Xenobiotica, 2009; 39(4): 323–334

informa
healthcare

RESEARCH ARTICLE

Intestinal flora induces the expression of Cyp3a in the mouse liver

T. Toda[1], N. Saito[1], N. Ikarashi[1], K. Ito[1], M. Yamamoto[2], A. Ishige[3], K. Watanabe[3], and K. Sugiyama[1]

[1]*Hoshi University, Tokyo, Japan,* [2]*Tsumura & Co., Ibaraki, Japan, and* [3]*Keio University, Tokyo, Japan*

Abstract

1. In order to determine the effects of intestinal flora on the expression of cytochrome P450 (CYP), the mRNA expression of CYP was compared between specific pathogen-free (SPF) and germ-free (GF) mice.
2. Most of the major CYP isozymes showed higher expression in the livers of SPF mice compared with GF mice.
3. Nuclear factors such as pregnane X receptor (PXR) and constitutive androstane receptor (CAR), as well as transporters and conjugation enzymes involved in the detoxification of lithocholic acid (LCA), also showed higher expression in SPF mice.
4. The findings suggest that in the livers of SPF mice, LCA produced by intestinal flora increases the expression of CYPs via activation of PXR and CAR.
5. Drugs such as antibiotics, some diseases and ageing, etc. are known to alter intestinal flora. The present findings suggest that such changes also affect CYP and are one of the factors responsible for individual differences in pharmacokinetics.

Keywords: *Intestinal flora; germ-free; Cyp3a; nuclear transcription factors; pregnane X receptor (PXR); lithocholic acid*

Introduction

There are individual differences in the body's ability to excrete xenobiotics, such as drugs and environmental pollutants. Because the individual differences can lead to the variability in drug concentration and effects, they are one of the issues that must be paid attention in drug therapy.

These individual differences are partially caused by qualitative and quantitative changes in cytochrome P450s (CYPs), a group of major drug-metabolizing enzymes. Genetic polymorphism is a typical qualitative change in CYPs. For example, it is known that the *in vivo* pharmacokinetics of omeprazole, a CYP2C19 substrate, are affected by the single nucleotide polymorphism of CYP2C19 (Ishizaki and Horai 1999). On the other hand, the quantity of CYP is affected by disease, gender, age and drugs. For example, studies have shown that rifampicin, an antibiotic, induces CYP3A4 (Pichard et al. 1990); obesity increases the expression of CYP2B in the liver (Yoshinari et al. 2006); and inflammatory bowel disease lowers the activity of CYP3A in the liver and small intestine (Masubuchi and Horie 2004). Furthermore, studies have shown that birth and age alter the expression of CYP in the liver (Itoh et al. 1994; Sakuma et al. 2000; Choudhary et al. 2003; Stevens et al. 2003).

In this manner, the expression of CYP changes depending on drug, disease and age, and such changes are known to act as one of the factors responsible for individual differences in pharmacokinetics, but its detailed mechanisms are unknown.

Several studies have documented findings suggesting the possibility that intestinal flora is involved in the changes in expression of CYP. For example, ciprofloxacin, a new quinolone antibacterial agent, lowers not only intestinal flora (Chin and Neu 1984), but also CYP expression (Xie et al. 2003). While CYP3A is down-

Address for Correspondence: K. Sugiyama, Department of Clinical Pharmacokinetics, Hoshi University, 2-4-41 Ebara, Shinagawa-ku, Tokyo 142-8501, Japan. E-mail: sugiyama@hoshi.ac.jp

(Received 28 October 2008; revised 26 November 2008; accepted 27 November 2008)

ISSN 0049-8254 print/ISSN 1366-5928 online
DOI: 10.1080/00498250802651984

http://www.informapharmascience.com/xen

regulated in inflammatory bowel disease (Masubuchi and Horie 2004), *Bacteroides* and *Escherichia coli* are elevated in such cases (Okayasu et al. 1990; Neut et al. 2002). Furthermore, birth and age alter not only CYP expression (Itoh et al. 1994; Sakuma et al. 2000; Choudhary et al. 2003; Stevens et al. 2003), but also intestinal flora (Savage et al. 1968). It is also known that there are marked individual differences in intestinal flora (Hayashi et al. 2002), and that psychogenic factors, such as stress, drastically affect intestinal flora (Morishita and Ogata 1970; Suzuki et al. 1983; Bailey and Coe 1999). Therefore, the present authors hypothesized that intestinal flora is involved in the changes in CYP expression and is one of the causes of individual differences in pharmacokinetics. The effects of intestinal flora on CYP expression at the mRNA level were investigated by GeneChip and real-time reverse transcriptase-polymerase chain reaction (RT-PCR) using specific pathogen-free (SPF) mice and germ-free (GF) mice lacking intestinal flora. Among the CYP subfamily, CYP3A is involved in the metabolism of the greatest number of drugs. Thus, the expression of murine Cyp3a, which is equivalent to human CYP3A, was measured at the protein level, and the changes in its activity were evaluated using triazolam, a specific substrate (Perloff et al. 2000).

In addition, in order to clarify that the changes in CYP expression are due to intestinal flora, we focused on the expression of nuclear transcription factors involved in CYP expression regulation, that is, aryl hydrocarbon receptor (AhR), constitutive androstane receptor (CAR), farnesoid X receptor (FXR), and pregnane X receptor (PXR), and the mRNA levels of these genes in the livers of GF and SPF mice were quantified. Among the biological components affected by intestinal flora, we focused on lithocholic acid (LCA), a secondary bile acid produced by bacterial enzymes (Ridlon et al. 2006), which act as an activator of FXR and PXR (Staudinger et al. 2001; Xie et al. 2001). A possibility of its involvement in the CYP expression changes observed in this study was tested by quantifying the enzymes and transporters which are responsible for its detoxification and are reported to be induced by LCA.

Materials and methods

Chemicals

RNAiso was purchased from Takara Bio, Inc. (Tokyo, Japan). The high-capacity cDNA synthesis kit was purchased from Applied Biosystems (Tokyo, Japan). The RNeasy Mini Kit, RNeasy plus Mini Kit and RNase-free Water were purchased from Qiagen, Inc. (Valencia, CA, USA). The GeneChip Array (Mouse Genome 430 2.0 Array) was purchased from Affymetrix Japan (Tokyo, Japan). Tris-EDTA buffer (TE buffer) was purchased from Nacalai Tesque (Kyoto, Japan). The iQ SYBR Green Supermix and DC protein assay kit were purchased from Bio-Rad Laboratories (Hercules, CA, USA). Various primers were purchased from Invitrogen (Tokyo, Japan). The ECL Plus Western Blotting Detection System was purchased from GE Healthcare (Chalfont St Giles, UK). Anti-rat CYP3A2 (from rabbit) (Lot O12RGC) was purchased from Daiichi Pure Chemicals (Tokyo, Japan). Anti-rabbit IgG (whole molecule) peroxidase conjugate (Lot 053K4853), bovine serum albumin and α-hydroxytriazolam were purchased from Sigma-Aldrich Corp. (St Louis, MO, USA). Skim milk was purchased from Snow Brand Milk Products (Tokyo, Japan). Acetonitrile, triazolam, monobasic potassium phosphate and di-potassium hydrogen phosphate were purchased from Wako Pure Chemical Industries (Osaka, Japan). Four-hydroxytriazolam was purchased from BIOMOL (Exeter, UK). The NADPH regeneration system was purchased from Becton, Dickinson & Co. (Tokyo, Japan). With regard to other reagents, commercially available products of the highest grade were purchased.

Laboratory animals

Nine-week-old IQI male GF and SPF mice were purchased from the Central Institute for Experimental Animals (Tokyo, Japan). GF mice were kept in vinyl isolators using autoclaved equipment, materials, cages and bedding materials, and they had free access to food and water. Autoclaved water was given as drinking water, and autoclaved CL-2 (CLEA Japan) was given as food. Faeces, bedding materials and swabs were tested for sterility, and mice were used after confirming sterility (Maejima and Nomura 1975; Ikeda et al. 2007).

The present study was conducted in accordance with the Guiding Principles for the Care and Use of Laboratory Animals, as adopted by the Committee on Animal Research of Hoshi University.

RNA preparation from tissue samples

Mice were dissected under diethyl ether anaesthesia, and the liver and small intestine were excised and washed with PBS. The small intestine was divided into three sections and the middle section was used. The organs were weighed, recorded and frozen using liquid nitrogen.

RNA was extracted from about 15 mg of frozen liver using the RNeasy Plus Mini Kit. With each small intestine sample, 13 ml of RNAiso were used to crush frozen tissue quickly and completely. Next, 8 ml of RNAiso were added to 2 ml of the homogenate (5× dilution), followed by mixing, and RNA was extracted using 2 ml

of the homogenate. RNA extraction was carried out according to the protocol for the RNeasy Plus Mini Kit and RNAiso. The resulting solution was diluted 50-fold using Tris-EDTA buffer (TE buffer), and purity was confirmed and RNA concentration ($\mu g\ ml^{-1}$) was calculated by measuring absorbance at 260 and 280 nm using a spectrophotometer (U-2800, Hitachi High Technologies).

GeneChip

GeneChip operation and array image data acquisition

Starting with 5 μg of extracted RNA, cDNA was synthesized by the standard one-cycle method (see http://bioinf.picr.man.ac.uk/mbcf/miame_protocols.jsp), and biotinylated cRNA synthesis and spectrophotometry quantification were performed. Synthesized cRNA fragmentation and cRNA quality checks before and after fragmentation were performed. Hybridization to the Mouse Genome 430 2.0 Array (Affymetrix) and array scan and image data acquisition using the GeneChip 3000 Scanner (Affymetrix) were carried out.

Numerical data extraction

According to the protocol shown at http://bioinf.picr.man.ac.uk/mbcf/miame_protocols.jsp, the GCOS (Affymetrix), a data analysis system for GeneChip system standard, was used to confirm the array image data of each sample, and the data were converted into a file format that enabled extraction of gene expression as a numerical value. The signals were normalized using the average value of all probes, excluding the upper and lower 2% signal values. In addition, genes with corrected signal values from 500 to 10 000 were extracted.

Real-time RT-PCR

From 1 μg of RNA, a high-capacity cDNA synthesis kit was used to synthesize cDNA. TE buffer was used to dilute the cDNA 20-fold to prepare cDNA TE buffer solution. The expression of each gene was detected by preparing primers listed in Table 1 and performing real-time RT-PCR. To each well of a Multiplate® PCR Plates 96-well clear (Bio-Rad Laboratories), 25 μl of iQ SYBR Green Supermix, 3 μl of forward primer of target gene (5 pmol μl^{-1}), 3 μl of reverse primer (5 pmol μl^{-1}), 4 μl of cDNA TE buffer solution and 15 μl of RNase-free Water were added. With regard to 18S rRNA, a housekeeping gene, 4 μl of a cDNA TE buffer solution that was prepared by diluting above-mentioned solution 20-fold using TE buffer was used. Denaturation temperature was set at 95°C for 15 s, annealing temperature at 56°C for 30 s and elongation temperature at 72°C for 30 s. The fluorescence intensity of the amplification process was monitored using the My

Table 1. Primer sequences of mouse mRNA.

Target	Accession number	Forward primer (5′ to 3′)	Reverse primer (5′ to 3′)	Amplicon size (bp)
Cyp1a2	NM_009993	GACATGGCCTAACGTGCAG	GGTCAGAAAGCCGTGGTTG	83
Cyp2a4	NM_009997	GGAAGACGAACGGTGCTTTC	AAAGCGGTCCCCGAAGACG	92
Cyp2b9	NM_010000	CCCTGGATCCCACCTTTC	CAGCAGATGAAGGAACTGGTC	107
Cyp2b13	NM_007813	GCTTCTTGGCCCGCTGAC	GTTTCCAGCAGCTCTCTCAG	96
Cyp2c37	NM_010001	GCTACTAATGGAATGGGCCTTG	GCAACGTGCTTCTTCTTGAACG	138
Cyp3a11	NM_007818	CGCCTCTCCTTGCTGTCACA	CTTTGCCTTCTGCCTCAAGT	260
Cyp3a13	NM_007819	CCTCTGCCTTTCTTGGGGACGAT	CCGCCGGTTTGTGAAGGTAGAGT	192
Cyp3a16	NM_007820	CACACATCTGGAGGGAGAAC	GACCAATGTATCCAGTGAGAGC	93
Cyp3a25	NM_019792	CAAGCACTTCCATTTCCCTC	CTTATTGGGCAGAGTTCTGTC	98
Cyp3a41	NM_017396	GCACACTTTCCTTCACCCTGT	GGTGCCTTATTGGGCAGAGT	97
Cyp3a44	NM_177380	CTGAGCTTTCTCAGTGTCTGTG	GATCCCATGAGAAACGGTGAAG	102
AhR	NM_013464	CCTCATCCAGCAGGATGAG	TTGCTCACGGAGCCCATG	90
CAR	NM_009803	CCCTGACAGACCCGGAGTTA	GCCGAGACTGTTGTTCCATAAT	101
FXR	NM_009108	GCTTGATGTGCTACAAAAGCTG	CGTGGTGATGGTTGAATGTCC	110
PXR	NM_010936	GATGGAGGTCTTCAAATCTGCC	GGCCCTTCTGAAAAACCCCT	102
Oatp2	NM_030687	CTGGCTATCCCTGACTGAG	GAGGGTGCTGAACTCCTTC	110
Oct1	NM_009202	CGTATGGGAGCCACCATTG	GGCAGAGCACACCATCATC	99
Ntcp	NM_011387	GTGCTTTCCTTCTGGGCAAG	CAGGGTGAAGAGGTTAGACAG	107
Mrp3	NM_029600	GGTACTCCTGCTCGAAGAC	CTTGCGGACCTCGTAGATG	79
PAPSS2	NM_011864	GTGCAACAGGTGGTGGAAC	GCCTCAGCTCGGATTTGATC	116
Sult1d1	NM_016771	CAGGAGGGAGTTAGTGGATG	GGCCGGGCTTCAAATGAC	85
18S rRNA	X00686	GTCTGTGATGCCCTTAGATG	AGCTTATGACCCGCACTTAC	177

iQ™ Single Color Real-time RT-PCR Detection System (Bio-Rad Laboratories).

As target genes, CYP isozymes belonging to the Cyp3a subfamily (Cyp3a11, Cyp3a13, Cyp3a16, Cyp3a25, Cyp3a41 and Cyp3a44) and, of the isozymes belonging to the Cyp1 and 2 families, those having mRNA expression in the livers of SPF mice at least twice that of GF mice (Cyp1a2, Cyp2a4, Cyp2b9, Cyp2b13 and Cyp2c37) were selected. Furthermore, nuclear transcription factors involved in the regulation of CYP expression (aryl hydrocarbon receptor (AhR), CAR, FXR and PXR) and PXR target genes (Oatp2, Oct1, Ntcp, PAPSS2, Sult1d1 and Mrp3) were also investigated.

Western blotting

Microsome preparation

About 70 mg of frozen liver were homogenized using dissecting buffer (0.3 M sucrose, 25 mM imidazole, 1 mM EDTA, pH 7.2, containing 8.5 μM leupeptin, and 1 mM phenylmethylsulfonyl fluoride). The resulting suspension was centrifuged for 15 min at 9000g, and the supernatant was centrifuged for 1 h at 105 000g. The precipitate was resuspended using dissecting buffer. All procedures were carried out at 4°C. Protein concentrations were measured by the Lowry method (Lowry et al. 1951) using bovine serum albumin as a standard.

Immunoblot analysis (Levert et al. 2002)

Electrophoresis was performed by Laemmli's method (Laemmli 1970). Using the loading buffer (84 mM Tris, 20% glycerol, 0.004% bromophenol blue, pH 6.3, 4.6% SDS, and 10% 2-mercaptoethanol), 0.6 μg of microsomal protein were diluted two-fold, boiled for 5 min and applied to 7.5% polyacrylamide gel (ATTO Corp., Tokyo, Japan). After electrophoresis, the isolated proteins were transferred to a PVDF membrane (ATTO Corp.) using CompactBLOT (AE-7500, ATTO Corp.). After blocking for 1 h using 0.3% skim milk, the resulting membrane was reacted for 1 h at room temperature with anti-rat CYP3A2 (1:2000). After washing the membrane using TBS-TWEEN (Tris-HCl 20 mM, NaCl 137 mM and Tween 20 0.1%, pH 7.6), the resulting membrane was reacted for 1 h at room temperature with anti-rabbit IgG (whole molecule) peroxidase conjugate (1:10 000). After washing the membrane, the membrane was reacted with the ECLplus detection reagent and visualized with LAS-3000 mini (Fuji Film, Tokyo, Japan), a luminoimage analyser.

Triazolam metabolism (Perloff et al. 2000)

After pre-incubation for 15 min at 37°C, various concentrations of triazolam solution (final concentration = 0–750 μM), liver microsome suspension (final concentration = 0.27 mg ml^{-1}) and NADPH-regeneration system solution, 50, 47 and 3 μl, respectively, were placed in an Eppendorf tube and incubated for 30 min at 37°C. By adding 200 μl of acetonitrile and cooling with ice, the reaction was stopped, and deproteinization was performed. Centrifugation was then performed at 16 000g for 15 min at 4°C, and the supernatant (180 μl) was evaporated under the stream of nitrogen. The residue was dissolved in 60 μl of the mobile phase, and HPLC-UV was used to quantify 4-hydroxytriazolam and α-hydroxytriazolam. The initial linear rate conditions have been confirmed with regards to microsomal protein concentration (up to 0.5 mg ml^{-1}) and incubation time (up to 40 min).

HPLC

The HPLC apparatus consisted of the Waters 2695 Separation Module (Waters) and Waters 2489 UV/Visible Detector (Waters), and measured data were recorded and analysed using the analysis software Empower (Waters). Inertsil C18 ODS-3 was used as a column (mean particle size = 5 μm, 4.6 × 250 mm, GL Sciences, Inc.). As a mobile phase, acetonitrile:methanol:10 mM potassium phosphate buffer (4:7:9; pH 7.4) was used. Flow rate was 1.0 ml min^{-1}, temperature was 40°C and detection wavelength was 220 nm (Perloff et al. 2000). The detection limit for 4-hydroxytriazolam and α-hydroxytriazolam was 0.458 μM and 0.434 μM, respectively, and the retention time for 4-hydroxytriazolam, α-hydroxytriazolam and triazolam was 11 min, 10 min and 13 min, respectively.

Kinetics analysis of triazolam metabolism

The relationship between triazolam concentration and metabolic rate was fitted to the Michaelis–Menten equation (4-hydroxylation) or the Michaelis–Menten equation with substrate inhibition (α-hydroxylation) (Perloff et al. 2000) using the non-linear least-squares regression program MULTI (Yamaoka et al. 1981) in order to calculate kinetic parameters (maximum velocity: V_{max}; Michaelis constant: K_m; and substrate inhibition constant: K_s).

Furthermore, for each metabolic pathway, the intrinsic clearance ($CL_{int} = V_{max}/K_m$) was calculated, and the sum of CL_{int} for both metabolic pathways was calculated.

Statistical analysis

Numerical data were expressed as means ± standard deviation (SD). A Student's *t*-test was used for assessing statistical significance.

Results

CYP expression in liver

Livers were dissected from SPF mice (9-week-old male IQI mice) and GF mice (9-week-old male IQI mice) for which faeces were shown to be free of intestinal flora, and RNA was prepared in a conventional manner and was analysed by GeneChip assay and real-time RT-PCR. On GeneChip analysis, of the CYP isozymes belonging to the Cyp1-3 family and showing a certain level of expression, the mRNA expression of eight isozymes was at least twice as high in the SPF mice when compared with the GF mice. Cyp3a13 and 25 also showed higher expression in SPF mice though the difference was less than two-fold. For Cyp3a11, GeneChip analysis resulted in a signal intensity of above the detectable limit in both GF and SPF mice (Figure 1).

On real-time RT-PCR, when compared with the GF mice, the mRNA of the eight CYP isozymes (Cyp1a2, Cyp2a4, Cyp2b9, Cyp2b13, Cyp2c37, Cyp3a16, Cyp3a41 and Cyp3a44) having equal to or more than two-fold differences between the two groups as assessed by GeneChip analysis was significantly higher in SPF mice and the mRNA of Cyp3a11 and Cyp3a25 was also significantly higher in SPF mice when compared with GF mice. While no significant differences were seen for Cyp3a13, it tended to be high in SPF mice (Figure 2). Therefore, the results of GeneChip analysis and those of PCR largely corresponded except for Cyp3a11, which was over-expressed.

Expression of Cyp3a at the protein level

From the livers of GF and SPF mice, the microsome fraction was prepared and subjected to Western blotting. Using polyclonal antibodies for rat CYP3A2 and HRP-labelled secondary antibody, Cyp3a protein was detected by ECL, and two bands for Cyp3a were detected near 52 and 50 kDa. It has been known that the isozymes

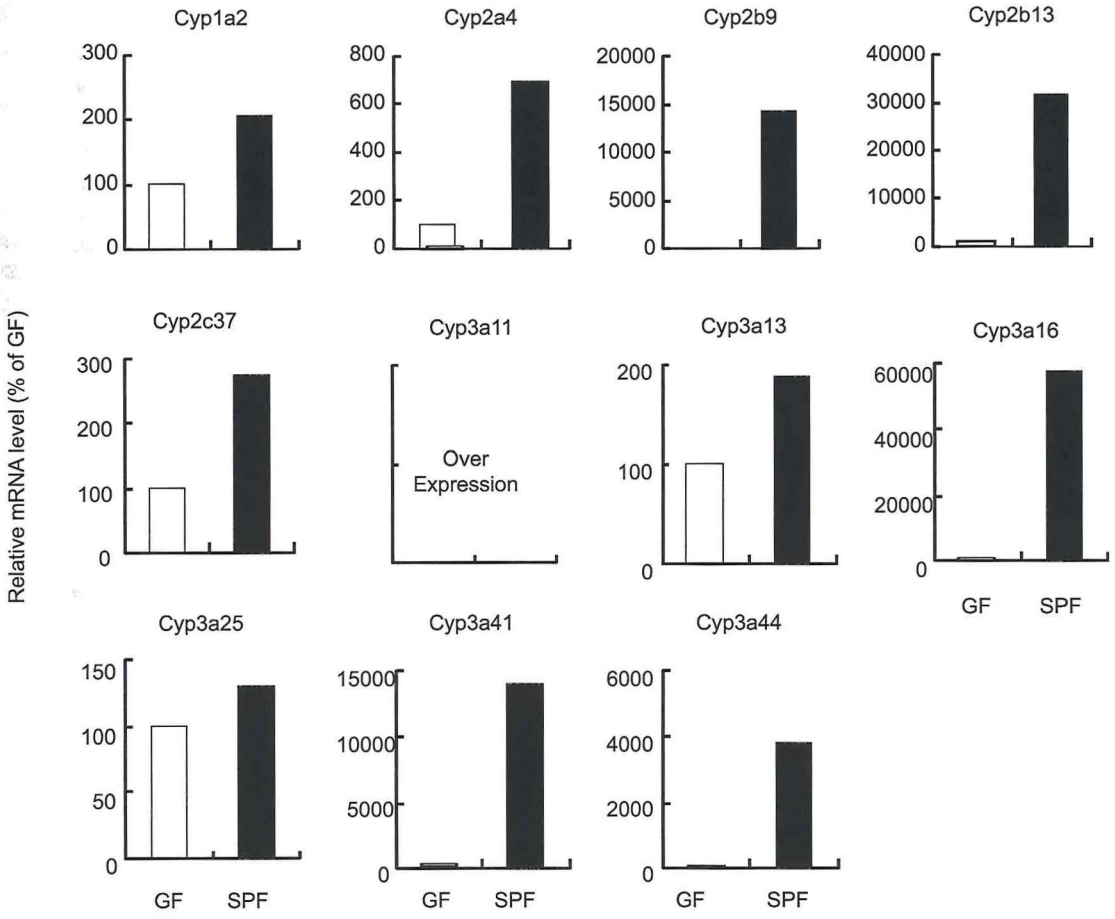

Figure 1. Cytochrome P450 (CYP) mRNA expression in the livers of germ-free (GF) and specific pathogen-free (SPF) mice analysed by GeneChip. RNA was extracted from the livers of GF (open columns, □) and SPF (closed columns, ■) mice, and the mRNA expression of the isozymes belonging to the Cyp1, Cyp2 and Cyp3 subfamilies was investigated by GeneChip analysis as described in the Materials and Methods section. The expression of each gene was compared in relation with GF mice (100%) ($n = 1$).

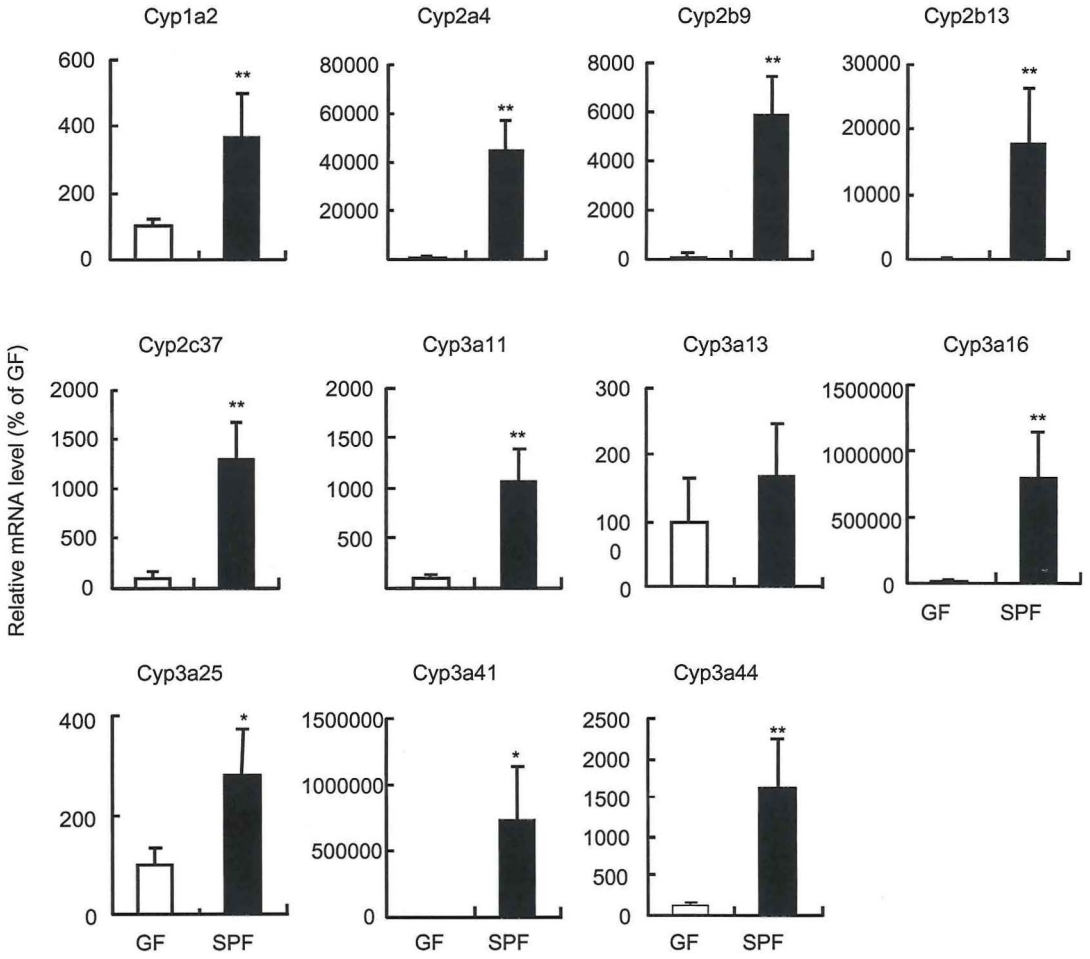

Figure 2. Cytochrome P450 (CYP) mRNA expression in the livers of germ-free (GF) and specific pathogen-free (SPF) mice analysed by real-time reverse transcriptase-polymerase chain reaction (RT-PCR). RNA was extracted from the livers of GF (open columns, □) and SPF (closed columns, ■) mice, and the mRNA expression of the isozymes belonging to the Cyp1, Cyp2 and Cyp3 subfamilies was investigated by PCR as described in the Materials and Methods section. Expression of each gene was corrected against 18S rRNA and compared in relation with the mean value in GF mice (100%). Values are the means ± standard deviation (SD), $n = 5$, $^{*}p < 0.05$, $^{**}p < 0.01$. Student's *t*-test.

of Cyp3a cannot be discriminated and are shown as two broad bands (Emoto et al. 2000). Therefore, the total concentration of the two bands was measured, and when compared with the GF mice, the total concentration for SPF mice was significantly higher (Figure 3). This confirmed that the expression of Cyp3a in the liver at the protein level in SPF mice was higher when compared with GF mice.

Triazolam metabolic activity in the liver

In order to compare Cyp3a activities between GF and SPF mice, the metabolic activity of triazolam (Cyp3a substrate) was investigated using the hepatic microsomes. When compared with GF mice, the Vmax for α-hydroxylation (α-OH) and 4-hydroxylation (4-OH) in SPF mice were higher. The Km for 4-OH in SPF mice was higher when compared with GF mice. With regard to α-OH, weak substrate inhibition was observed in both groups (GF: Ks = 3510 ± 1430 μM, and SPF: Ks = 21181 ± 24675 μM). The CLint for α-OH in SPF mice was higher when compared with GF mice. Total CLint for α-OH and 4-OH in SPF mice (17.8 ± 1.7 μl $min^{-1} mg^{-1}$ protein) was significantly higher than that in GF mice (14.0 ± 0.6 μl $min^{-1} mg^{-1}$ protein) (Figure 4).

CYP mRNA expression in the small intestine

From the small intestine of the GF and SPF mice, RNA was prepared, and CYP expression was analysed by GeneChip analysis and real-time RT-PCR. The results of GeneChip analysis showed no marked differences in the isozymes belonging to the Cyp1-3 family in the small intestine between GF and SPF mice (data not shown).

PCR was used to analyse the Cyp3a isozymes in the small intestine, but no significant differences were seen in the mRNA expression of any isozymes in the small intestine between GF and SPF mice (Figure 5).

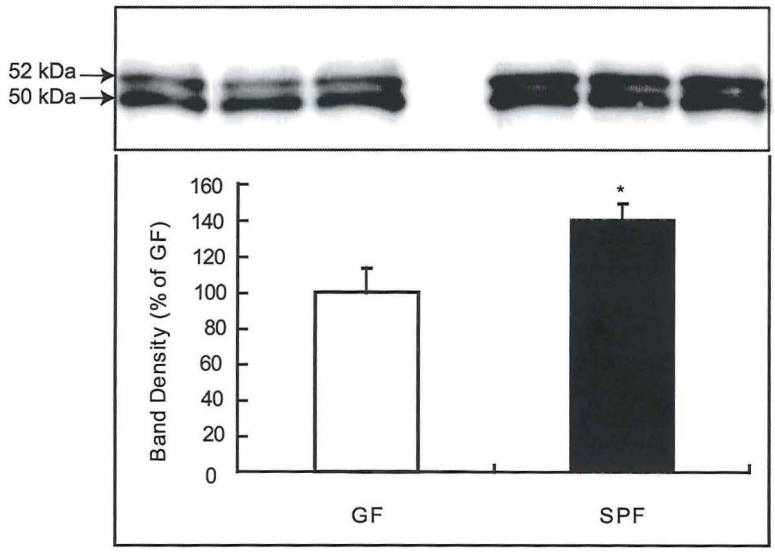

Figure 3. Expression of Cyp3a protein in the livers of germ-free (GF) and specific pathogen-free (SPF) mice. After extracting the microsome fraction from the livers of GF (open columns, □) and SPF (closed columns, ■) mice, Western blotting was performed using the rat polyclonal antibody for CYP3A2. The total concentrations of the two bands were compared in relation with the mean value in GF mice (100%). Values are the means ± standard deviation (SD), $n = 3$, $^*p < 0.05$. Student's t-test.

Nuclear transcription factor mRNA expression in liver

From the livers of GF and SPF mice, RNA was prepared, and the expression of nuclear transcription factors regulating the expression of CYP isozymes that showed intergroup differences was analysed by real-time RT-PCR. The results showed that the mRNA expression of CYP transcription regulators, AhR, CAR, FXR and PXR, in the livers of SPF mice was significantly higher when compared with GF mice (Figure 6). In particular, the expression of CAR in SPF mice was about five times higher than that in GF mice.

PXR target gene mRNA expression in liver

The expression of transporters and conjugation enzymes involved in the detoxification of lithocholic acid that is specifically produced by intestinal flora was investigated at the mRNA level. The results of real-time RT-PCR showed that the mRNA levels of PXR target genes, Oatp2, Oct1, Ntcp, PAPSS2, Sult1d1 and Mrp3, were significantly higher in the livers of SPF mice compared with GF mice (Figure 7).

Discussion

Drug-metabolizing enzymes and transporters are known to change due to disease, age and drugs, but there have been no reports documenting the changes due to intestinal flora. The authors hypothesized that intestinal flora is involved in the changes in enzymes and transporters, thus contributing to the individual differences in pharmacokinetics, and this issue was investigated using germ-free (GF) mice.

In the present study, when compared with the livers of GF mice, the mRNA expressions of Cyp1a2, Cyp2a4, Cyp2b9, Cyp2b13, Cyp2c37, Cyp3a11, Cyp3a13, Cyp3a16, Cyp3a25, Cyp3a41 and Cyp3a44 were higher for the livers of specific pathogen-free (SPF) mice. Cyp3a is the most important enzyme for drug metabolism, and its expression at the protein level was significantly higher in SPF mice. Although the difference in the Cyp3a protein level was relatively small (about 1.4-fold), the biological significance of the difference was confirmed by comparing the metabolic activity of triazolam, a mouse Cyp3a substrate (Perloff et al. 2000), which showed a significantly higher value in the SPF mice compared with GF mice. This was because the V_{max} values for both 4-hydroxylation and α-hydroxylation were greater in SPF mice, which reflected the difference in Cyp3a expression. Although the reason is unknown for the difference in K_m for 4-hydroxylation between the GF and SPF mice, this could possibly reflect the difference in the ratio of Cyp3a subfamily members expressed in the livers of these mice. Next, nuclear transcription factors related to the expression of CYP isozymes exhibiting expression differences between the livers of GF and SPF mice were investigated. The results showed that the expression of aryl hydrocarbon receptor (AhR), constitutive androstane receptor (CAR), farnesoid X receptor (FXR) and pregnane X receptor (PXR) in the livers of SPF mice were higher than in GF mice.

The results of previous studies have shown that increased level or activity of FXR leads to increased

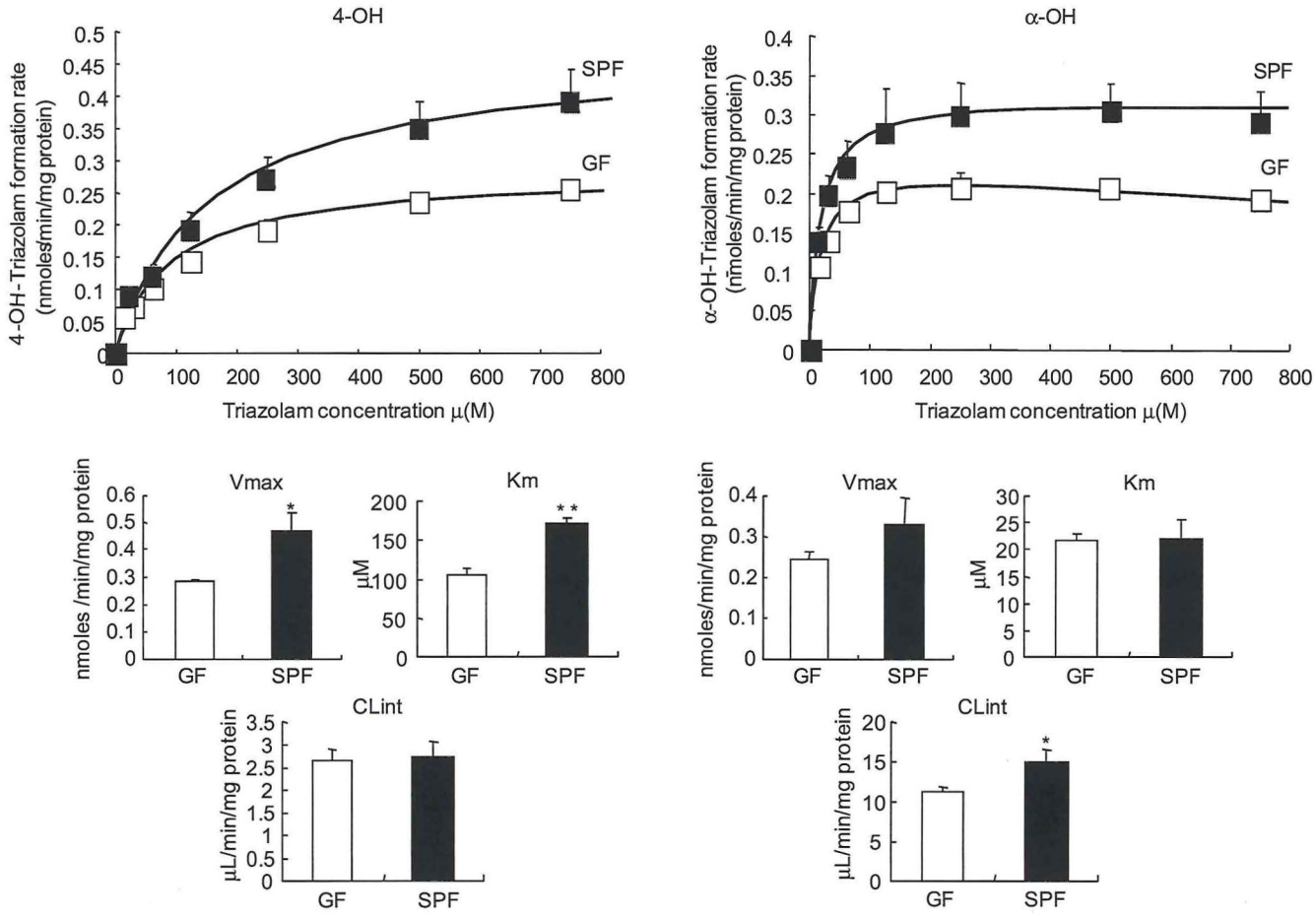

Figure 4. Metabolic activity of triazolam in the livers of germ-free (GF) and specific pathogen-free (SPF) mice. The microsome fraction was extracted from the livers of GF and SPF mice in order to measure the α-hydroxylation (α-OH) and 4-hydroxylation (4-OH) activities of triazolam. Values are the means ± standard deviation (SD), $n = 3$, $^{*}p < 0.05$. $^{**}p < 0.01$. Student's *t*-test.

PXR level (Jung et al. 2006), increased level or activity of PXR leads to increased CAR level (Maglich et al. 2002; Lim and Huang 2008), and increased level or activity of CAR leads to increased AhR level (Maglich et al. 2002; Lim and Huang 2008). Studies have also shown that PXR activation increases Cyp1a2, Cyp3a11 and Cyp3a25 (Lee et al. 1996; Xie et al. 2001), CAR activation increases Cyp2a4, Cyp2b9, Cyp2c37 and Cyp3a11 (Hernandez et al. 2006; Jackson et al. 2006), and AhR activation increases Cyp1a2 (Lee et al. 1996) (Figure 8). Furthermore, recent studies have documented very interesting findings: a secondary bile acid lithocholic acid (LCA), which is produced only by intestinal flora and is detected in the faeces from conventional rats but not from GF rats (Madsen et al. 1976), is a potent ligand for FXR and PXR, and increases the expression of PXR, which elevates the expression of Cyp3a in the liver (Staudinger et al. 2001;] Xie et al. 2004; Zhang et al. 2004). Other studies have reported that LCA induces the expression of transporters that transport LCA from the portal vein to the liver [organic anion-transporting polypeptide 2 (Oatp2), organic cation transporter 1 (Oct1), and sodium taurocholate-transporting polypeptide (Ntcp)] (Müller and Jansen 1997; Staudinger et al. 2001; Teng and Piquette-Miller 2007); LCA induces LCA conjugating enzymes [3′-phosphoadenosine 5′-phosphosulfate synthase 2 (PAPSS2) and sulfotransferase family 1D, member 1 (Sult1d1)] (Sonoda et al. 2002); and LCA induces multidrug resistance-associated protein 3 (Mrp3) (Teng and Piquette-Miller 2007), a transporter that eliminates LCA conjugates from the liver to the hepatic vein (Staudinger et al. 2001;Xie et al. 2001; Sonoda et al. 2002; Kitada et al. 2003). The expression of these transporters is regulated by PXR (Staudinger et al. 2001; Sonoda et al. 2002; Klaassen and Slitt 2005; Teng and Piquette-Miller 2007). To determine the involvement of intestinal flora in the increased expression of

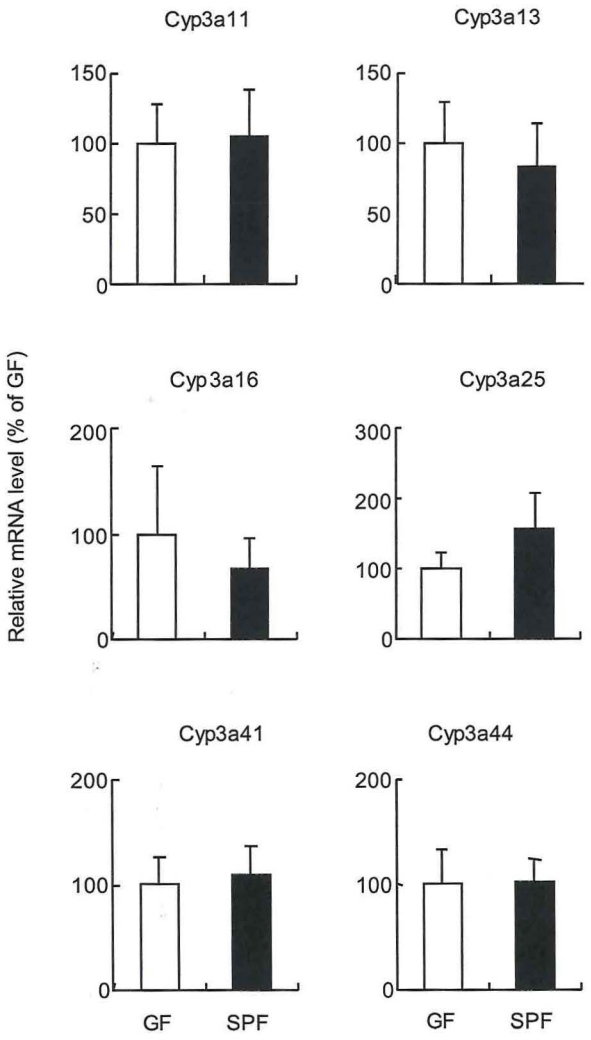

Figure 5. Cyp3a mRNA expression in the small intestines of germ-free (GF) and specific pathogen-free (SPF) mice. RNA was extracted from the small intestines of GF (open columns, □) and SPF (closed columns, ■) mice, and the mRNA expression of the isozymes belonging to the Cyp3a subfamily was measured by polymerase chain reaction (PCR) as described in the Materials and Methods section. The expression of each gene was corrected against 18S rRNA and was compared in relation with the mean value of GF mice (100%). Values are the means ± standard deviation (SD), $n = 5$.

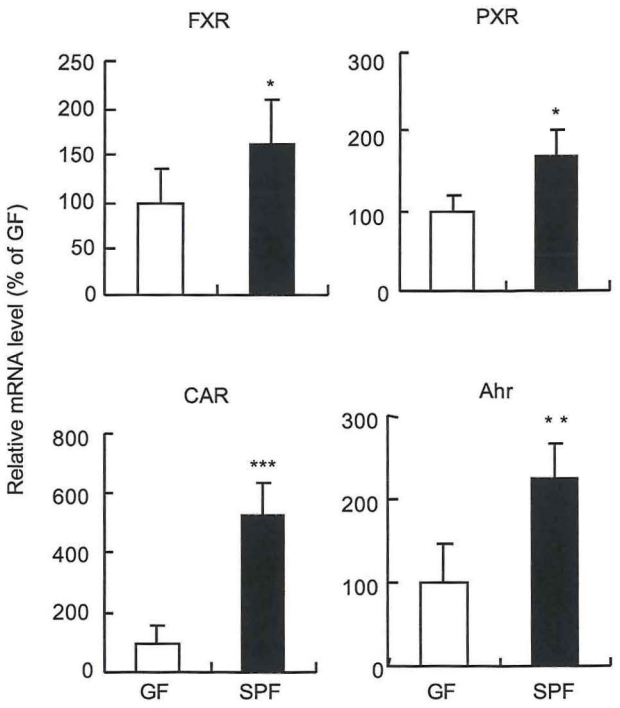

Figure 6. Nuclear transcription factor mRNA expression in the livers of germ-free (GF) and specific pathogen-free (SPF) mice. RNA was extracted from the livers of GF (open columns, □) and SPF (closed columns, ■) mice, and the mRNA expression of nuclear transcription factors was measured by polymerase chain reaction (PCR) as described in the Materials and Methods section. The expression of each gene was corrected against 18S rRNA and was compared in relation with the mean value of GF mice (100%). Values are the means ± standard deviation (SD), $n = 5$, $^{*}p < 0.05$ and $^{***}p < 0.001$. Student's *t*-test.

FXR, PXR, CAR and AhR, the authors focused on LCA, which is specifically produced by intestinal flora, and then the expression of transporters and conjugation enzymes involved in LCA detoxification was measured at the mRNA level. The results clarified that expression of Oatp2, Oct1, Ntcp, PAPSS2, Sult1d1 and Mrp3 was increased in the livers of SPF mice (Figure 7). The expression of CAR, which is directly regulated by PXR, was also markedly elevated. These findings indicate that LCA, which is produced by intestinal flora, activates FXR and PXR to increase the expression of Cyp1a2, Cyp2a4, Cyp2b9, Cyp2b13, Cyp2c37, Cyp3a11, Cyp3a13, Cyp3a16, Cyp3a25, Cyp3a41 and Cyp3a44 in the livers of SPF mice.

Studies have reported the possibility that the nuclear transcription factors that exhibited expression changes in the present study (AhR, CAR, FXR and PXR) act as xenobiotics sensors and protect the body from toxicity and damage due to xenobiotics and toxic substances by regulating the expression of various genes, including CYP and transporters (Staudinger et al. 2001; Xie et al. 2004; Zhang et al. 2004). LCA is a factor for bile stasis and it damages the liver due to its high toxicity (Radominska et al. 1993). In SPF mice, therefore, it is possible that the levels of Cyp3a, transporters and conjugation enzymes involved in LCA disposition are elevated to detoxify more efficiently LCA in the liver. Thus, the changes in the expression of nuclear transcription factors seen in the present study might represent part of the bodily defences against toxic substances, such as LCA, produced by intestinal flora.

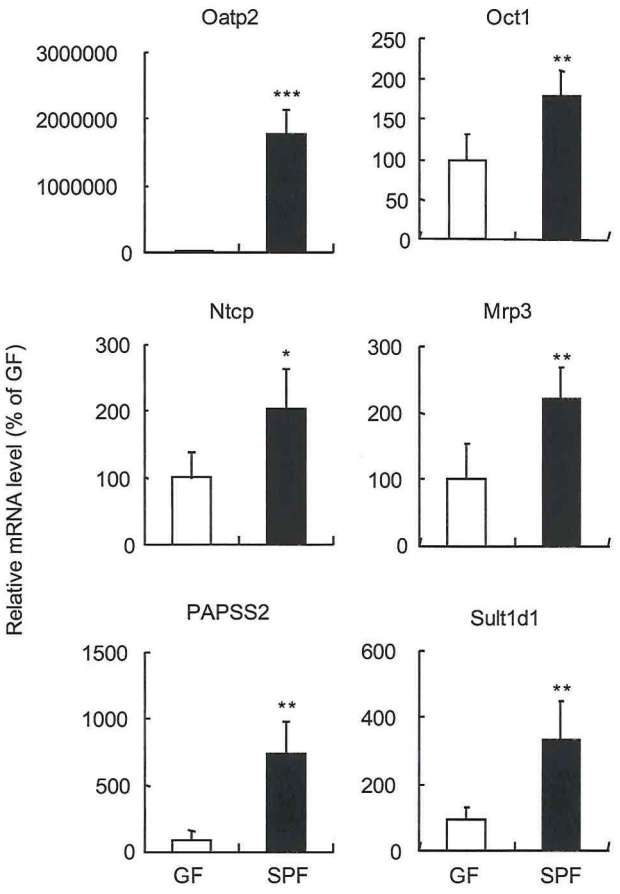

Figure 7. Pregnane X receptor (PXR) target gene mRNA expression in the livers of germ-free (GF) and specific pathogen-free (SPF) mice. RNA was extracted from the livers of GF (open columns, □) and SPF (closed columns, ■) mice, and the mRNA expression of PXR target genes was measured by polymerase chain reaction (PCR) as described in the Materials and Methods section. The expression of each gene was corrected against 18S rRNA and was compared in relation with the mean value of GF mice (100%). Values are the means ± standard deviation (SD), $n = 5$, $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$. Student's *t*-test.

The reason for the lack of difference in the expression of Cyp3a in the small intestine between GF and SPF mice may be that the regulation mechanisms of Cyp3a expression differ between the liver and small intestine. While PXR is closely involved in the expression of Cyp3a in the liver, the involvement of vitamin D receptor (VDR), which has vitamin D_3 as a ligand is very high in the small intestine (Fukumori et al. 2007). In the present study, both GF and SPF mice were fed the same food, and no differences were seen in food intake between the two groups. It is possible that the food contained excessive amounts of vitamin D_3, which played a large role in the regulation of Cyp3a expression in the small intestine, masking the effects of LCA.

LCA is produced from chenodeoxycholic acid by 7α-dehydroxylase in *Clostridium* or *Bacteroides* (Ridlon et al. 2006). Because ciprofloxacin is known to reduce these bacteria (Chin and Neu 1984), it is possible that the down-regulation of CYP2C and CYP3A by ciprofloxacin in rats (Xie et al. 2003) is attributed to LCA reduction due to its bactericidal effect. This suggests that the administration of other antibacterial agents, having similar bactericidal effects, could also lower the expression of CYP isozymes. Furthermore, the expression of CYP3A4, a major drug metabolizing enzyme in the human liver, is also regulated by PXR, which shows higher activation by LCA compared with the mouse PXR (Staudinger et al. 2001). With regard to the composition of human intestinal flora, LCA-producing *Clostridium* and *Bacteroides* are also dominant bacterial strains (Mitsuoka and Ono 1977), suggesting that administration of antibiotics such as ciprofloxacin can cause a decrease in the LCA level in human livers that may lower CYP3A4 expression.

Not only drugs such as antibiotics, but also stress-related diseases, inflammatory bowel disease and age have been shown to alter intestinal flora. The results of the present study suggest that the changes in intestinal flora alter CYP, thus suggesting that the changes in intestinal flora are one of the causes of individual differences in pharmacokinetics.

Acknowledgements

***Declaration of interest*:** The authors report no conflicts of interest.

References

Bailey MT, Coe CL. 1999. Maternal separation disrupts the integrity of the intestinal microflora in infant rhesus monkeys. *Dev Psychobiol* 35:146-155.

Chin NX, Neu HC. 1984. Ciprofloxacin, a quinolone carboxylic acid compound active against aerobic and anaerobic bacteria. *Antimicrob Agents Chemother* 25:319-326.

Choudhary D, Jansson I, Schenkman JB, Sarfarazi M, Stoilov I. 2003. Comparative expression profiling of 40 mouse cytochrome P450 genes in embryonic and adult tissues. *Arch Biochem Biophys* 414:91-100.

Emoto C, Yamazaki H, Yamasaki S, Shimada N, Nakajima M, Yokoi T. 2000. Characterization of cytochrome P450 enzymes involved in drug oxidations in mouse intestinal microsomes. *Xenobiotica* 30:943-953.

Fukumori S, Murata T, Taguchi M, Hashimoto Y. 2007. Rapid and drastic induction of CYP3A4 mRNA expression via vitamin D receptor in human intestinal LS180 cells. *Drug Metab Pharmacokinet* 22:377-381.

Hayashi H, Sakamoto M, Benno Y. 2002. Fecal microbial diversity in a strict vegetarian as determined by molecular analysis and cultivation. *Microbiol Immunol* 46:819-831.

Hernandez JP, Chapman LM, Kretschmer XC, Baldwin WS. 2006. Gender-specific induction of cytochrome P450s in

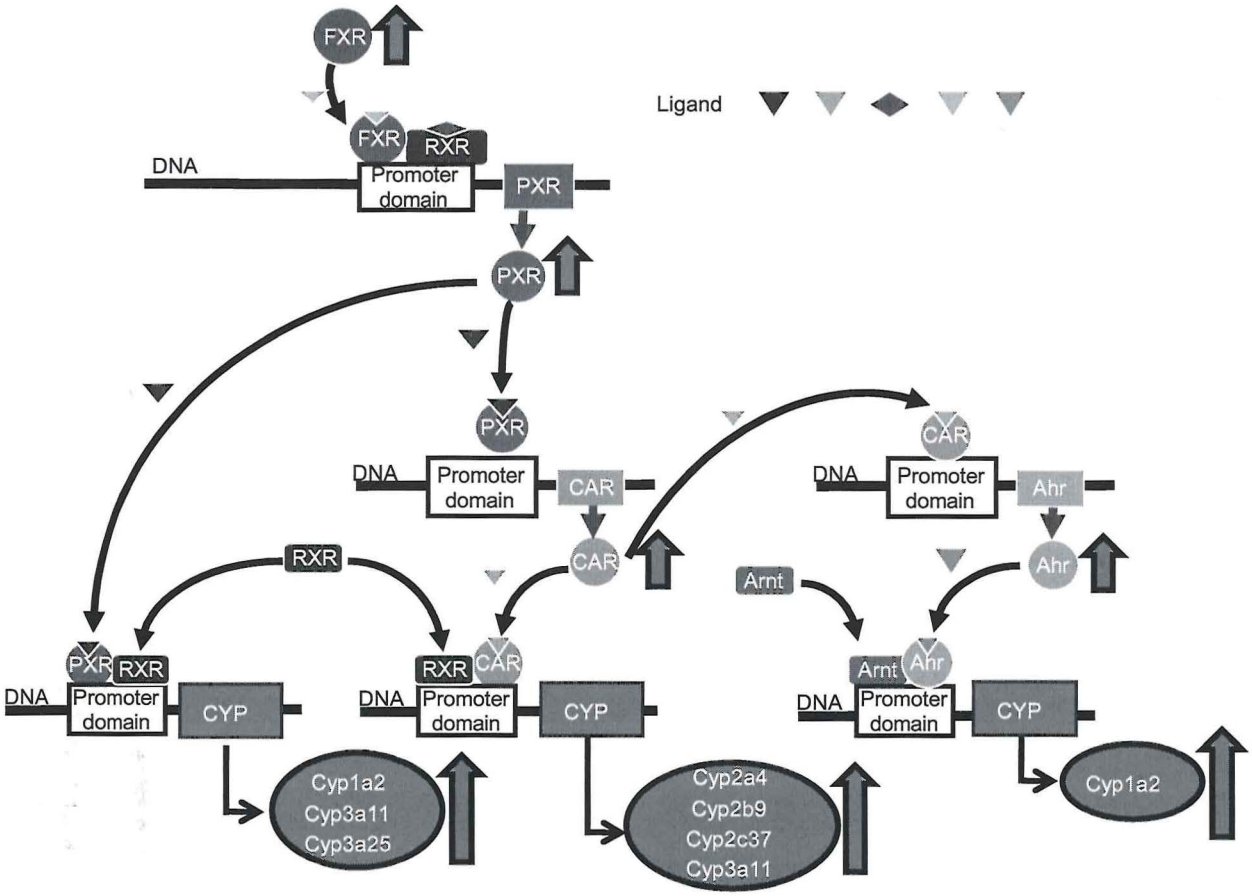

Figure 8. Expression regulation mechanism of various cytochrome P450 (CYP) isozymes. When activated by endogenous or exogenous ligands, aryl hydrocarbon receptor (AhR), constitutive androstane receptor (CAR) and pregnane X receptor (PXR) migrate inside the nucleus and form heterodimers with either aryl hydrocarbon receptor nuclear translocator (Arnt) or retinoid X receptor (RXR), and when such heterodimers bind to the promoter domain upstream of CYP DNA, CYP transcription is elevated. The expression of AhR is elevated by activation of CAR bound to its promoter domain. The expression of CAR is elevated by the activation of PXR bound to its promoter domain. Moreover, the expression of PXR is elevated by activation of FXR.

nonylphenol-treated FVB/NJ mice. *Toxicol Appl Pharmacol* 216:186–196.

Ikeda M, Ohira H, Toyama Y, Katagiri T, Sakakibara B. 2007. Effect of intestinal microflora on the production of interleukin 10 and prostaglandin e(2) in serum and Kupffer cells from germfree and conventional mice. *J Clin Biochem Nutr* 41:169–174.

Ishizaki T, Horai Y. 1999. Review article: cytochrome P450 and the metabolism of proton pump inhibitors—emphasis on rabeprazole. *Aliment Pharmacol Ther* 13:27–36.

Itoh S, Satoh M, Abe Y, Hashimoto H, Yanagimoto T, Kamataki T. 1994. A novel form of mouse cytochrome P450 3A (Cyp3a-16). Its cDNA cloning and expression in fetal liver. *Eur J Biochem* 226:877–882.

Jackson JP, Ferguson SS, Negishi M, Goldstein JA. 2006. Phenytoin induction of the cyp2c37 gene is mediated by the constitutive androstane receptor. *Drug Metab Dispos* 34:2003–2010.

Jung D, Mangelsdorf DJ, Meyer UA. 2006. Pregnane X receptor is a target of farnesoid X receptor. *J Biol Chem* 281:19081–19091.

Kitada H, Miyata M, Nakamura T, Tozawa A, Honma W, Shimada M, Nagata K, Sinal CJ, Guo GL, Gonzalez FJ, Yamazoe Y. 2003. Protective role of hydroxysteroid sulfotransferase in lithocholic acid-induced liver toxicity. *J Biol Chem* 278:17838–17844.

Klaassen CD, Slitt AL. 2005. Regulation of hepatic transporters by xenobiotic receptors. *Curr Drug Metab* 6:309–328.

Laemmli UK. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 277:680–685.

Lee IJ, Jeong KS, Roberts BJ, Kallarakal AT, Fernandez-Salguero P, Gonzalez FJ, Song BJ. 1996. Transcriptional induction of the cytochrome P4501A1 gene by a thiazolium compound, YH439. *Mol Pharmacol* 49:980–988.

Levert KL, Waldrop GL, Stephens JM. 2002. A biotin analog inhibits acetyl-CoA carboxylase activity and adipogenesis. *J Biol Chem* 277:16347–16350.

Lim YP, Huang JD. 2008. Interplay of pregnane X receptor with other nuclear receptors on gene regulation. *Drug Metab Pharmacokinet* 23:14–21.

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. 1951. Protein measurement with the Folin phenol reagent. *J Biol Chem* 193:265–275.

Madsen D, Beaver M, Chang L, Bruckner-Kardoss E, Wostmann B. 1976. Analysis of bile acids in conventional and germfree rats. *J Lipid Res* 17:107–111.

Maejima K, Nomura T. 1975. An experience of application of sterility test of germfree mice and rats recommended by JEARA. *Jikken Dobutsu* 24:177–181. [in Japanese]

Maglich JM, Stoltz CM, Goodwin B, Hawkins-Brown D, Moore JT, Kliewer SA. 2002. Nuclear pregnane x receptor and constitutive androstane receptor regulate overlapping but distinct sets

of genes involved in xenobiotic detoxification. *Mol Pharmacol* 62:638–646.

Masubuchi Y, Horie T. 2004. Endotoxin-mediated disturbance of hepatic cytochrome P450 function and development of endotoxin tolerance in the rat model of dextran sulfate sodium-induced experimental colitis. *Drug Metab Dispos* 32:437–441.

Mitsuoka T, Ono K. 1977. Fecal flora of man. V. Communication: the fluctuation of the fecal flora of the healthy adult. *Zentralbl Bakteriol [Orig A]* 238:228–236.

Morishita Y, Ogata M. 1970. Studies on the alimentary flora of pig. V. Influence of starvation on the microbial flora. *Nippon Juigaku Zasshi* 32:19–24.

Müller M, Jansen PL. 1997. Molecular aspects of hepatobiliary transport. *Am J Physiol* 272:G1285–G1303.

Neut C, Bulois P, Desreumaux P, Membré JM, Lederman E, Gambiez L, Cortot A, Quandalle P, Van Kruiningen H, Colombel JF. 2002. Changes in the bacterial flora of the neoterminal ileum after ileocolonic resection for Crohn's disease. *Am J Gastroenterol* 97:939–946.

Okayasu I, Hatakeyama S, Yamada M, Ohkusa T, Inagaki Y, Nakaya R. 1990. A novel method in the induction of reliable experimental acute and chronic ulcerative colitis in mice. *Gastroenterology* 98:694–702.

Perloff MD, Von Moltke LL, Court MH, Kotegawa T, Shader RI, Greenblatt DJ. 2000. Midazolam and triazolam biotransformation in mouse and human liver microsomes: relative contribution of CYP3A and CYP2C isoforms. *J Pharmacol Exp Ther* 292:618–628.

Pichard L, Fabre I, Fabre G, Domergue J, Saint Aubert B, Mourad G, Maurel P. 1990. Cyclosporin A drug interactions. Screening for inducers and inhibitors of cytochrome P-450 (cyclosporin A oxidase) in primary cultures of human hepatocytes and in liver microsomes. *Drug Metab Dispos* 18:595–606.

Radominska A, Treat S, Little J. 1993. Bile acid metabolism and the pathophysiology of cholestasis. *Semin Liver Dis* 13:219–234.

Ridlon JM, Kang DJ, Hylemon PB. 2006. Bile salt biotransformations by human intestinal bacteria. *J Lipid Res* 47:241–259.

Sakuma T, Takai M, Endo Y, Kuroiwa M, Ohara A, Jarukamjorn K, Honma R, Nemoto N. 2000. A novel female-specific member of the CYP3A gene subfamily in the mouse liver. *Arch Biochem Biophys* 377:153–162.

Savage DC, Dubos R, Schaedler RW. 1968. The gastrointestinal epithelium and its autochthonous bacterial flora. *J Exp Med* 127:67–76.

Sonoda J, Xie W, Rosenfeld JM, Barwick JL, Guzelian PS, Evans RM. 2002. Regulation of a xenobiotic sulfonation cascade by nuclear pregnane X receptor (PXR). *Proc Natl Acad Sci USA* 99:13801–13806.

Staudinger JL, Goodwin B, Jones SA, Hawkins-Brown D, MacKenzie KI, LaTour A, Liu Y, Klaassen CD, Brown KK, Reinhard J, Willson TM, Koller BH, Kliewer SA. 2001. The nuclear receptor PXR is a lithocholic acid sensor that protects against liver toxicity. *Proc Natl Acad Sci USA* 98:3369–3374.

Stevens JC, Hines RN, Gu C, Koukouritaki SB, Manro JR, Tandler PJ, Zaya MJ. 2003. Developmental expression of the major human hepatic CYP3A enzymes. *J Pharmacol Exp Ther* 307:573–582.

Suzuki K, Harasawa R, Yoshitake Y, Mitsuoka T. 1983. Effects of crowding and heat stress on intestinal flora, body weight gain, feed efficiency of growing rats and chicks. *Nippon Juigaku Zasshi* 45:331–338.

Teng S, Piquette-Miller M. 2007. Hepatoprotective role of PXR activation and MRP3 in cholic acid-induced cholestasis. *Br J Pharmacol* 151:367–376.

Xie HJ, Broberg U, Griskevicius L, Lundgren S, Carlens S, Meurling L, Paul C, Rane A, Hassan M. 2003. Alteration of pharmacokinetics of cyclophosphamide and suppression of the cytochrome p450 genes by ciprofloxacin. *Bone Marr Transpl* 31:197–203.

Xie W, Radominska-Pandya A, Shi Y, Simon CM, Nelson MC, Ong ES, Waxman DJ, Evans RM. 2001. An essential role for nuclear receptors SXR/PXR in detoxification of cholestatic bile acids. *Proc Natl Acad Sci USA* 98:3375–3380.

Yamaoka K, Tanigawara Y, Nakagawa T, Uno T. 1981. A pharmacokinetic analysis program (multi) for microcomputer. *J Pharmacobiodyn* 4:879–885.

Yoshinari K, Takagi S, Sugatani J, Miwa M. 2006. Changes in the expression of cytochromes P450 and nuclear receptors in the liver of genetically diabetic db/db mice. *Biol Pharm Bull* 29:1634–1638.

Zhang J, Huang W, Qatanani M, Evans RM, Moore DD. 2004. The constitutive androstane receptor and pregnane X receptor function coordinately to prevent bile acid-induced hepatotoxicity. *J Biol Chem* 279:49517–49522.