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Pharmacokinetic Properties of CYP3A Substrate Midazolam Associated with Xenobiotic-Mediated CYP Induction in Inflammation Model Mice

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Inflammation can downregulate CYP isoforms and alter the pharmacokinetics of certain drugs. The expression of CYP isoforms is induced by the activation of nuclear receptors, including the pregnane X receptor (PXR). This study aimed to clarify the changes in the inducibility of CYP and *in vivo* relevance to pharmacokinetics in an inflammatory state. We investigated the changes in the mRNA expression of Cyp3a11 induced by pregnenolone-16α-carbonitrile (PCN), a rodent PXR agonist, in the liver and small intestine of inflammation model mice generated by injection of lipopolysaccharide (LPS). The systemic effects of inflammation on CYP induction were also investigated by measuring changes in the plasma concentration of midazolam, a CYP3A substrate. Cyp3a11 mRNA expression levels in the liver and small intestine of mice were decreased by LPS treatment and increased by PCN administration. Hepatic Cyp3a11 mRNA expression levels were significantly lower in LPS- and PCN-administered mice than in PCN-administered mice, whereas intestinal Cyp3a11 mRNA expression levels were significantly higher in LPS- and PCN-administered mice than in PCN-administered mice. The plasma concentration-time profiles and area under the concentration-time curve of midazolam and its metabolite 1'-hydroxymidazolam in LPS- and PCN-administered mice tended to be higher than those in PCN-administered mice. LPS-induced inflammation may suppress the increased metabolism of midazolam by PCN, which is consistent with the hepatic Cyp3a11 mRNA expression results. Therefore, CYP inducibility via PXR activation may be attenuated in an inflammatory state. Pharmacokinetic drug-drug interactions caused by CYP induction may be altered in patients with inflammation.

Key words CYP induction, inflammation, pregnane X receptor

INTRODUCTION

Pharmacokinetics of certain drugs are variable in patients with inflammatory diseases, mainly attributed to the downregulation of drug-metabolizing enzymes, such as CYP isoforms.[1,2] For example, the plasma concentration of CYP3A substrate darunavir is high in patients with coronavirus disease 2019 (COVID-19).[3] Inflammatory responses are often severe in COVID-19, and inflammatory cytokines increase with disease severity.[4,5] Inflammatory cytokines, including interleukin-6 (IL-6) and tumor necrosis factor (TNF)-α, have been shown to decrease basal expression of CYP isoforms in human, mouse, and rat hepatocytes.[6–8] In the population pharmacokinetic analysis, IL-6 was suggested to be the clinical covariate that affects the high plasma concentration of darunavir in patients with COVID-19.[3] Moreover, transcriptional regulation is involved in the CYP downregulation under inflammatory state.[1,2,9–11] Increase of inflammatory cytokines activate cellular signaling pathways or nuclear factor (NF)-κB, leading to suppression of transcriptional factors that regulate the CYP gene expression including pregnane X receptor (PXR), constitutive androstane receptor (CAR), and their dimerization partner retinoid X receptor (RXR).

PXR and CAR are members of the nuclear receptor superfamily, and activation of these receptors induces the expression of drug-metabolizing enzymes, including CYP isoforms.[12] A typical human PXR agonist, rifampicin, mainly induces CYP3A4, which causes an increase in metabolism and decreases the blood concentration of co-administered drugs that are metabolized by CYP3A4.[13] Rifampicin-induced drug-drug interactions (DDI) have been reported in clinical studies.[13] Species differences in the ligand-binding region of PXR and CAR are well known.[14] Rifampicin activates human PXR but hardly activates rodent PXR; while, pregnenolone-16α-carbonitrile (PCN) activates rodent PXR but hardly activates human PXR.[15] Administration of PCN into mice induces the hepatic and intestinal expression of Cyp3a11, which is a generally comparable isoform to human CYP3A4.[16]

In *in vitro* studies, transcriptional activation of PXR and CYP3A4 mRNA induction by rifampicin has been found to be suppressed by TNF-α treatment.[17,18] Phenobarbital-mediated CAR transactivation and induction of its target genes is suppressed by the treatment of IL-1β in human hepatocytes.[19] In *in vivo* studies, PCN-mediated CYP3A induction has been found to be suppressed in the liver of inflammation model mice generated by injecting lipopolysaccharide (LPS).[20,21] These reports suggest that the inducibility of CYP varies under inflammatory state. However, there are various clinical reports demonstrating the changes in the blood concentration of CYP substrates when inducers and substrates are co-administered

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to patients with inflammation. Rifampicin co-administration is predicted to reduce the levels of hydroxychloroquine in patients with COVID-19.[22] The plasma concentration of clindamycin was dramatically reduced by rifampicin co-administration in patients with osteoarticular infections.[23] Another clinical report suggested that CYP3A inducibility by rifampicin is approximately two times lower in patients infected with human immunodeficiency virus (HIV) than in healthy individuals, based on the changes in the AUC of saquinavir, an HIV protease inhibitor and CYP3A substrate.[24] Therefore, it is important to clarify *in vivo* relevance to pharmacokinetics associated with changes in the CYP inducibility in the inflammatory state. We investigated the changes in PCN-mediated gene expression of Cyp3a11 in the liver and small intestine of mice with LPS-induced inflammation. The systemic effects of inflammation on CYP induction were also investigated by measuring changes in the plasma concentration of midazolam, a typical CYP3A substrate.

MATERIALS AND METHODS

Animals The animal experiments in this study were conducted in accordance with the Ritsumeikan Animal Experiment Rules after approval by the Animal Care Committee of Ritsumeikan University. Six-week-old male C57BL/6J mice were purchased from Japan SLC Inc. (Shizuoka, Japan). Mice (6–7 weeks old) were randomly assigned to each group and intraperitoneally injected with 1.0 mg/kg LPS (Chondrex, Redmond, WA, USA) to induce acute systemic inflammation. Phosphate-buffered saline (PBS) was administered instead of LPS as a vehicle control. PCN (Cayman Chemical, Ann Arbor, MI, USA) was intraperitoneally administered at 25 mg/kg, 3 h after LPS injection. Corn oil was used to suspend PCN and was administered instead of PCN as a vehicle control. Midazolam (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) was orally administered to all groups at 5.0 mg/kg 24 h after LPS injection. Blood samples were collected 5, 15, 30, 45, 60, 90, and 120 min after midazolam administration. The mice were, then, anesthetized with isoflurane, and the blood (with heparin), liver, and small intestinal epithelium (jejunum) were collected.

Measurement of Plasma Cytokine Levels Plasma IL-6 and TNF-α levels were estimated using ELISA MAX™ Deiuxe Set Mouse IL-6/TNF-α (BioLegend, Inc., San Diego, CA, USA), according to the manufacturer's instructions.

RT-qPCR The mRNA expression levels of Cyp3a11 in the liver and small intestine were determined using RT-qPCR. Details were described in a previous study.[25] Briefly, RNAlater Solution (Thermo Fisher Scientific, Waltham, MA, USA), TRIzol reagent (Thermo Fisher Scientific), and High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific) was used for collection of the tissues, isolation of total RNA, and reverse transcription, respectively. qPCR was carried out using StepOne Real-Time PCR System (Thermo Fisher Scientific) and PowerUp SYBR Green Master Mix (Thermo Fisher Scientific). The relative expression levels of Cyp3a11 were calculated by the $\Delta\Delta C_T$ method using glyceraldehyde 3-phosphate dehydrogenase as the reference gene.

LC-MS/MS The plasma concentrations of midazolam and its major metabolite 1'-hydroxymidazolam, were determined using LCMS-8060NX (Shimadzu Corporation, Kyoto, Japan). Plasma samples were mixed with methanol and acetonitrile containing phenacetin (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan) as an internal standard. After centrifugation for 5 min at 12,000 rpm, the supernatant was filtered and injected into the LC-MS/MS system. COSMOCORE $2.6C_{18}$ Packed Column (2.1 mmI.D. × 50 mm; Nacalai Tesque Inc., Kyoto, Japan) was used for LC separation. The mobile phase was 0.1% formic acid in ultrapure water (A) and acetonitrile (B). The gradient condition (A:B) at the flow rate of 200 μL/min was as follows: 0 min (95:5), 4 min (5:95), and 4.01–5 min (95:5). The precursor ion and product ions (*m/z*, precursor ion → product ion) under positive-ion mode are described as follows: midazolam (326.3 → 291.15), 1'-hydroxymidazolam (342.30 → 203.10), and phenacetin (180.15 → 110.20). Standard product of 1'-hydroxymidazolam were purchased from Cayman Chemical.

Statistical Analysis Data are presented as the mean ± S.D. Statistical significance of differences was evaluated by one-way ANOVA with Tukey's multiple comparison test using GraphPad Prism version 8.4.3 (GraphPad Software, La Jolla, CA). The area under the concentration-time curve (AUC) were calculated using the trapezoidal rule. $P < 0.05$ was set as indicative of statistical significance.

RESULTS AND DISCUSSION

To evaluate the degree of inflammation caused by LPS treatment, plasma levels of IL-6 and TNF-α, inflammatory cytokines, were measured using ELISA method. Plasma IL-6 and TNF-α levels were markedly increased in LPS-injected mice (Fig. 1); these levels did not differ between LPS-injected mice with or without PCN administration (Fig. 1). These results show that treatment with LPS caused inflammation, and PCN did not affect LPS-induced inflammation. PCN is a typical rodent PXR agonist, and previous reports have suggested that PXR activation suppresses inflammatory responses through suppression of NF-κB.[26,27] PCN was administered 3 h after LPS injection in our experiments. Therefore, PXR may not suppress the LPS-induced inflammation that had already occurred.

The mRNA expression levels of Cyp3a11 were measured in the liver and small intestine (Fig. 2). In the liver, Cyp3a11 mRNA expression was significantly decreased by LPS treatment and increased by PCN administration. In the small intestine, Cyp3a11 mRNA expression was decreased following LPS treatment and increased following PCN administration (Fig. 2). These results indicated LPS-mediated downregulation and PCN-mediated induction of Cyp3a11, in agreement with previous reports.[16,28–31] Weak induction of Cyp3a11 in the small intestine may be due to administration route in which PCN does not pass through the lumen of small intestine. We confirmed that PXR mRNA expression is abundant in both liver and small intestine of mice (data not shown). Cyp3a11 mRNA expression levels were lower in the livers of LPS- and PCN-administered mice than in PCN-administered mice (Fig. 2). In previous reports, PCN-induced expression of Cyp3a11 decreased in the livers of LPS-injected mice,[20,21] which is similar to our results. These results suggested that PCN-mediated CYP3A induction was attenuated by inflammation. In contrast, Cyp3a11 mRNA expression levels were significantly higher in the small intestines of LPS- and PCN-administered mice than in PCN-administered mice, unlike in the liver (Fig. 2). Administration of dexamethasone, a gluco-

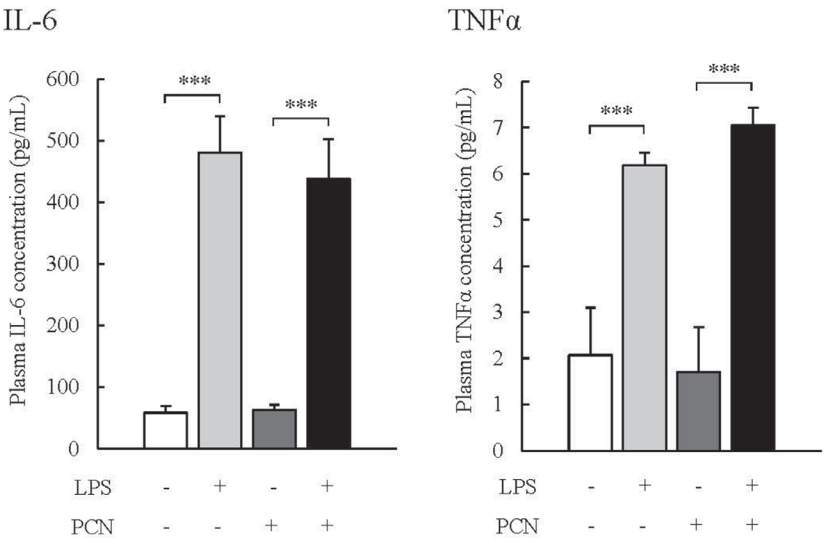

Fig. 1. Plasma Concentration of Inflammatory Cytokines in Each Mouse.

The degree of inflammation was evaluated based on plasma IL-6 and TNF-α levels measured by ELISA method. Data are expressed as mean ± S.D. (n=4); ***P<0.001 (Tukey's multiple comparison test).

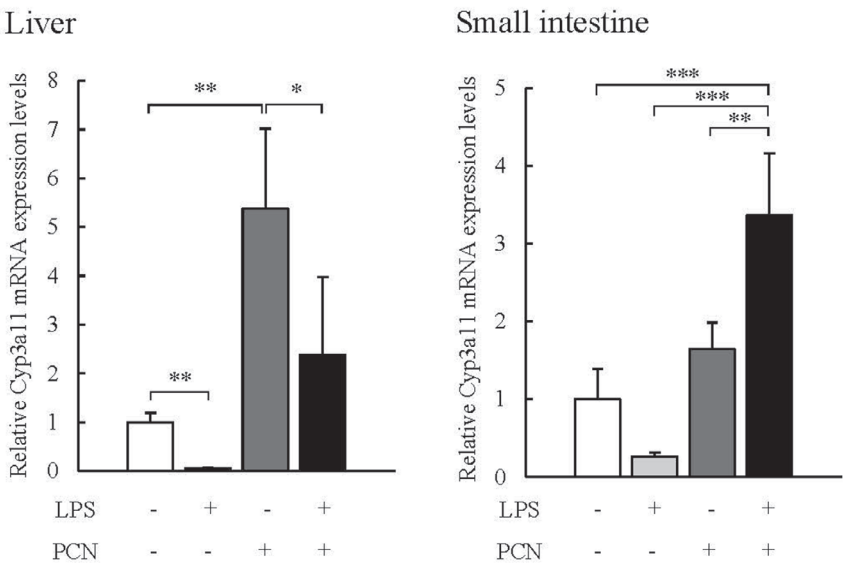

Fig. 2. mRNA Expression Levels of Cyp3a11 in the Liver and Small Intestine of Each Mouse.

The mRNA expression levels of Cyp3a11 were evaluated using RT-qPCR. The relative expression levels of the target genes were normalized to the expression level of glyceraldehyde 3-phosphate dehydrogenase as the reference gene and calculated relative to those in control mice (LPS-/PCN-). Data are expressed as mean ± S.D. (n=4); *P<0.05, **P<0.01, **P<0.001 (Tukey's multiple comparison test).

corticoid receptor and PXR agonist, has been reported to significantly increase Cyp3a11 mRNA expression in the small intestine of mice, and the increase was significantly suppressed when co-administered with LPS.[28] The reasons for the difference between the results of this study and those of our study are unclear; however, differences in the anti-inflammatory effects of dexamethasone and the dosing schedule are possible reasons. From the mRNA expression results shown in Fig. 2, to determine the systemic effects of LPS and PCN co-administration, it was necessary to clarify the changes in the pharmacokinetics of the CYP3A substrate.

The pharmacokinetics of midazolam were evaluated to investigate the systemic effects of changes in Cyp3a mRNA expression. The plasma concentration-time profiles of midazolam and 1'-hydroxymidazolam, a major metabolite formed by CYP3A, is shown in Fig. 3. The AUC from 0 to 2 h ($AUC_{0\text{-}2h}$) of midazolam and 1'-hydroxymidazolam are shown in Table 1. The plasma concentration-time profiles and $AUC_{0\text{-}2h}$ of midazolam tended to increase in LPS-administered mice and decrease in PCN-administered mice. In LPS- and PCN-administered mice, the plasma concentration-time profiles and $AUC_{0\text{-}2h}$ tended to be higher than those in PCN-administered mice, and lower than those in control mice (LPS-/PCN-). These results support the observed changes in hepatic Cyp3a11 mRNA expression (Fig. 2). Nevertheless, the plasma concentration-time profiles and $AUC_{0\text{-}2h}$ of 1'-hydroxymidazolam

Table 1. Pharmacokinetic Parameters of Midazolam and 1'-Hydroxymidazolam in Each Mouse.

LPS	-	+	-	+
PCN	-	-	+	+
Midazolam				
$AUC_{0\text{-}2h}$(ng·h/mL)	218.4 ± 268.1	621.7 ± 373.5	10.71 ± 8.858	141.1 ± 158.0
1'-Hydroxymidazolam				
$AUC_{0\text{-}2h}$(ng·h/mL)	1479 ± 651.9	1618 ± 630.1	553.6 ± 465.7	923.8 ± 408.3
AUC ratio ($AUC_{1'\text{-hydroxymidazolam}}$ / $AUC_{midazolam}$)	6.774	2.603	51.70	6.549

The $AUC_{0\text{-}2h}$ were calculated using the trapezoidal rule. Data are expressed as mean ± S.D. (n=4).

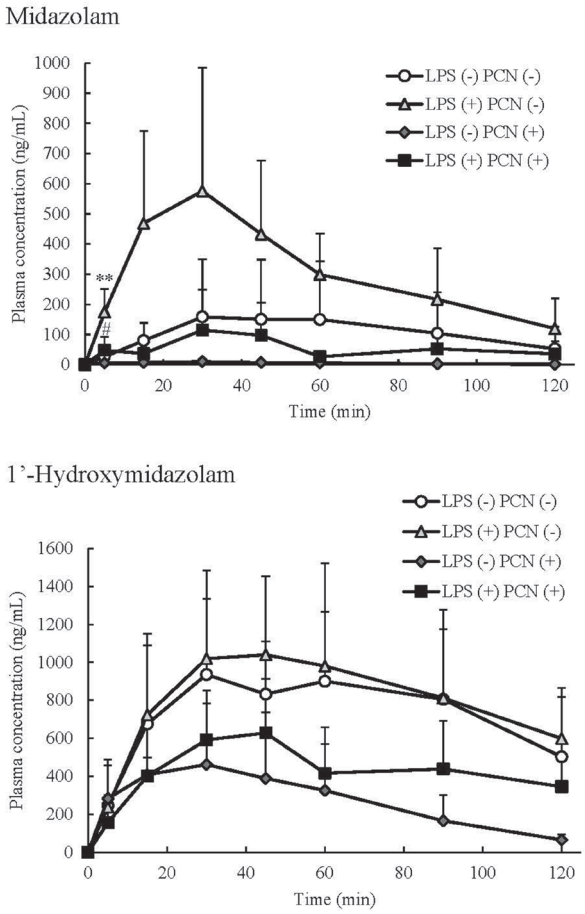

Fig. 3. Plasma Concentration-Time Profiles of Midazolam and 1'-Hydroxymidazolam in Each Mouse.

Midazolam, a CYP3A-specific substrate, was orally administered to the mice at 5.0 mg/kg after LPS and PCN administration. The plasma concentrations of midazolam and 1'-hydroxymidazolam, a CYP3A-specific metabolite, were measured using LC-MS/MS. Data are expressed as mean ± S.D. (n=4); $^{**}P<0.01$ *vs* LPS (-) PCN (-), $^{\#}P<0.05$ *vs* LPS (+) PCN (-) (Tukey's multiple comparison test).

were not significantly higher in PCN-administered mice compared with those in control mice (Fig. 3 and Table 1). Midazolam is metabolized by CYP3A4 to 1'-hydroxymidazolam as well as 4-hydroxymidazolam and 1',4-dihydroxymidazolam, and their glucuronide conjugates are generated by UDP-glucuronosyltransferase (UGT) 1A and UGT2B isoforms in the human liver.[32,33] PCN also induces Ugt1a and Ugt2b isoforms in mice.[34,35] Therefore, in PCN-administered mice, more secondary metabolites may be formed following the formation of 1'-hydroxymidazolam than that in control mice. The plasma concentration and $AUC_{0\text{-}2h}$ of midazolam and 1'-hydroxymidazolam in LPS- and PCN-administered mice tended to be higher than those in PCN-administered mice and lower than those in control mice (Fig. 3 and Table 1). These results suggest that the elevation of the metabolite levels of midazolam and 1'-hydroxymidazolam by PCN is suppressed by inflammation. In previous reports of *in vitro* metabolism experiments, intrinsic clearance in human and mouse liver microsomes for the reaction of midazolam to 1'-hydroxymidazolam was comparable or higher than that in small intestine microsomes.[36–39] Considering the abundance of microsomes in the liver, PCN-induced Cyp3a11 mRNA expression in the small intestine was enhanced by LPS co-administration (Fig. 2); however, the changes in Cyp3a11 expression in the liver may have affected the pharmacokinetics more than that in the small intestine. The AUC ratio ($AUC_{1'\text{-hydroxymidazolam}}/AUC_{midazolam}$) tended to be lower in LPS-administered mice and higher in PCN-administered mice than that in control mice. In LPS- and PCN-administered mice, the AUC ratio was comparable to that of control mice. These results suggest that the metabolism of midazolam to 1'-hydroxymidazolam was suppressed by LPS treatment and enhanced by PCN administration, and PCN-induced metabolism was canceled by LPS treatment.

Our results suggest that CYP inducibility through PXR activation by xenobiotics may be attenuated in an inflammatory state, leading to variations in pharmacokinetic DDI in patients with inflammation. In clinical reports, there are different findings regarding the changes in the blood concentration of CYP3A substrates when inducers and substrates are co-administered to patients with inflammation.[22–24] Further investigation is needed to determine the effects of the pathological condition, the degree of inflammation and induction, and type of inducers on CYP inducibility. Finally, we investigated the changes in the plasma concentration of CYP3A probe substrate midazolam when inducer PCN is co-administered to mice with LPS-induced inflammation. We highlight the importance of the *in vivo* relevance to pharmacokinetics associated with changes in the CYP inducibility in the inflammatory state. In patients with inflammation, the pharmacokinetic DDI may vary and will be further characterized in future studies.

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Conflict of interest The authors declare no conflict of interest.

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