer with RXR. In mice and rats, PPAR\textalpha agonists elicit a predictable course of adaptive responses in the liver including peroxisome proliferation, induction of lipid-metabolizing genes, and hepatomegaly.\textsuperscript{25} Aryl hydrocarbon receptor (AhR) regulates the transcription of CYP1A1.\textsuperscript{26} Hepatocyte nuclear factor (HNF) 4\alpha constitutively regulates the transcription of CYP isoforms in the liver.\textsuperscript{27,28} Accordingly, in addition to CYP isoforms, the further investigation of the mRNA levels of nuclear receptors, transcription factors for CYP, is needed in liver of SD and DA rats. Furthermore, although the small intestines are also the principal organ involved in first-pass metabolism during gastrointestinal absorption, there is no available information on strain differences in intestinal mRNA levels of CYP isoforms and nuclear receptors in rats. Much effort is needed to characterize CYP isoforms with nuclear receptors based on the mRNA levels in the liver and small intestine of both strains.

It has also been reported that the gender and age affect the profile of CYP expression. For example, the expression of CYP3A1 or CYP3A2 was generally higher in male Wistar rat than in female Wistar rat.\textsuperscript{29} CYP activities showed gender differences in rat.\textsuperscript{30} The hepatic microsomes of male rats have relatively higher activities of CYP3A than those of female rats.

This study aimed to clarify the strain differences in mRNA levels of hepatic and intestinal CYP isoforms and nuclear receptors between CYP2D-deficient DA rats and CYP2D-expressing SD rats. We examined the mRNA levels of CYP1A1, 1A2, 2A1, 2B1, 2C11, 2D1, 2D2, 2E1, 3A1, 3A2, 3A9, 3A18, and 3A62 as CYP isoforms; and those of PXR, CAR, RXR, PPAR\textalpha, AhR, HNF-4\alpha, and CCRP as nuclear receptors or associated proteins. Furthermore, we preliminary examined the strain differences of CYP mRNA expression in female as well as male rats.

The results suggested that CYP isoforms and one nuclear receptor have some strain differences at the mRNA level in the liver and not in small intestine of SD and DA rats. Finally, we briefly discussed whether SD and DA rats are suitable as preclinical models of extensive and poor metabolizers for CYP2D6.

Methods

Materials: Trypsin inhibitor and diazepam were obtained from Sigma (St. Louis, MO, USA) and midazolam injection (Dormicum\textsuperscript{®}) from Astellas Pharma (Tokyo, Japan). NADP\textsuperscript{+}, glucose-6-phosphate, and glucose-6-phosphate dehydrogenase were purchased from Oriental Yeast (Tokyo, Japan). All other chemicals used were of the highest purity available.

Animals: Eight-wk-old male and female SD or DA rats were purchased from CLEA Japan, Inc. (Tokyo, Japan), and maintained under conventional housing conditions until use at 9-wk-old. The experiments were approved by the Committee for the Care and Use of Laboratory Animals at Kinki University School of Pharmacy.

Sample Preparation: The liver and small intestines were removed from SD or DA rats after anesthesia by inhalation of diethyl ether, and euthanasia by cervical dislocation. The excised small intestines were flushed with 50 ml ice-cold saline. The length of the small intestine sample was about 5 cm, which was removed from about 5 cm below the ligament of Treitz. Each sample was preserved at −80°C until use after flash freezing with liquid nitrogen.

Real-Time Reverse Transcriptase Polymerase Chain Reaction: Total RNA was extracted from approximately 100 mg of each rat liver and small intestines using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. Following RNase-free DNase I treatment (TaKaRa, Shiga, Japan), approximately 500 ng total RNA, as evaluated by UV absorption at 260 nm, was reverse-transcribed to complementary DNA (cDNA) using a PrimeScript-RT reagent Kit (TaKaRa), 0.2 \muM primer set of target gene or ribosome 18S ribosomal RNA (18S rRNA) as endogenous reference. Amplification was performed in 50-\muL reaction mixtures containing 2 × SYBR Premix Ex Taq (TaKaRa), 0.2 \muM primer set of target gene or ribosome 18S ribosomal RNA (18S rRNA) as endogenous reference. Amplification and detection were performed with an ABI PRISM 7000 (Applied Biosystems, Foster City, CA, USA). The PCR reactions were incubated at 95°C for 10 s, and amplified by a 40 three-step cycles at 95°C for 5 s, 55°C for 20 s, and 72°C for 31 s. The amount of 18S rRNA in each sample was also measured for normalization. For all PCR amplifications, we used oligonucleotide sequences designed by Primer Express 2.0 (Applied Biosystems), as shown in Table 1. Data were analyzed using the ABI Prism 7000 SDS Software (Applied Biosystems) particularly for the multiplex comparative method. The relative quantitation of the amount of target mRNA in the tested tissue samples was accomplished by measuring Cycle thresholds (Ct). To determine the quantity of the target gene-specific transcripts present in the liver and small intestines, their respective Ct values were first normalized by subtracting the Ct value obtained from the ribosome 18S rRNA control (\DeltaCt = Ct, target − Ct, control). The concentration of gene-specific mRNA in the liver and small intestines of DA rats relative to each tissue of SD rats was calculated by subtracting the normalized Ct values obtained for each tissue of SD rat from those obtained from each tissue of DA rat (\DeltaCt = \DeltaCt, DA − \DeltaCt, SD) and the relative concentration was determined (2^−\DeltaCt).

Microsomal preparation of the intestines: Microsomal preparation of the intestines was performed with modifications of the procedure described by Komura et al.\textsuperscript{31}
briefly, the three intestinal segments (25 cm each) were flushed with 5 ml of iced-cold 50 mM HEPES buffer pH 7.4 containing 0.1 mM EDTA and 0.5 mg/ml trypsin inhibitor. each intestinal segment was cut longitudinally, and the mucosal side was exposed. The mucosa was scraped with the cover glass (18 × 18 mm, MATSUNAMI, Osaka, Japan) to obtain an epithelial cell-enriched fraction. The homogenized mucosa was centrifuged at 9,000 g for 60 min, and the pellet was resuspended in 50 mM HEPES buffer, pH 7.4 without trypsin. Protein concentrations were determined using a BCA protein assay kit (Pierce Biotechnology, Rockford, IL, USA).

Incubation conditions: Activities of midazolam 1-hydroxylation (MZ1H) were measured to estimate CYP3A activity based on a modified method of Komura and Iwaki.[10] The incubation mixture (total volume, 0.2 ml) contained 100 mM potassium phosphate buffer (pH 7.4), an NADPH-generating system [1.0 mM NADP+, 10 mM glucose-6-phosphate, 1.0 units/ml glucose-6-phosphate dehydrogenase, and 10 mM MgCl2], rat intestinal microsomes, and a substrate. Midazolam (25 µM) was incubated with 0.5 mg/ml microsomal protein for 15 min. the reaction was initiated by the addition of the NADPH-generation system following a 5-min pre-incubation at 37 °C. The MZ1H reaction was later stopped by the addition of 0.5 ml 0.1 M Na2CO3 and 0.1 ml 3.0 M NaOH and analyzed by a high-performance liquid chromatograph (HPLC) as described below.

### HPLC Analysis
Determination of the MZ1H was performed using a Shimadzu HPLC SCL-10A system and an YMC-Pack Pro C18 column (5.0 µm, 4.6 mm × 150 mm; YMC, Kyoto, Japan). The mobile phase consisted of 10 mM sodium acetate buffer (pH 4.7) and acetonitrile (55:45, v/v). the eluent was monitored at 254 nm. the flow rate was 1.0 ml/min for all analyses.

### Statistical Analysis
Significant differences between SD and DA rats were estimated using a Student’s unpaired t-test.

## Results
We examined the differences in mRNA expression levels of CYP isoforms and nuclear receptors in the liver and small intestines between SD and DA rats. Figure 1 shows the mRNA levels of CYP1A1, 1A2, 2A1, 2B1, 2C11, 2D1, 2D2, 2E1, 3A1, and 3A2 in liver of male
Fig. 1. mRNA expression levels of CYP1A1, 1A2, 2A1, 2B1, 2C11, 2D1, 2D2, 2E1, 3A1, and 3A2 in the liver of male SD and DA rats. The results are expressed as means ± S.D. of 3 rats. *p < 0.05 and **p < 0.01, significant differences between values for SD and DA rats.

Fig. 2. mRNA expression levels of CYP1A1, 2B1, 2C11, 2D1, 2D2, 3A9, 3A18, and 3A62 in the small intestines of male SD and DA rats. The results are expressed as means ± S.D. of 3 rats.
DA rats compared to those in SD rats. In DA rats, the mRNA levels of CYP2D2 were significantly low compared to the significant high expression levels of CYP3A1, 3A2, and 1A1 mRNAs. Particularly, the CYP1A1 mRNA level was approximately 10 times higher in SD rats than that in DA rats. For the other CYP isoforms examined, there were little differences in mRNA levels between the SD and DA rats. **Figure 2** shows the mRNA levels of CYP1A1, 2B1, 2C11, 2D1, 2D2, and the main intestinal CYP3A family, CYP3A9, 3A18, and 3A62 in the small intestines of male SD and DA rats. With regards to the CYP isoforms (CYP1A2, 2A1, 2E1, and 3A2) examined in the liver, we confirmed their extremely low mRNA levels in the small intestines (data not shown). The intestinal mRNA expression levels of the CYP isoforms examined including CYP3A9, 3A18, and 3A62 were within similar ranges in both strains unlike the hepatic CYP3A1 and 3A2 isoforms. The activity of MZ1H as an indicator of CYP3A activity did not differ between the male SD and DA rat intestinal microsomes, being consistent with the CYP3A mRNA level results (**Fig. 3**).

Nuclear receptors such as PXR and CAR regulate the transcription of CYP isoforms. To clarify whether strain differences of nuclear receptors exist between male SD and DA rats, we determined the mRNA levels of PXR, CAR, RXR, PPARα, CCRP, AhR, and HNF4α in the liver and small intestines of male SD and DA rats. **Figure 4** shows that the hepatic mRNA levels of CAR were 4 times higher in DA rats than those in SD rats. Other nuclear receptors exhibited little strain difference between SD and DA rats. The levels of CCRP, which maintains the cytoplasmic localization of CAR by forming a complex with CAR were not significantly different between the 2 rat strains. **Figure 5** shows the mRNA levels of the nuclear receptors in the small intestines of male SD and DA rats. The all examined nuclear receptors including CAR showed similar expression levels in both SD and DA rats. **Figures 6 and 7** show the results of CYP mRNA expression in female DA and SD rats.
Fig. 5. mRNA expression levels of PXR, CAR, RXR, PPARα, and CCRP in the small intestines of male SD and DA rats. The results are expressed as means ± S.D. of 3 rats.

Fig. 6. mRNA expression levels of CYP1A1, 1A2, 2A1, 2B1, 2C11, 2D1, 2D2, 2E1, 3A1, and 3A2 in the liver of female SD and DA rats. The results are expressed as means ± S.D. of 3–4 rats. There are significant differences (*p < 0.05 and **p < 0.01) between SD and DA rats.
DA rats also exhibited significantly higher mRNA levels of CYP3A1 and 3A2 than female SD rats. The strain difference of CYP3A1 and 3A2 mRNA expression was larger in female rats than in male rats. However, the mRNA levels of CYP2D2 and 1A1 were similar in female SD and DA rats, while the mRNA levels of CYP2B1 was increased in DA rats. Female DA rats also exhibited the significantly higher mRNA expression of CAR than female SD rats (data not shown). In Figure 7, little strain differences between female SD and DA rats were also observed in small intestinal CYP mRNA expression.

**Discussion**

The present study showed that there are strain differences in the mRNA levels of CYP1A1/3A and nuclear receptors in liver but not in small intestine.

Among CYP isoforms examined in the liver of male rats, CYP1A1, 2B2, 1A1 and 3A2 showed a strain difference. The lower mRNA levels of CYP2D2 in DA rats were almost congruent with the results of a previous report on the deficient CYP2D2 activity in DA rats. The mRNA levels of CYP3A1, 3A2, and 1A1 were significantly higher in DA rats than those in SD rats. Komura and Iwaki have previously demonstrated strain differences in metabolic activities using probe substrates of each CYP isoform using DA and Wistar rats as model animals for extensive and poor metabolizers, respectively. Dextromethorphan O-demethylation activity in hepatic microsome for an indicator for CYP2D was lower in DA rats than that in Wistar rats, which was in contrast to the higher levels for ethoxyresorfin O-deethylation and midazolam 4-hydroxylation (MZ4H) activity catalyzed by CYP1A and CYP3A isoforms, respectively. Hence, the mRNA expression levels in the present study were consistent with previously reported metabolic activities of CYP2D, 1A and 3A although the strains (Wistar vs SD rats) used in both studies were different. Saito et al. have investigated the levels of metabolic activities for dextromethorphan and CYP expression in hepatic microsome among 4 rat strains and have reported that the CYP2D1 activity in SD rat is significantly higher than that in Wistar rats in vivo. In vitro and in vivo characteristics of a model of CYP2D6 poor metabolizer. In Figures 1 and 2, the lower mRNA levels of CYP2D in male DA rat were shown compared to those in
SD rat in liver but not in small intestine. Our results of mRNA levels also suggested that the liver of male DA rat could be appropriate model for CYP2D poor metabolizer.

First-pass metabolism in the small intestines has received a great deal of attention due to its impact on the oral bioavailability of drugs such as cyclosporine A and midazolam in humans.\(^{41-43}\) and some attempts have been made to evaluate small intestinal metabolism using rats. However, there is no information on strain differences in intestinal CYP isoforms. We examined the mRNA levels of CYP isoforms and nuclear receptors that would be the determinant factors to the expression levels of CYP isoforms in the small intestines. We chose to focus on the mRNA levels of CYP1A1, 2B1, 2C11, 2D1, 2D2, 3A9, 3A18, and 3A62 in the present study because of the following reasons: (1) the mRNA expression levels of CYP1A2, 2A1, 2E1, 3A1, and 3A2 in the small intestines have been shown to be relatively low in previous reports,\(^{8,9}\) (2) CYP3A isoforms are the key factors in intestinal metabolism and their mRNA expression levels in the liver show strain differences, (3) recently, a new CYP3A (CYP3A62) cDNA was isolated from the cDNA library of rat liver,\(^{12}\) and CYP3A62 is a major isofrom in the small intestines of both sexes unlike in the liver of male rats, 4) both CYP3A9 and 3A18 in addition to CYP3A62 are expressed in the small intestines. CYP3A isoforms such as CYP3A9, 3A18 and 3A62 are mainly expressed in small intestine, and their protein levels were higher than those of CYP3A1 and 3A2.\(^{15}\) There were no significant differences in the mRNA levels of the CYP isoforms in the small intestines unlike in the liver, which indicated that there is no strain difference in protein levels of CYP isoforms. This was also proven by the similar MZ1H activity in the small intestinal microsomes of both strains. Midazolam is mainly metabolized by CYP3A. In humans, CYP3A4 and CYP3A5 catalyze the metabolism of midazolam to two different metabolites, the major metabolite 1′-OH-midazolam) and the minor metabolite 4-OH-midazolam.\(^{44}\) In rat liver and small intestine, there is a preferential formation of 4-OH-midazolam over 1′-OH-midazolam. The formation of both metabolites is inhibited to a great extent by addition of an anti-CYP3A2 antibody.\(^{45,46}\) However, the involvement of CYP3A9, CYP3A18 and CYP3A62 in midazolam metabolism in small intestine is unclear. In this study, we used the proximal part of the small intestines because the mRNA levels and activities of CYP in the proximal portion were higher compared to those in the distal part.\(^{47,48}\)

In addition, our preliminary findings in female rats indicated that the mRNA expression of CYP3A1 and 3A2 was increased in DA rats and that the mRNA expression of CYP2D2 was similar in female SD and DA rats. The finding regarding the mRNA level of CYP2D2 is inconsistent with the previous report about the CYP activities. Komura and Iwaki demonstrated that the activities of CYP2D in both male and female DA rats were significantly decreased compared to Wistar rat and that the activities of CYP1A1 were facilitated in male DA rats.\(^{30}\) Further studies are needed to clarify the gender effects on strain difference in CYP activities corresponding to mRNA expression.

We investigated the mRNA levels of nuclear receptors in the liver of SD and DA rats to reveal the strain differences in nuclear receptors and the relationships of mRNA levels between the CYP isoforms and nuclear receptors because nuclear receptors play a role in the regulation of transcription of CYP isoforms. The higher CAR mRNA levels were noted in the liver of male and female DA rats compared to those in SD rats. Little changes were observed in mRNA levels of other nuclear receptors, suggesting that CAR is involved in the strain differences of CYP in both male and female DA rats. Furthermore, the nuclear translocation of CAR may differ because the mRNA levels of CCRP showed little strain difference between SD and DA rats. CAR regulates the transcriptions of CYP2B,\(^{49}\) CYP3A\(^{49,50}\) and CYP2C\(^{51}\) through DR4 motifs and the enoyl-CoA hydratase/3-hydroxy acyl CoA-dehydrogenase gene through a DR1 motif.\(^{52}\) Therefore, increased levels of CAR could be partially involved in CYP3A upregulation in the liver of DA rats. However, the mRNA levels of CYP2B and CYP2C regulated by CAR were not different between male SD and DA rats. It has been reported that HNF4α constitutively regulates CYP isoforms in the liver.\(^{27,28}\) Thus, we speculate that this disagreement between CYP3A and CYP2B and 2C is arisen from the difference of transcriptional regulation for each CYP isoform, namely, the involvement of HNF4α could differ between CYP isoforms. However, the mRNA levels of HNF4α showed little differences between SD and DA rats. To clarify the cause of this disagreement in CAR, the nuclear receptors including activities for CYP transcription are needed to investigate the strain difference. Without exogenous ligand, it is possible that CAR is activated by endogenous ligands such as steroid or bile acid. The mRNA levels of CYP1A1 in DA rats were significantly increased compared to those in SD rats. The mRNA levels of AhR, which regulates the transcription of CYP1A1, also exhibited little strain difference. Further studies are needed to clarify the mechanisms of the upregulated levels of the CYP isoforms. This upregulation for CYP1A1, CYP3A1 and CYP3A2 in DA rats may work as an adjunct to drug metabolism because DA rats were deficient in CYP2D2, although the substrate specificities of CYP2D and those of CYP3A and 1A are less overlapping. Its implication for metabolism is unclear but warrants examination. In the small intestines, the mRNA levels of the nuclear receptors and CYP did not change, indicating that there were little strain differences of CYP and nuclear receptors in the small intestines between SD and DA rats. These results suggested that first pass effects of drugs in the small intestines did not alter between SD and DA rats. Similar mRNA levels of CYP were observed in the small intestines of SD and DA rats although the liver of DA rats might exhibit the characteristics of a CYP2D6 poor metabolizer. However, it is possible
that the inducibility of CYP in contacting ingested xenobiotics exhibits the different effects on CYP activities between liver and small intestine of SD and DA rats. CYP1A1 has been reported as the most prominent inducible form in rat small intestines.\(^8\)\(^9\) Inducing agents such as \(\beta\)-naphthoflavone can induce the protein level of small intestinal CYP1A1 up to 17 folds. For the CYP1A1, CYP3A1 and CYP3A2 observed strain differences in mRNA levels, the inducibility of CYP may exhibit the different patterns between SD and DA rats. These strain differences are an important factor that may clarify the differences in drug-drug interactions between SD and DA rats. In the case of using the common nuclear receptor ligand between rat and human, SD and DA rats might be used as models of intestinal drug metabolism in humans during the induction of CYPs. It is also possible that the inducibility of CYP could exhibit site specificity in the small intestines because the CYP activities changed from the proximal to the distal part.

In conclusion, strain differences of CYP isoforms between SD and DA rats were observed in the liver, but not in the small intestines. We showed that the mRNA expression of CYP3A1 and 3A2 were improved in male and female DA rats although it was unclear whether similar strain differences existed among other strains.

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**References**

(2004).


