Department of Biopharmaceutics[1], Hyogo University of Health Sciences, Kobe, Hyogo; School of Pharmacy and Pharmaceutical Sciences[2], Mukogawa Women's University, Nishinomiya, Hyogo, Japan

Different expression patterns of hepatic cytochrome P450 s during anaphylactic or lipopolysaccharide-induced inflammation

N. Moriya[1], H. Kataoka[2], H. Fujino[1], J. Nishikawa[2], F. Kugawa[1]

Received May 27, 2013, accepted July 7, 2013

Fumihiko Kugawa, Department of Biopharmaceutics1, School of Pharmacy, Hyogo University of Health Sciences, 1-3-6 Minatojima, Chuo-Ku, Kobe, Hyogo 650-8530, Japan
jfkugawa@huhs.ac.jp

Dedicated to Dr. Masanori Sakamoto, Professor Emeritus of Meiji Pharmaceutical University, on the occasion of his 77th birthday (KIJU).

Pharmazie 69: 142–147 (2014) *doi: 10.1691/ph.2014.3725*

Certain physiological states and diseases can alter the expression and activity of cytochrome P450 s (CYPs), which have the potential to cause unexpected adverse effects. We previously demonstrated that lipopolysaccharide (LPS)–induced inflammation attenuates the induction of CYPs by xenobiotics in mouse liver. In this study, to investigate whether anaphylaxis-induced inflammation affects the hepatic CYPs' expression, we examined the effects of ovalbumin (OVA)-induced anaphylaxis on constitutive CYP mRNA and protein expressions. We also compared these effects with those obtained with LPS treatment. In addition, we examined the tumor necrosis factor (TNF) α and interleukin (IL)-1β mRNA levels, because these cytokines are known to be induced by LPS treatment and anaphylactic reactions. LPS treatment decreased the constitutively expressed Cyp1a2, Cyp2c29, and Cyp3a11 mRNAs, and increased the TNFα and IL-1β mRNAs. LPS treatment also decreased the CYP1A2 and CYP3A protein levels. Anaphylaxis, on the other hand, did not change the levels of the constitutively expressed Cyp1a2, Cyp2c29, or Cyp3a11 mRNAs, although it increased the TNFα and IL-1β mRNAs, as observed in the LPS-treated mice. These results suggest that anaphylaxis-induced inflammation had less effect than LPS-induced inflammation on these CYPs in the liver. In contrast, we observed that the expressions of Cyp2b10 mRNA and its protein were quite different from those of the other CYPs in both the anaphylactic and LPS-treated mice. Our findings strongly suggest that the alteration of the constitutive CYPs' expression levels during inflammation varies according to the immunostimulation pathway.

1. Introduction

Several drugs used clinically are known to induce or inhibit cytochrome P450 (CYP) enzymes (Ogu and Maxa 2000). Drug-drug interactions mediated by CYP enzymes can cause plasma drug concentrations to fluctuate, sometimes (in worst-case scenarios) causing death. The mechanisms by which rifampicin, erythromycin, itraconazole, and cimetidine induce or inhibit CYP enzymes have been studied extensively (Pelkonen et al. 1998), and thus it is clear that certain drugs can alter CYP enzyme activity.

Recent reports have shown that some diseases alter drug metabolism (Mann 2006; Morgan 1997, 2001), and patients with these diseases may experience a phenomenon termed "disease-drug interactions" (Kulmatycki and Jamali 2005). This theme, which is increasingly important in the field of molecular metabolism, was addressed in the symposium "Regulation of drug-metabolizing enzymes and transporters in infection, inflammation, and cancer", presented in 2008 by the American Society of Pharmacology and Experimental Therapeutics (Morgan et al. 2008). In this symposium, Morgan et al. reported that CYP gene expression and enzymatic activity can be altered by inflammation caused by bacterial infection or cancer. Such disease-drug interactions are reported to affect CYP expression through transcriptional and/or translational pathways (Aitken et al. 2006; Lee et al. 2008). It is increasingly clear that the effect of diseases themselves on the therapeutic effects of drugs must be taken into account by treating physicians, particularly because CYP fluctuations could unexpectedly cause drug therapies to fail or trigger adverse side effects (Kraemer et al. 1982; Morgan 2009).

Fluctuations in hepatic CYP expression are commonly responses to infection and inflammation in humans, rats, and mice (Iber et al. 1999; Aitken and Morgan 2007; Yang and Lee 2008). Morgan (1989) reported that levels of constitutively expressed CYP genes were downregulated in the rat liver when inflammation was induced with lipopolysaccharide (LPS). In a previous study, we evaluated the effect of LPS-induced inflammation on inducible hepatic *Cyp* gene expression in mice (Moriya et al. 2012). We observed that LPS downregulated the xenobiotic-induced expression of representative *Cyp* genes, and increased the levels of mRNAs for tumor necrosis factor (TNF) α and interleukin (IL)-1β. The changes in these cytokines, which are well-known pro-inflammatory mediators, correlate with the changes in CYP expression and enzymatic activity during infection and inflammation (Li et al. 2008; Warren et al. 1999; Siewert

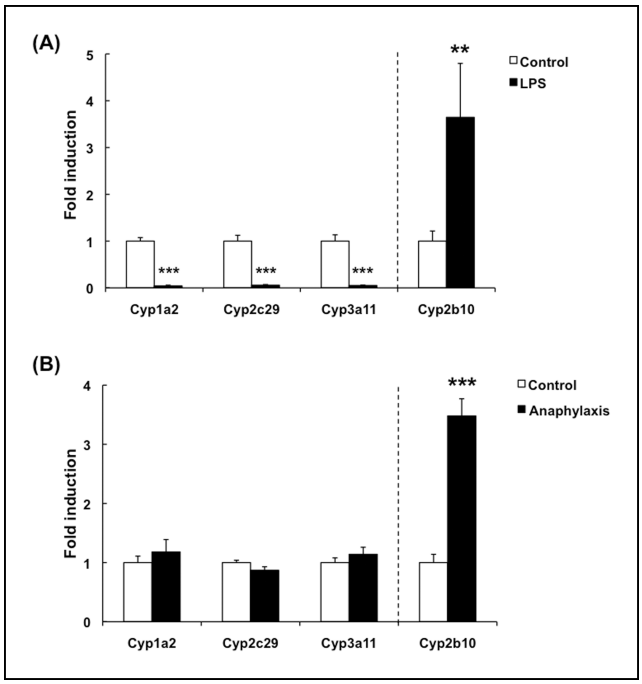

Fig. 1: Changes of hepatic Cyp mRNA levels in mice in response to induced anaphylaxis or LPS. Cyp mRNAs were measured by real-time PCR and normalized to the *Gapdh* mRNA level in the same sample. (A) Mice were injected with saline or LPS (1 mg/kg, i.p.) once per day on two consecutive days. The livers were excised and total RNA extracted 3 h after the second LPS injection. Values show ratios relative to control as mean ± S.E. (n = 5–6). The significance of differences between the groups was assessed by Student's *t*-test (**$p < 0.01$ and ***$p < 0.001$). (B) Mice were sensitized by OVA (i.p.) on day 0, and anaphylaxis was induced on day 21 by OVA challenge (i.v.). Control mice received saline. The livers were excised 3 hr after the challenge, and total RNA was extracted. Values show ratios relative to the corresponding controls as mean ± S.E. (n = 5). Differences were assessed for significance by Student's *t*-test (***$p < 0.001$).

et al. 2000). Thus, increasing these cytokines appears to strongly affect the pathways regulating constitutive and/or inducible CYP expression. Therefore, we speculated that diseases that increase the expression of inflammatory cytokines might also cause CYP expression to fluctuate.

In this study, we used anaphylaxis (a very rapid and severe allergic reaction) as a model of inflammatory disease (Simons et al. 2007). Since serum cytokines, including TNFα and IL-6, are elevated in patients with severe anaphylactic reactions (Stone et al. 2009), we hypothesized that anaphylactic reactions might affect the pathways that regulate CYPs. For this study, we compared the effects of LPS treatment on constitutive CYP mRNA and protein expression and on pro-inflammatory cytokines with the effects of induced systemic anaphylaxis. We found that the effects on CYP expression differed for inflammation induced by LPS or anaphylaxis, suggesting that the effect on CYP gene expression is most likely regulated differentially by inflammatory pathways activated by specific disease states.

2. Investigations and results

2.1. Expression of hepatic Cyp genes in mice with LPS- or anaphylaxis-induced inflammation

We used real-time PCR to examine the levels of the constitutively expressed *Cyp* genes *Cyp1a2*, *Cyp2b10*, *Cyp2c29,* and *Cyp3a11* after LPS treatment. Mice were treated with LPS (1 mg/kg) on two consecutive days and sacrificed 3 h after the second LPS treatment. Changes in the mRNA expression of these genes are shown in Fig. 1A. LPS treatment markedly decreased *Cyp1a2*, *Cyp2c29* and *Cyp3a11* mRNA to about

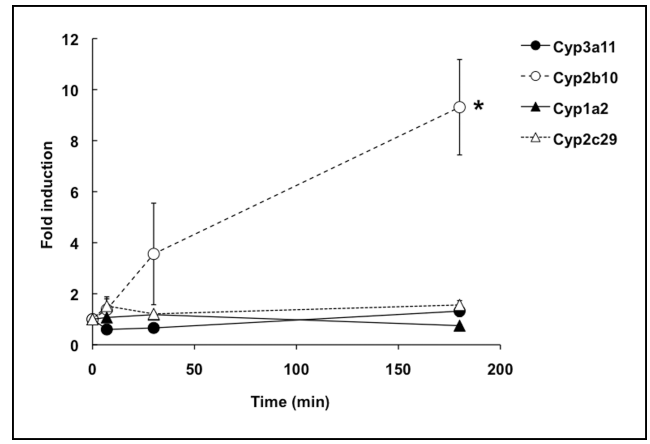

Fig. 2: Hepatic Cyp mRNA expression in early anaphylaxis. Mice were sensitized by OVA (i.p.) on day 0. Anaphylaxis was induced on day 14 by OVA challenge (i.v.). Untreated mice were used as a control. The livers were excised 7, 30, and 180 min after the challenge, and total RNA extracted. Cyp mRNAs were measured by real-time PCR and normalized to the *Gapdh* mRNA level in the same sample. Values show ratios relative to control mice as mean ± S.E. (n = 5). Differences were assessed for significance by Dunnett's test (*$p < 0.05$).

10% of their respective controls. However, *Cyp2b10* mRNA increased about 3.5-fold over the control (Fig. 1A). These results are very similar to those in our previous study, in which we examined the effect of LPS on xenobiotic-induced *Cyp* gene expression.

We induced anaphylaxis in mice to determine whether anaphylactic reaction affects the transcription of *Cyp* genes. In this experiment, we confirmed that all mice exhibited typical symptoms (lying down, reddening of the ears and tail, and dilated ear vessels) of anaphylaxis soon after an ovalbumin (OVA) challenge; this response was not observed in mice that were only sensitized with OVA of the same dose (data not shown). The mice were sacrificed 3 h after the OVA challenge on day 21, and the liver was excised. Unlike our results from the LPS-treated mice (Fig. 1A), there was no change in *Cyp1a2*, *Cyp2c29,* or *Cyp3a11* mRNA levels between the anaphylactic and control mice (Fig. 1B). However, we observed a significant increase of *Cyp2b10* mRNA in anaphylactic mice, just as with LPS-treated mice.

To investigate the effect of OVA-sensitization alone, we also examined the expression levels of these *Cyp* mRNAs in OVA-sensitized mice sacrificed on days 2, 3, 4, 5, 6, 9, and 21. However, there was no clear change in *Cyp* mRNA levels (data not shown). Accordingly, we concluded that only OVA-sensitization did not influence *Cyp* gene expression; *Cyp2b10* mRNA increased in these mice only after inducing anaphylaxis. These results clearly indicated that the hepatic *Cyp* genes examined, with the exception of *Cyp2b10,* have completely different patterns of expression in LPS-treated and anaphylactic mice.

2.2. Hepatic Cyp gene expression in early anaphylaxis

Anaphylaxis progresses so rapidly after onset and causes such severe physical symptoms that it must be treated as soon as possible. Therefore, we were interested in the levels of constitutively expressed *Cyp* genes at the early stages of anaphylaxis. For this experiment, the liver was excised from anaphylactic mice at 7, 30, and 180 min after an OVA challenge on day 14, and the expression of *Cyp* genes was quantified.

There was little change in the levels of *Cyp1a2*, *Cyp2c29*, or *Cyp3a11* mRNAs at the 180 min time point. However, *Cyp2b10* expression increased 1.4-, 3.7-, and 9.3-fold relative to that in non-treated mice at 7, 30, and 180 min, respectively (Fig. 2).

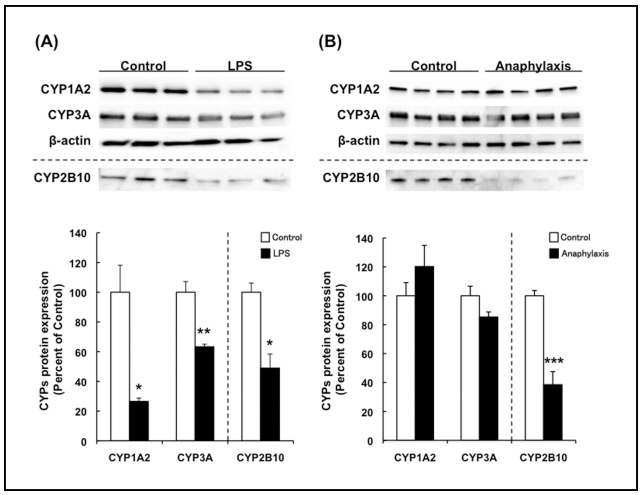

Fig. 3: Changes of CYP protein expression in LPS-treated or anaphylactic mice.Hepatic microsomal protein was isolated from livers and analyzed by western blot as described in Materials and Methods. Upper panels: western blots. The lanes labeled Control, LPS, and Anaphylaxis show results from experiments simultaneously conducted with three to four independent samples; relative protein levels are shown in bar graphs below. (A) Mice were given an injection of saline or LPS (1 mg/kg, i.p.) on two consecutive days; livers were excised and total RNA extracted 3 hr after the second injection. Values in the lower bar graph show protein as a percentage of that in control mice as mean ± S.E. (n = 3). Differences between the groups were analyzed for significance by Student's *t*-test (*$p < 0.05$ and **$p < 0.01$). (B) Mice were sensitized by OVA-administration (i.p.) on day 0, and anaphylaxis was induced on day 21 by OVA challenge (i.v.). Control mice were treated with saline. The livers were excised and total RNA was extracted 3 hr after the challenge. Values show protein as a percentage of that in control mice, shown as mean ± S.E. (n = 4). The significance of differences between the groups was assessed by Student's *t*-test (***$p < 0.001$).

Therefore, we concluded that the pathway regulating *Cyp2b10* expression responds differently to inflammation than the pathways of the other three *Cyp* genes tested.

2.3. Hepatic CYP proteins in LPS-treated and anaphylactic mice

To examine correlations between mRNA and protein expression for the CYPs tested in this study, we analyzed CYP1A2, CYP2B10, and CYP3A by western blot using liver microsomes. If the mRNA and protein expression patterns matched, we could exclude the possibility of post-translational regulation under inflammatory conditions (Lee et al. 2008). The western blot results and relative protein levels for CYP1A2, CYP2B10, and CYP3A are shown in Fig. 3.

The CYP1A2 and CYP3A protein levels in the liver of LPS-treated mice were 30–60% lower than in the control mice (Fig. 3), and decreases in these CYP proteins clearly correlated with the decreases in their respective mRNA (Fig. 1). However, in mice with induced anaphylaxis (Fig. 3B), there was almost no alteration in CYP1A2 or CYP3A protein levels. Interestingly, although CYP2B10 levels decreased in both LPS-treated and anaphylactic mice—50% and 35% of control levels, respectively—its mRNA increased. Significant differences observed between LPS-treated and anaphylactic mice are summarized in the Table.

2.4. TNFα and IL-1β cytokines in the liver of anaphylactic and LPS-treated mice

We previously observed that the levels of two inflammatory cytokines, TNFα and IL-1β, increased in LPS-induced inflammation in a study of inducible *Cyp* expression (Moriya et al. 2012). Other research suggested that TNFα and IL-1β may downregulate hepatic *Cyp* mRNAs. To determine whether TNFα and IL-1β were upregulated in the anaphylactic mice, we measured the mRNAs for hepatic TNFα and IL-1β in the liver by real-time PCR.

In LPS-treated mice, we observed significant increases in *TNFα* and *IL-1β* mRNA (Fig. 4A). The mRNAs of these cytokines also increased significantly in the anaphylactic mice (Fig. 4B). Thus, we observed qualitatively responses in the hepatic expression of *TNFα* and *IL-1β* genes in LPS-treated and anaphylactic mice, although the *Cyp* gene expression patterns differed.

Table: Expression patterns of three CYPs in LPS-treated and anaphylactic mice

	LPS		Anaphylaxis	
	mRNA	Protein	mRNA	Protein
Cyp1a2	↓	↓	—	—
Cyp3a11	↓	↓	—	—
Cyp2b10	↑	↓	↑	↓

Changes in expression: ↓ indicates decrease; ↑ indicates increase; - indicates no change.

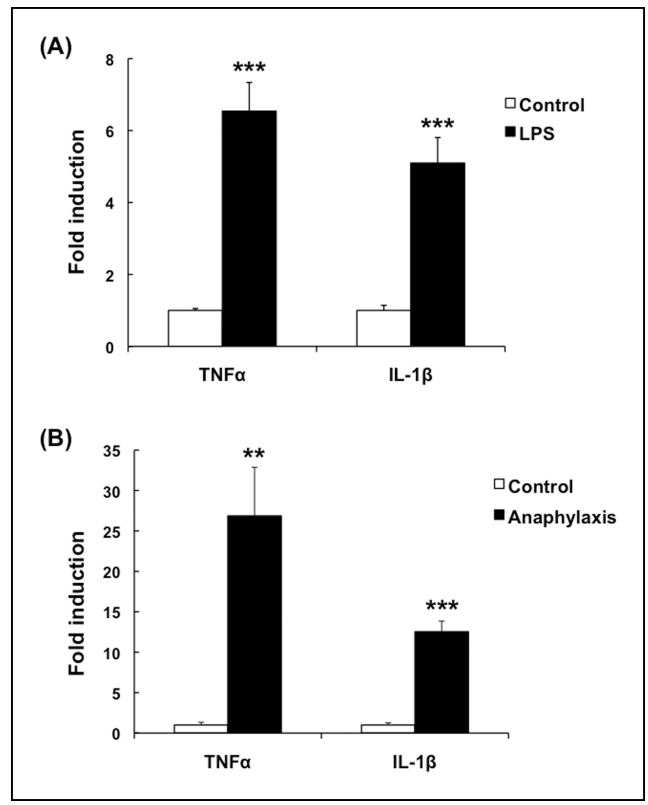

Fig. 4: Changes in hepatic TNFα and IL-1β mRNA expression in LPS-treated or anaphylactic mice. TNFα and IL-1β mRNA levels were measured by real-time PCR, normalized to the Gapdh mRNA in the same sample. (A) Mice were given an injection of saline or LPS (1 mg/kg, i.p.) on two consecutive days. The livers were excised and total RNA extracted 3 hr after the second injection. Values given are ratios relative to the corresponding controls, shown as mean ± S.E. (n = 5–6). Differences were assessed for significance by Student's *t*-test (***$p < 0.001$). (B) Mice were sensitized by OVA (i.p.) on day 0. Anaphylaxis was induced on day 21 by OVA challenge (i.v.). Control mice were injected with saline. The livers were excised and total RNA extracted 3 hr after the challenge. Values given are ratios relative to the corresponding controls, shown as mean ± S.E. (n = 5). Differences were assessed for significance by Student's *t*-test (** < 0.01 and ***$p < 0.001$).

3. Discussion

In the last 15 years, it has become widely recognized that hepatic CYP enzymes are influenced by disease states such as bacterial infection (Chaluvadi et al. 2009), rheumatoid arthritis (Sanada et al. 2011; Ashino et al. 2007), and cancer (Assenat et al. 2006). Slaviero et al. (2003) reported that cancer patients have significantly reduced CYP3A4 activity associated with increased plasma concentration of inflammatory cytokines. Because of the potential for serious repercussions on the patient, it is important to investigate the molecular biology behind the influence of particular diseases on CYP enzyme activity. In this study, we chose anaphylaxis as a disease model and investigated its effects on CYP enzyme expression. We expected that comparing CYP expression patterns during inflammation caused by LPS with their levels in the early stages of experimentally induced anaphylaxis would suggest a hypothesis to improve our understanding of CYP gene expression in mice.

First, we examined how LPS treatment affected the constitutive hepatic expression of CYP genes in mice. LPS markedly decreased the *Cyp1a2*, *Cyp2c29,* and *Cyp3a11* mRNA levels, and the increased *TNFα* and *IL-1β* mRNAs (Figs. 1A and 4A). These findings were consistent with our previous findings (Moriya et al. 2012), in which we examined the inducible expression of CYP genes. In the present study, we also examined the levels of the CYP3A and CYP1A2 proteins, and found that both proteins consistently decreased in parallel with their respective mRNA (Fig. 3A). The role of pro-inflammatory cytokines in regulating CYP expression has already been predicted in previous reports: Kinloch et al. (2011) demonstrated that *Cyp3a11* mRNA levels were downregulated during infection in wild-type mice but not in TNFα receptor 1-null mice, and Wu et al. (2006) reported that pro-inflammatory cytokines derived from Kupffer cells might downregulate CYP1A2 in sepsis. These data on the effect of inflammatory conditions on CYP mRNAs and proteins suggested that the inflammatory cytokines TNFα and IL-1β were good candidates for down-regulating the expression levels of *Cyp1a2*, *Cyp2c29*, and *Cyp3a11*.

To test this hypothesis, we also examined the effects of induced anaphylaxis on the expression of CYP and cytokine genes. Interestingly, there was hardly any change in the constitutive expression levels of *Cyp1a2*, *Cyp2c29*, or *Cyp3a11* mRNA in the anaphylactic mice (Fig. 1B), even early in anaphylaxis (Fig. 2), although the pattern of TNFα and IL-1β expression was the same as in LPS-treated mice (compare Fig. 4A and 4B). Furthermore, there was almost no difference in CYP3A and CYP1A2 enzyme levels between the anaphylactic and control mice (Fig. 3B). This pattern was quite different from that seen in LPS-treated mice. Thus, the CYPs tested in this study showed very different patterns of mRNA and protein expression in response to LPS treatment versus induced anaphylaxis.

To understand the details of how inflammatory cytokines regulate CYP expression in response to LPS treatment or induced anaphylaxis, we must look closely at the inflammatory signaling pathways involved. In the case of LPS-induced inflammation, Toll-like receptor 4 (TLR4) recognizes LPS and activates interferon regulatory factors and the transcription factors NF-κB and AP-1, thus inducing potent innate immune responses (Takeda and Akira 2005). In the liver, Kupffer cells respond to LPS through TLR4 and produce various inflammatory cytokines, such as TNFα, IL-1β, IL-6, IL-12, and IL-18 (Aoyama et al. 2010). Anaphylaxis, on the other hand, is a type I allergic reaction that is mostly mediated by IgE. Once IgE binds to mast cells, they degranulate and release pro-inflammatory mediators, causing immediate symptoms of allergic disease (Finkelman et al. 2005, 2007). In particular, Stone et al. (2009) reported that some serum cytokines, including TNFα and IL-6, are elevated during the onset of anaphylaxis. In our present study, we compared TNFα and IL-1β in LPS-treated and anaphylactic mice (Fig. 4), and found that both cytokines increased greatly in response to anaphylaxis and LPS. However, the *Cyp1a2* and *Cyp3a11* expression patterns were completely different under the two conditions, indicating that the hepatic CYP expression pattern probably varies according to the inflammatory signaling pathway. Currently, we have no additional data to support this tentative conclusion. Based on the facts presented above, however, we speculate that the anaphylaxis-induction pathway is more complicated than the LPS-induction pathway. The anaphylaxis reaction is associated with many inflammatory chemical mediators, including histamine, tryptase, serotonin, platelet-activating factor, leukotrienes, prostaglandins, and nitric oxide (NO) (Osada et al. 1994; Kemp and Lockey 2002; Ogawa and Grant 2007). Some of these mediators likely antagonize or counteract the effect of pro-inflammatory cytokines on CYP expression.

Cyp2b10 expression characteristics were unique among the CYPs tested. Compared to its level in control mice, the expression of *Cyp2b10* was about 4-fold higher in both LPS-treated and anaphylactic mice (Fig. 1A, B), even in early anaphylaxis (Fig. 2). Interestingly, the expression patterns of *Cyp2b10* mRNA and the CYP2B10 protein did not correlate in either LPS-treated or anaphylactic mice. Compared to control mice, CYP2B10 protein levels were about 50% lower in both groups of mice (Fig. 3A, B). Similar results have been reported in some previous studies. From findings in rat hepatocytes, Ferrari et al. (2001) predicted the existence of individual regulatory pathways for the expression of the *Cyp2b1* gene and its transcripts when exposed to LPS. Their study found that LPS suppressed CYP2B1 protein levels in a NO-dependent manner, despite the transient upregulation of *Cyp2b1* mRNA shortly after LPS treatment. Sun et al. (2012) revealed that IL-1β degraded CYP2B proteins in rat hepatocytes, and that the immunoproteasome subunit large multifunctional peptidase 2 contributed to the NO-dependent degradation of CYP2B (Sun et al. 2012). Taking these previous studies into consideration, we speculate that the inconsistency between the levels of *Cyp2b10*'s mRNA and protein expression may be NO-dependent.

In the present study, we found two interesting phenomena. The first is that, for both *Cyp1a2* and *Cyp3a11,* their mRNA and protein levels differed during inflammation depending on whether the inflammation was caused by LPS or anaphylaxis. The second is the unique behavior of *Cyp2b10* expression at the mRNA and protein levels. These findings are helpful in revealing not only disease-based fluctuations in CYP activity, but also the fundamental mechanisms of CYP gene regulation. The fact that some diseases may drastically affect drug metabolism deserves serious consideration, and a deeper understanding of the molecular basis of CYP regulatory mechanisms is likely to suggest future clinical therapies.

4. Experimental

4.1. Chemicals

LPS (0128:B12 from *Escherichia coli*) and OVA (Grade V) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Freund's Incomplete Adjuvant (FIA) was purchased from Difco Laboratories (Detroit, MI, USA). Other chemicals used were of super-fine grade. Rabbit anti-rat CYP3A1, rabbit anti-mouse CYP2B10, rabbit anti-mouse CYP1A2, and rabbit anti-β-actin antibodies were purchased from Enzo Life Sciences (Farmingdale, NY, USA), Merck Millipore (Billerica, MA, USA), Proteintech Group (Chicago, IL, USA), and GeneTex (San Antonio, TX, USA), respectively. Goat anti-rabbit IgG-horseradish peroxidase (HRP) antibody, purchased from R&D Systems (Minneapolis, MN, USA), was used as secondary antibody.

4.2. Animals

Eight-week-old male Balb/c mice weighing 23 g were purchased from Japan SLC Inc. (Shizuoka, Japan). Mice were fed standard rodent chow and given water *ad libitum*. All animals were treated in accord with the Guiding Principles of the "Care and Use of Laboratory Animals Code", approved by Mukogawa Women's University.

4.3. Administration of LPS

To investigate the effects of LPS on constitutive CYP mRNA and protein expression, we induced inflammation with two intraperitoneal injections, administered 24 h apart, of 1 mg/kg LPS dissolved in saline (Honkakoski and Negishi 2000). Control mice received saline. The mice were sacrificed 3 h after the second LPS injection, and the liver was excised and used for total RNA isolation and microsomal preparation.

4.4. Inducing systemic anaphylaxis in mice

Immunization with OVA was performed as reported previously (Osada et al. 1994). Briefly, mice were sensitized on day 0 by an intraperitoneal injection of 50 μg OVA emulsified in FIA. Control mice were not treated. To investigate the effect of sensitization alone, three OVA-sensitized mice were sacrificed on days 2, 3, 4, 5, 6, 9, and 21, and the liver was excised. For experiments with anaphylaxis induction, a single intravenous injection (challenge) with 10 μg OVA in 100 μl saline was given on day 14 or 21. Control mice were injected with saline. The mice were sacrificed 7, 30, or 180 min after the challenge. The liver was then excised and used for total RNA isolation and microsomal preparation.

4.5. Isolation of total RNA, and reverse transcription

Total RNA was isolated from mouse liver using Sepasol RNA I Super (Nacalai Tesque, Kyoto, Japan) following the manufacturer's protocol. The RNA concentration was determined spectrophotometrically by measuring the absorbance at 260 nm, and was adjusted to 77 μg/ml. Total RNA extracts were stored at -80 °C, and cDNA was synthesized from total RNA using the PrimeScript RT reagent Kit (TaKaRa Bio, Shiga, Japan) according to the manufacturer's protocol. Briefly, 1 μg of total RNA from each sample was re-suspended in 20 μl of reaction buffer with PrimeScript RT enzyme, and the mixture was incubated for 15 min at 37 °C. Reverse transcription was stopped by denaturing the enzyme at 85 °C.

4.6. Real-time PCR

We measured mRNA expression in the mouse liver by real-time PCR using StepOnePlus (Applied Biosystems, Foster City, CA, USA). All primers for mouse *Cyps*, *cytokine,* and *glyceraldehyde-3-phosphate dehydrogenase (Gapdh)* genes were used as described previously (Moriya et al. 2012). Real-time PCR was conducted using 10 μM (forward and reverse) gene-specific primers and SYBR Premix Ex TaqII (TaKaRa Bio) with 2 μl cDNA/sample as the template. The number of cycles and annealing temperature were optimized for each primer pair. All experiments were conducted in duplicate using the same sample.

We calculated mRNA expression from a relative standard curve, normalized to that of *Gapdh* in the same sample. Results were expressed as the relative fold induction of the target gene mRNA compared with that of a control sample. The specificity of the PCR product was routinely monitored by checking the product melting curves (dissociation curves) for each reaction.

4.7. Preparation of liver microsomes

The excised liver was homogenized with a Polytron homogenizer in 100 mM sodium phosphate buffer (pH 7.4) containing 3.3 mM magnesium chloride, and then centrifuged at 9,000 × g for 10 min. The supernatant was collected and centrifuged at 105,000 × g for 1 h. The pellet was re-suspended in the same buffer and centrifuged again at 105,000 × g for 1 h. The resulting microsomal pellet was re-suspended in the same buffer and stored at -80 °C until use. Protein concentrations of the microsome samples were determined using the Bradford method, with bovine serum albumin as the standard reference protein (Bradford 1976). All these procedures were conducted at 4 °C.

4.8. Western blot analysis of CYP proteins

CYP proteins were detected by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by western blot. Each hepatic microsomal preparation (3–6 μg protein/lane) was separated by 4–15% SDS-PAGE. The separated proteins were transferred from the gel to a PVDF membrane (BioRad Laboratories, Hercules, CA, USA) at 350 mA for 1 h. Blots were immunolabeled with a primary antibody and a HRP-conjugated secondary antibody. Signals were detected with ECL western blotting detection reagent (GE Healthcare Japan, Tokyo, Japan) according to the manufacturer's protocol.

Rabbit anti-rat CYP3A1 (1,000 folds diluted), rabbit anti-mouse CYP2B10 (1,000 folds diluted), and rabbit anti-mouse CYP1A2 antibodies (1,000 folds diluted) were used as primary antibodies for the CYP3A subfamily enzymes, CYP2B10 enzymes, and CYP1A2 enzymes, respectively. Rabbit anti-β-actins antibody (1,000 folds diluted) was used as the primary antibody to normalize to CYP proteins. Goat anti-rabbit IgG-HRP antibody (2,000 folds diluted) was used as a secondary antibody for CYP3A, CYP2B10, CYP1A2, and β-actin. Relative densities were analyzed using ImageQuant TL (GE Healthcare Japan). Protein levels were normalized to β-actin in the same sample, and results were expressed as a percent of the control.

4.9. Statistical analysis

All results are presented as the mean ± S.E. The results from the various experimental groups and their corresponding controls were compared using Student's *t*-test. Differences were considered significant for $p < 0.05$.

Acknowledgments: This work supported by a KAKEN Grant-in-Aid for Scientific Research (C) 22590159 to F.K. in 2010 and 24659077 to N.M. in 2012.

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