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Changes in mRNA Expression and Activity of Xenobiotic Metabolizing Enzymes in Livers from Adjuvant-Induced Arthritis Rats

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ABSTRACT

Pathophysiological changes in human patients and in animal models of infection or inflammation are associated with alterations in the production of numerous liver-derived proteins including metabolizing enzymes. In this study, the effects of adjuvant-induced arthritis (AA) in rats on the levels of mRNA and activity of hepatic xenobiotic metabolizing enzymes were determined during the inflammatory response. The mRNA levels of cytochrome P450 (CYP) 1A2, CYP2C12, CYP2D1, CYP2D2, and CYP3A1 were significantly decreased compared with control levels in almost all phases of inflammation. A reduction in the activity of CYP2C and CYP3A, which are abundantly expressed in the liver, was also observed. For phase II metabolizing enzymes, mRNA levels of uridine 5'-diphospho-glucuronosyltransferase (UGT) 1A1, UGT1A6, sulfotransferase (SULT) 2A1, and glutathione S-transferase 2 were significantly decreased compared with control levels. However, the mRNA levels of UGT2B and SULT1A1 returned to control levels during the subacute (7 d after adjuvant treatment) and chronic (21 d after adjuvant treatment) phases although these levels decreased during the acute (3 d after adjuvant treatment) phase. These results suggest that the effects of inflammation on the expression of xenobiotic metabolizing enzymes differ depending on the isoform of the enzyme and could affect the pharmacokinetics of each substrate.

Keywords: Inflammation; Arthritis; Enzyme; Cytochrome; Metabolism

1. Introduction

Pathophysiological changes in human patients and in animal models of infection or inflammation are associated with immediate and often dramatic alterations in the production of numerous liver-derived proteins, including metabolizing enzymes such as cytochrome P450s (CYP), UDP-glucuronosyltransferases (UGT), sulfotransferases (SULT), and glutathione S-transferases (GST) [1,2]. Inflammatory conditions such as rheumatoid arthritis and Crohn's disease have been shown to reduce hepatic clearance of several highly cleared drugs [3-5]. Adjuvant-induced arthritis (AA) in rats has been used as an animal model for rheumatoid arthritis in the development of new anti-inflammatory medicines because rats exhibit a systemic inflammatory disease with similar bone and cartilage alterations to those observed in rheumatoid arthritis on day 3 (acute), day 7 (subacute) and day 21

(chronic) after adjuvant treatment [6]. Changes in the pharmacokinetics and pharmacological effects of several drugs via altered CYP activities and serum protein binding have been reported in AA rats, including elevated plasma concentrations of cyclosporine A, acebutolol and propranolol [4,7], and prolongation of sleeping time with pentobarbital [8]. We also demonstrated that flurbiprofen glucuronidation activity and CYP content in liver microsomes were reduced [9] and intestinal CYP3A activity was decreased in AA rats [10]. Inflammatory cytokines, for example, tumor necrosis factor (TNF)-α, interleukin (IL)-6, and IL-1 could be involved in the decrease of metabolizing enzymes [11-13]. The nuclear receptor NR1I2, which is a pregnane X receptor (PXR) and NR1I3, which is a constitutive androstane receptor (CAR), is involved in the regulation of CYP transcription by interacting with xenobiotics and endogenous toxins [14-16].

However, a comprehensive understanding of the changing profile of xenobiotic metabolizing enzymes in acute

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(3 d after adjuvant treatment), subacute (7 d after adjuvant treatment) and chronic (21 d after adjuvant treatment) phases of inflammation remains elusive, despite their importance on the pharmacokinetics of drug-related substrates. In this study, we examined the influence that the inflammatory response in an AA rat model has on hepatic enzymes involved in phase I (CYP1A2, CYP2C11, CYP2D1, CYP2D2, and CYP3A1) and phase II (SULT1A1, SULT2A1, UGT1A1, UGT1A6, UGT2B, and GSTP2) metabolism.

2. Materials and Methods

2.1. Preparation of AA Rats

Female Sprague-Dawley rats (seven weeks old), weighing 180 - 240 g, were purchased from CLEA Japan, Inc. (Tokyo, Japan). The animals were housed in a temperature-controlled room with free access to standard laboratory chow and water. Adjuvant was prepared from 100 mg heat-killed *Mycobacterium butyricum* (Difco Laboratories, Detroit, MI, USA) suspended in 10 mL of Bayol F oil. Hindpaw volumes were measured by liquid plethysmometry. Animals were studied 5, 10, 24 and 3 d (acute phase), 7 d (subacute phase), and 14 and 21 d (chronic phase) after the injection of adjuvant or Bayol F. AA rats in the acute phase exhibit local inflammation at the treated site. In the chronic phase, severe inflammation was observed in local and systemic sites. The experiments were approved by the Committee for the Care and Use of Laboratory Animals at Kinki University School of Pharmacy.

2.2. Measurement of mRNA

After the animals were anesthetized with diethyl ether, the liver was perfused with ice-cold saline and then removed. After flash freezing in liquid nitrogen, each sample was preserved at -80°C until used for RNA extraction.

Determination of mRNA levels was performed using real-time reverse transcriptase polymerase chain reaction (RT-PCR) as previously described [17]. Total RNA (500 ng) was extracted from each liver and reverse-transcribed to complementary DNA (cDNA) using a PrimeScript-RT reagent Kit (TaKaRa, Shiga, Japan). Reactions were incubated for 15 min at 37°C and 5 sec at 85°C. The reverse-transcribed cDNA was used as a template for real-time RT-PCR. Amplification was performed in 50 μL reaction mixtures containing 2 × SYBR Premix Ex Taq (TaKaRa) and 0.2 mM of each primer set shown in **Table 1**. PCRs were incubated at 95°C for 10 sec, and then amplified at 95°C for 5 sec, 55°C for 20 sec, and 72°C for 31 sec for 40 cycles. Data was normalized to the amount of 18S rRNA in each sample. The data were analyzed

Table 1. Primer sequences used in PCR assays.

Gene		Primer sequence (5'-3')
CYP1A2	Forward:	ACGTGAGCAAAGAGGCTAACCA
	Reverse:	ATTAGCCACCGATTCCACCAC
CYP2C11	Forward:	CGCACGGAGCTGTTTTTGTT
	Reverse:	GCAAATGGCCAAATCCACTG
CYP2D1	Forward:	GCAAAGTCTTCCCCAAGCTCA
	Reverse:	GGAAGGCATCAGTCATGTCTCG
CYP3A1	Forward:	GCCTTTTTTTGGCACTGTGCT
	Reverse:	GCATTTGACCATCAAACAACCC
SULT1A1	Forward:	GCCCGAAATGCAAAGGATG
	Reverse:	TGCAGCTTGGCCATGTTGT
SULT2A1	Forward:	CAGTAGCCCAAGCTGAAGCCTT
	Reverse:	CGGCCATTTTCTCCTGGAAA
UGT1A1	Forward:	CGGAGTTATTCAGCAGCTCCAG
	Reverse:	GGTGCTATGACCACCACTTCGT
UGT1A6	Forward:	AACCTAGAAGAGTTGCGGACCC
	Reverse:	CAGCAAAGTGGTTGTTCCCAA
UGT2B	Forward:	TCCCCACCCAACATTACCAA
	Reverse:	AGCAGGTTTGCAATGGAGTCC
GSTP2	Forward:	GCAGCTCCCCAAGTTTGAAGA
	Reverse:	GGTGCCTCAAGATGGCATTAGA
18S rRNA	Forward:	CGCCGCTAGAGGTGAAATTC
	Reverse:	CCAGTCGGCATCGTTTATGG

with ABI Prism 7000 SDS Software (Applied Biosystems) using the multiplex comparative method.

2.3. Preparation of Hepatic Microsomes

Livers were perfused with ice-cold saline and chopped into small pieces. A 25% (w/v) homogenate was made in ice-cold 1.15% KCl solution using a Physcotron homogenizer. The homogenate was centrifuged at 12,000 g for 20 min, and the supernatant was further centrifuged at 105,000 g for 60 min to obtain a microsomal pellet. The microsomal pellet was washed by resuspending it in 3 mL of 1.15% KCl, and the suspension was centrifuged at 105,000 g for 30 min to obtain the final microsomal pellet, which was resuspended in 1.5 mL of 1.15% KCl and stored at -80°C until use. All procedures were carried out at 4°C. Protein concentrations were determined using a BCA protein assay kit (Pierce Biotechnology, Rockford, IL, USA).

2.4. CYP Activity Measurements

CYP3A activity was determined using a P450-Glo CYP3A4 assay (Promega, Madison, WI, USA). P450-Glo CYP3A4 was used to determine CYP3A1 and

CYP3A2 activities in rats and CYP3A4 activity in humans as per the manufacturer's instructions. In brief, CYP3A reactions were performed in a 96-well plate (OptiPlate-96 (PerkinElmer, Waltham, MA, USA)). An incubation mixture (50 μL total volume) was prepared, containing 200 mM potassium phosphate buffer (pH 7.4), NADPH regeneration system (Promega), 20 μg rat liver microsomes and 50 μM of luciferin 6' benzyl ether (luciferin-BE) as a substrate for CYP3A1 and CYP3A2. The concentrations of luciferin-BE were around the K_m values (50 μM). After preincubation for 10 min at 37°C, the reaction was initiated by addition of the NADPH regeneration system and then incubated for 30 min at 37°C with constant shaking. The reconstituted luciferin detection reagent (50 μL) was then added to stop the reaction and to generate chemiluminescence. CYP3A converts luciferin-BE to luciferin by a debenzylation reaction and the production of luciferin by CYP3A1 and CYP3A2 was determined using a luciferase assay. Luminescence was measured using the FLUOstar Optima (Moritex, Tokyo, Japan). CYP2C9 activity was determined using a P450-Glo CYP2C9 assay (Promega). 100 μM of 6'-deoxyluciferin (luciferin-H) was used as a substrate for CYP2C9. All other conditions were the same as for the CYP3A assay. CYP2C9 converts 6'-deoxyluciferin (luciferin-H) to luciferin and the production of luciferin by CYP2C9 was determined using a luciferase assay. All CYP isoform activity determinations were performed in duplicate.

2.5. Statistical Analysis

Separate control groups were made for acute, subacute and chronic phases. The differences between the AA and control groups for (each of) the three phases were estimated using the Student's unpaired *t*-test.
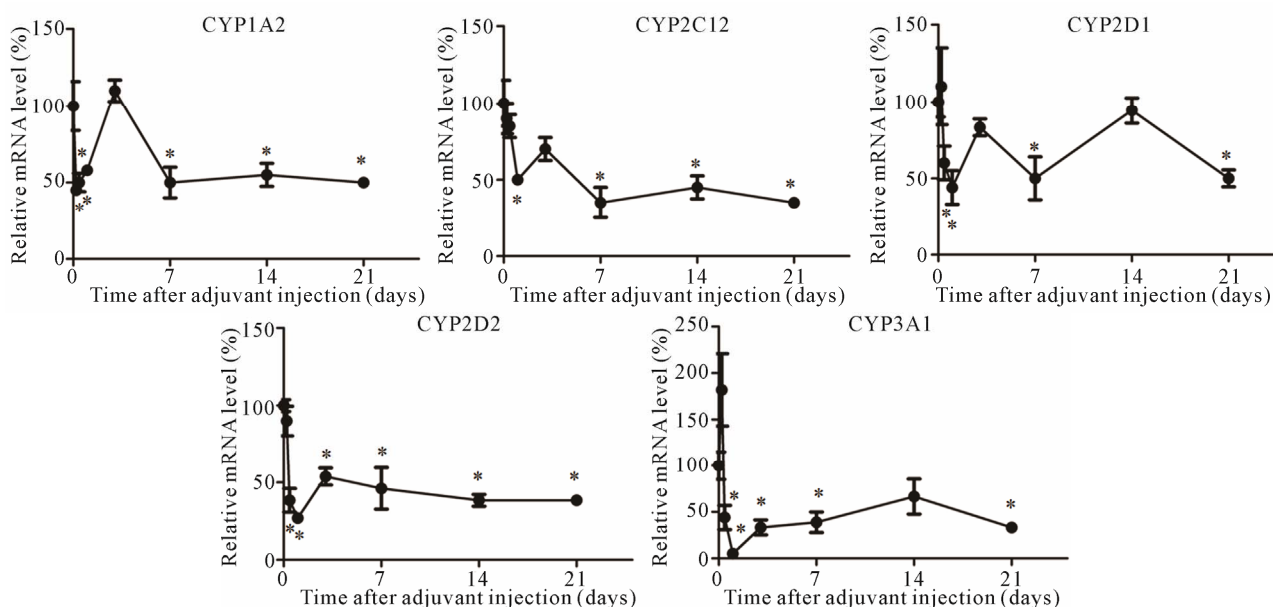
3. Results and Discussion

Changes in mRNA levels of various xenobiotic metabolizing enzymes from the CYP, UGT and SULT families and GSTP during each response phase of inflammation were determined. CYP1A2, CYP2C12, CYP2D1, CYP2D2, and CYP3A1 mRNA levels are shown in **Figure 1**. The mRNA level of all examined CYPs exhibited significant decreases 24 h after adjuvant treatment. Sanada *et al*. demonstrated that the hepatic mRNA and protein levels of inflammatory cytokines such as TNF-*α*, IL-6, and IL-1 significantly increased by 24 h after adjuvant treatment in rats [18]. The increased cytokines in the early stage of inflammation could cause a reduction in CYP mRNA levels. It is reported that IL-1*β* inhibits the expression of various hepatic CYP isoforms [19]. The recovery of CYP1A2, CYP2C12, and CYP2D1 mRNA occurred by day 3. All examined CYP mRNAs were re-

duced to approximately half of control levels by day 21 (chronic phase).

These results showed that almost all examined CYP isoforms significantly decreased in the acute, and subacute and the chronic phases in the arthritic (rats) compared with control rats. In particular, CYP3A1 mRNA decreased to low levels 24 h after adjuvant treatment. It is possible that the diminished expression of CYP3A1 could affect the pharmacokinetics of substrates because CYP3A participates mainly in the metabolism of various drugs. **Figure 2** shows the alterations in the activities of CYP2C and CYP3A, which have relatively high protein content in the liver in each phase of inflammation. The activity of CYP2C decreased going from the acute to the chronic phase of inflammation and was less than 10% of control levels in the subacute and chronic phases of inflammation. The activity of CYP3A also significantly decreased at 3, 14 and 21 d, suggesting that both protein and mRNA levels had decreased. Total CYP2C metabolizing activity showed a further decrease compared with the mRNA level of CYP2C12 in AA rats. It could be that the changes in expression of these other CYP2C isoforms, such as CYP2C6 and CYP2C7, accounted for this difference between activity and mRNA level.

These results could be interpreted to mean that the expression of all examined CYP isoforms were suppressed during inflammation and this decreased activity could affect the pharmacokinetics of various drugs. The transcription of CYPs is regulated by nuclear receptors such as PXR and CAR. For example, the transcription of CYP2B and CYP3A is known to be regulated through CAR and PXR, respectively [20,21]. CAR and PXR show overlapping regulation of transcription of CYPs and transporters [22]. The effects of the phases of inflammation on the expression of nuclear receptors are unclear but warrant examination.

To further clarify the effects of AA on other metabolizing enzymes, we examined the alterations of SULTs, UGTs and GSTP involved in the phase II metabolic pathway. The changes in mRNA of three isoforms of UGTs (UGT1A1, UGT1A6, and UGT2B) are shown in **Figure 3**. UGT1A1 and UGT1A6 mRNAs exhibited significant decreases in the acute, subacute, and chronic phases of inflammation. On the other hand, UGT2B showed little change from control levels except on day one (acute phase). Interestingly, the distinct effects of AA on the mRNA levels of UGT unlike CYP isoforms were presented. The UGT1 locus is located on chromosome 2q37 and the UGT2 family is located on chromosome 4q13. UGT1A participates in the metabolism of endobiotic substrates such as bilirubin and estrogens and drug substrates such as irinotecan, imipramine and cyproheptadine [23]. It is possible that the metabolism of substrates by UGT1A was affected by the inflammatory

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Figure 1. Changes in relative mRNA levels of CYP1A2, 2C12, 2D1, 2D2, and 3A1 in the liver of AA rats. The results are expressed as the mean ± S.D. (n = 4). There were significant differences between control and AA rats ($^{*}p < 0.05$).
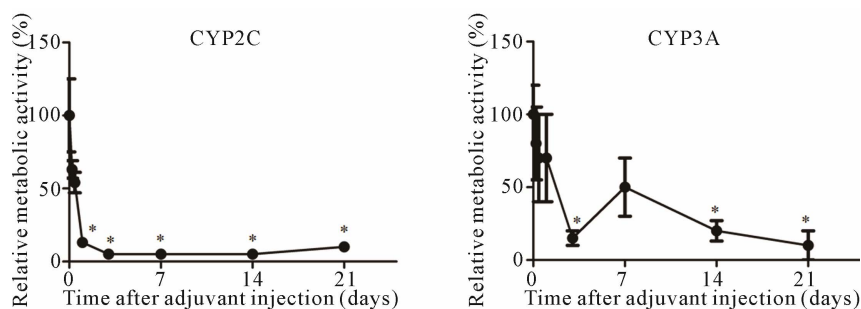
Figure 2. Changes in relative metabolic activities of CYP2C and 3A in the liver of AA rats. The results are expressed as the mean ± S.D. (n = 4). There were significant differences between control and AA rats ($^{*}p < 0.05$).
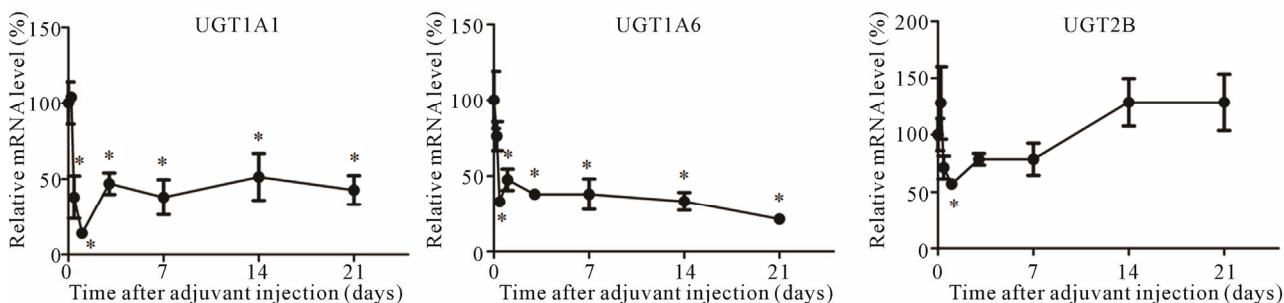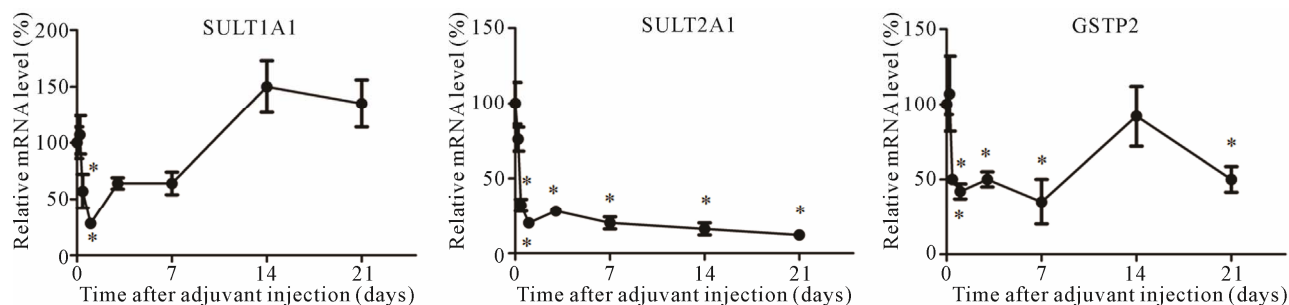

Figure 3. Changes in relative mRNA levels of UGT1A1, 1A6, and 2B in the liver of AA rats. The results are expressed as the mean ± S.D. (n = 4). There were significant differences between control and AA rats ($^{*}p < 0.05$).

response in AA rats. It has been reported that UGT1A and UGT2B are regulated by the aryl hydrocarbon receptor and NF-E2-related factor 2, respectively [24,25]. These different mechanisms in transcriptional regulation could lead to differences in the expression of UGT isoforms. Our future research will be focused on investigat-

ing alterations between UGTs and transcription factors.

The changes in mRNA of SULT1A1, SULT2A1 and GSTP2 are shown in **Figure 4**. The SULTs are categorized into two major groups, the arylsulfotransferases (SULT1 family) and the hydroxysteroid sulfotransferases (SULT2 family) [26]. Although both SULT1A1 and

Figure 4. Changes in relative mRNA levels of SULT1A1, 2A1, and GSTP2 in the liver of AA rats. The results are expressed as the mean ± S.D. (n = 4). There were significant differences between control and AA rats ($^*p < 0.05$).

SULT2A1 mRNAs decreased in the acute phase, the mRNA level of SULT1A1 but not SULT2A1 recovered to control levels in the subacute and chronic phases. It has been reported that SULT2A1 is predominantly regulated by PXR [27,28]. In our previous report, we demonstrated that the mRNA level of PXR but not CAR was significantly decreased in AA rats [29]. Therefore, it is possible that the inflammatory response could lead to the inhibition of transcription of SULT2A1 through regulation of PXR levels. Further research is needed to better understand the differences in regulation between SULT1-A1 and SULT2A1. The mRNA level of GSTP2 decreased by close to 50% in all phases. These results suggest that phase II enzymes could have more distinct patterns of changes in mRNA for each isoform compared with CYPs.

In conclusion, the mRNA level of almost all metabolizing enzymes examined were decreased in all three response phases in AA rats, suggesting that the inflammatory condition could affect the pharmacokinetics of substrates used by these enzymes, most likely as a result of decreased protein expression. However, some enzymes such as UGT2B and SULT1A1 showed a relatively quick recovery to control mRNA levels, indicating that the effects of inflammation on mRNA levels of metabolizing enzymes differ depending on the isoform.

4. Acknowledgements

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