Downregulation of Hepatic Cytochrome P450 3A in Mice Infected with *Babesia microti*

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ABSTRACT. To investigate effects of *Babesia* infection on drug metabolism, we intraperitoneally inoculated *B. microti* into ICR mice and measured the expression and activity of hepatic cytochrome P450 (CYP) 3A, a major drug-metabolizing enzyme. Twelve days after infection, CYP3A11 mRNA, CYP3A protein and activity and mRNAs of nuclear receptors, which participate in CYP3A expression, were significantly reduced. These results suggest that *B. microti* infection suppresses CYP3A-dependent drug metabolism. Additionally, tumor necrosis factor (TNF)-α and nitric oxide synthase (NOS) 2 mRNAs were induced in the infected mouse liver. Since TNF-α is one of the potent mediators that induce NOS2 and repress CYP3A transcription, the possible involvement of TNF-α in this downregulation of CYP3A was discussed.
KEY WORDS: *Babesia microti*, CYP3A, cytochrome P450, downregulation, drug metabolism.

Babesia sp. is a tick-borne protozoan that parasitizes the erythrocytes in a wide range of animals. It is prevalent worldwide, mainly in tropical and subtropical areas. Infection with *Babesia* causes fever, fatigue and hemolytic anemia lasting from several days to several months in the host, resulting in hyperbilirubinuria, hemoglobinuria and possibly organ failure [15]. Babesiosis is a well-recognized veterinary disease of importance in cattle, horses and dogs and has gained increasing attention as an emerging zoonotic disease issue.

The alteration of drug metabolism under a diseased condition is of clinical importance. Since most drugs are partially or completely biotransformed by hepatic metabolism prior to their elimination from the body, the alteration of hepatic drug metabolism amplifies the clinical toxicity of drugs with a low therapeutic index [19]. Thus, side effects observed in anti-babesiosis chemotherapy [27] might be attributed to an overdose of the drugs caused by repression of metabolism in the liver. It is therefore necessary to evaluate the state of hepatic drug metabolism during a period of anemia caused by *Babesia* infection.

Hepatic cytochrome P450 (CYP) 3A is considered to be the most important and abundantly expressed CYP subfamily and is responsible for the phase I metabolism of 45–60% of clinically administered drugs [2, 13]. CYP3A in humans and animals is also involved in the metabolism of clindamycin and quinine used in the standard treatment for human babesiosis [29, 33]. Among six CYP3A isoforms in mice [10], CYP3A11 is predominantly expressed in the male liver and is the most studied isoform [32]. Expression of CYP3A is regulated by nuclear receptors, pregnane X receptor (PXR), constitutive androstane receptor (CAR) and retinoid X receptor (RXR) α [7, 25, 31].

So far, there have been many reports on the downregulation of CYPs during infections and inflammations induced by endotoxin or proinflammatory cytokines [19]. Among the proinflammatory cytokines, TNF-α is principally produced by the monocyte lineage cells [14] and plays a key role in the development of liver injury [21]. In *Babesia* infection, TNF-α has been presumed to be involved in the pathogenesis of liver necrosis [16]. On the other hand, TNF-α is also one of the potent mediators that suppress hepatic CYP3A expression during inflammation [19]. In addition, TNF-α induces NOS2 in the liver [17] and thereby generates NO that participates in the inhibition of CYP3A activity during inflammation [22].

In this study, we examined the effect of *B. microti* infection on the levels of CYP3A11 mRNA, protein and activity of CYP3A in the mouse liver. We also measured the levels

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Table 1.   Primer pairs used in qRT-PCR

	NCBI accession No.	Forward primer (5'–3')	Exon(s)	Reverse primer (5'–3')	Exon(s)	Product size (bp)	Annea- ling
CYP3A11	NM_007818.3	ATTCCTGGGCCCAAACCTCTGCCA	2	TGGGTCTGTGACAGCAAGGAGAGGC	4	150	60°C
PXR	NM_010936.3	ACGGAAGAACAGCAGGCGCTGAT	4	AACACTGCAGGCAGCCGGAAATC	4/5	104	60°C
CAR	NM_009803.4	CGGCGGGCAGAGAAAGCATCTTT	4	GCCGGAGGCTTGAACTGCACAA	4/5	128	60°C
RXRα	NM_011305.3	TCAATGGCATGGGTCCGCCCTT	2	TGGGTGAATTGAGCTGGGGGCT	2/3	120	60°C
TNF-α	NM_013693.2	ACTTCGGGGTGATCGGTCCCCAAA	1	TTGGTGGTTTGCTACGACGTGGGC	3/4	146	60°C
NOS2	NM_010927.3	GTTCTCAGCCCAACAATACAAGA	1	GTGGACGGGTCGATGTCAC	2	127	60°C
β-actin	NM_007393.3	CACCCGCGAGCACAGCTTCTTT	1	TTGTCGACGACCAGCGCAGCGATA	2	99	60°C

of PXR, CAR and RXRα mRNA and the levels of TNF-α and NOS2 mRNA in the liver.

Male ICR mice (aged 6 weeks, Charles River, Yokohama, Japan) were intraperitoneally inoculated with red blood cells (RBCs) containing 1×10^6 of *B. microti* (Munich strain) obtained from infected mice or RBCs from normal donor mice. The percentage of parasitemia was determined by counting the number of parasitemic RBCs in tail blood smears stained with Giemsa. The hematocrit value of the blood was measured with Celltac α (NIHON KODEN, Tokyo, Japan). In our preliminary experiment, a significant change in CYP 3A activity was observed only 12 days after infection among 3 points in time (10, 12 and 21 days after infection, corresponding to the most severe anemia phase, early phase in recovery from the most severe anemia and recovery phase, respectively). Therefore, mice were sacrificed by cervical dislocation 12 days after inoculation. All the experimental procedures were approved by, and conducted in accordance with, the animal experimentation guidelines of the Kitasato University Animal Ethics Committee. Total RNA was isolated from the liver with TRIzol (Invitrogen, Carlsbad, CA, U.S.A.) and was treated with DNase I (Invitrogen). One microgram of RNA was reverse transcribed to cDNA with MMLV reverse transcriptase (Invitrogen). Quantitative polymerase chain reaction (qPCR) was performed with a KAPA™ SYBR® FAST qPCR Kit (KAPA Biosystems, Woburn, MA, U.S.A.) using a StepOnePlus™ Real-Time PCR System (Applied Biosystems, Carlsbad, CA, U.S.A.). The specific primers used in the present study are listed in Table 1. Primers spanning at least one intron were chosen. A standard curve for each transcript was constructed using the purified PCR product generated for each specific primer pair. All samples were amplified in duplicate. For each PCR reaction, the absence of genomic DNA was confirmed by a reverse transcription negative control. To normalize expression data, β-actin was used as an internal control. Liver microsomal proteins (15.6 μg) were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane. Western blot analysis was performed as previously described [23] using a polyclonal rabbit antibody against rat CYP3A2 (Daiichi Chemical Co., Ltd.. Tokyo, Japan) and horseradish peroxidase conjugated anti-rabbit goat antibody (Cell Signaling Technology, Danvers, MA, U.S.A.), as primary and secondary antibodies, respectively. The bands of CYP3A protein were visualized
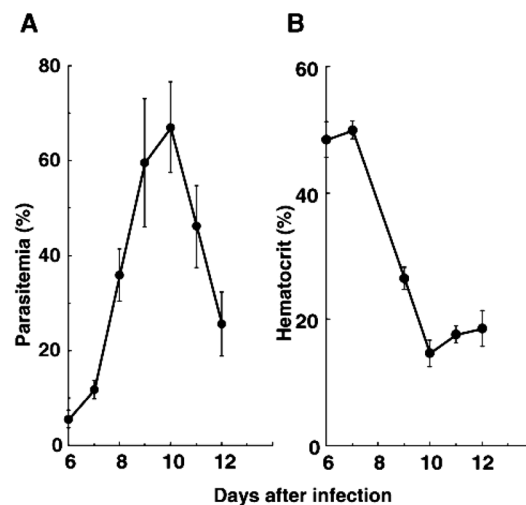

Fig. 1.   Time courses of parasitemia (A) and hematocrit (B) after *B. microti* infection. Representative data from two sets of experiments are shown. Data are means ± SD of 4 mice for an infected group.

with ECL Plus Western Blotting Detection Reagent (GE Healthcare, Buckinghamshire, U.K.) and scanned using a LumiCube (Liponics, Tokyo, Japan). The intensity of the bands was analyzed using the ImageJ software version 1.44l [1]. The preparation of hepatic microsomal protein was performed as previously described [20]. The activity of erythromycin N-demethylase (ERND), representative of CYP3A, was measured according to the method of Arlotto *et al.* [3]. All the data are means ± SD. Statistical comparisons were made by Student's-*t* test.

B. microti infection successfully caused parasitemia and anemia. Parasitemia achieved a peak (67 ± 9.5%) 10 days after infection and then went into a decline (26 ± 6.7%) 12 days (Fig. 1A). Conversely, hematocrit showed the lowest value (hematocrit; Ht 15 ± 2.0%) 10 days after infection and then a slight recovery (Ht 18 ± 2.8%) 12 days (Fig. 1B). The variations of these parameters were consistent with the results of a previous study by Igarashi *et al.* [16].

In this study, we demonstrated that *B. microti* infection caused the downregulation of hepatic CYP3A in mice at an early phase in recovery from the most severe anemia. qPCR and Western blot analyses revealed that CYP3A11 mRNA and CYP3A protein were significantly repressed to 27 ± 8.0
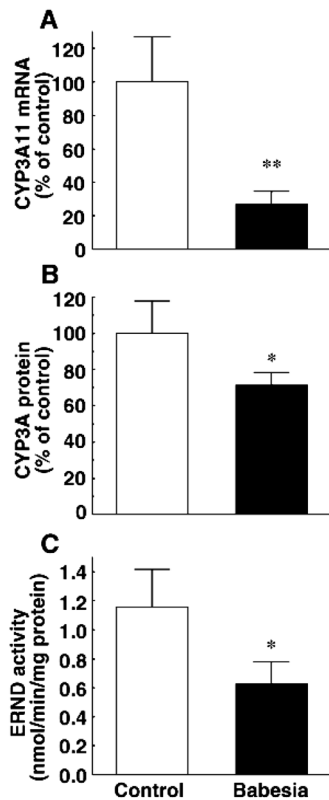
Fig. 2.   Effects of *B. microti* infection on CYP3A in the mouse liver 12 days after inoculation: A: CYP3A11 mRNA, B: CYP3A protein, C: ERND activity. Representative data from two sets of experiments are shown. Data are means ± SD of 4 mice for each treatment group. * and **: Significantly different from the control performed concurrently (*P*<0.05 and *P*<0.01, respectively).

Fig. 3.   Effects of *B. microti* infection on mRNA of nuclear receptors, PXR, CAR and RXRα, in the mouse liver 12 days after inoculation. Representative data from two sets of experiments are shown. Data are means ± SD of 4 mice for each treatment group. **: Significantly different from the control performed concurrently (*P*<0.01).

Fig. 4.   Effects of *B. microti* infection on mRNA of TNF-α and NOS2 in the mouse liver 12 days after inoculation. Representative data from two sets of experiments are shown. Data are means ± SD of 4 mice for each treatment group. Significantly different from the control performed concurrently (*P*<0.01).

and 71 ± 7.2% of the control values, respectively, 12 days after *B. microti* infection (Fig. 2A and 2B). At this point in time, ERND (CYP3A) activity was also significantly decreased to 54 ± 13% of the control values (Fig. 2C). These observations indicate that the decrease in CYP3A activity results from the expressional repression of CYP3A by *B. microti* infection.

We also observed the transcriptional repression of nuclear receptors involved in CYP3A expression. The levels of PXR, CAR and RXRα mRNA were markedly reduced to 47 ± 9.8, 32 ± 7.6 and 47 ± 5.8% of the control values, respectively, in the infected mouse liver (Fig. 3). CYP3A expression is regulated by nuclear receptors, PXR and CAR, which share RXRα as their heterodimeric partner [7, 25, 30]. PXR activity in liver nuclear extracts assayed by gel EMSA exhibit a strict correlation with mRNA levels [4]. These reports suggest that alteration of CYP3A mRNA is positively correlated with that of mRNAs of these nuclear receptors. In fact, Beigneux *et al*. reported that reduction in cytochrome P450 enzyme expression is associated with reduction of CAR and PXR mRNA levels in a lipopolysac-
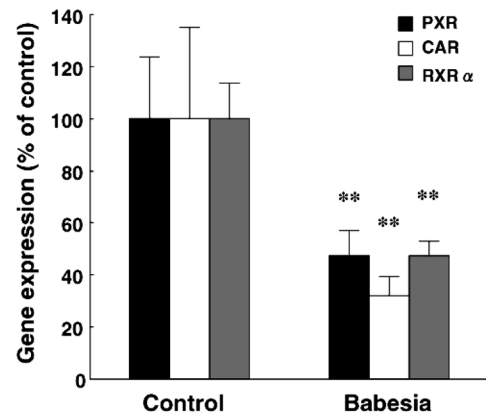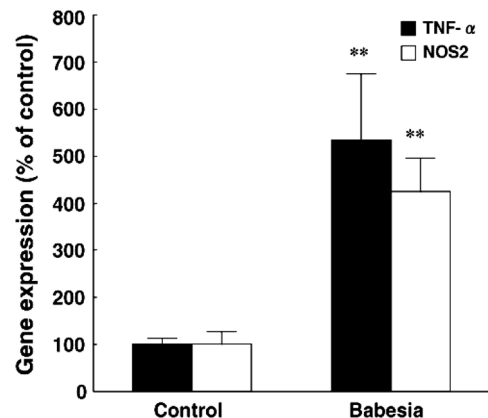
charide (LPS)-induced inflammation model [5]. Thus, reduction in the levels of PXR, CAR and RXRα mRNA observed in our study may contribute to the expressional repression of CYP3A by *B. microti* infection.

As shown in Fig. 4, the levels of TNF-α and NOS2 mRNA in the infected mouse liver were increased to 533 ± 140 and 424 ± 72% of the control value, respectively. TNF-α is principally produced by monocyte lineage cells such as macrophages and Kupffer cells [14]. Activation of these cells by infections and LPS administration leads to the induction of TNF-α, and then TNF-α elicits the induction of NOS2 through the activation of NFκB, a transcription fac-

tor, after binding of TNF-α to its receptor on the surface of Kupffer cells and hepatocytes in the liver [28]. Therefore, it is speculated that TNF-α is increased following the induction of its mRNA in Kupffer cells in *B. microti*-infected mouse liver and actually elicits the increase in NOS2 mRNA through NFκB activation in Kupffer cells and hepatocytes. The speculation of NFκB activation in *B. microti* infection would be supported by our finding of the decrease in RXRα mRNA in the infected mouse liver (Fig. 3) because NFκB activation could decrease the mRNA of RXR isoforms through transcriptional and posttranscriptional mechanisms [34].

TNF-α is also one of the potent mediators that downregulate hepatic CYP3A during infection and inflammation [19]. Recently, it has been demonstrated that NFκB activated by TNF-α directly disrupts the binding of PXR-RXRα complex to regulatory elements of CYP3A4 and thereby represses its expression in HepG2 cells [12]. In this study, we observed increases in TNF-α and NOS2 mRNA (Fig. 4) and a decrease in RXRα mRNA (Fig. 2) in the infected mouse liver, implying NFκB activation by TNF-α in Kupffer cells and hepatocytes. Hence, it is probable that TNF-α is involved in the expressional repression of CYP3A by *B. microti* infection through the direct disruptive action of activated NFκB and/or through the indirect action that mediates the repression of RXRα.

As another candidate mediator that could suppress CYP3A expression by *B. microti* infection, we take notice of interferon (IFN)-γ. *B. microti* infection increases the serum level of IFN-γ through the activation of T cells in association with parasitemia [9]. IFN-γ has the ability to downregulate CYP3A in rat hepatocytes [26]. Thus, IFN-γ might also participate in the downregulation of CYP3A during babesiosis in a different manner from TNF-α.

Recently, it has been reported that murine malaria, which causes hemolytic anemia and subsequently bilirubinemia, transcriptionally represses hepatic CYP1A2, CYP2E1 and CYP3A11 [8]. Carvalho *et al.* has speculated that toll-like receptor (TLR) and NFκB may be involved in repression of these CYPs after malaria infection. In *Babesia* infection, it has been more recently reported that lipids from *B. bovis* induce TLR2-mediated release of proinflammatory cytokines such as TNF-α and interleukin-6 (IL-6) from activated macrophages [11]. IL-6 is also one of the proinflammatory cytokines that downregulate hepatic CYP3A [19]. It seems that upregulation of TNF-α in the liver by *B. microti* infection results from TLR-mediated Kupffer cell activation. Therefore, it is predicted that the downregulation of CYP3A by *B. microti* infection may be caused by proinflammatory cytokines produced through TLR-mediated Kupffer cell activation similar to *B. bovis*.

Although we could not demonstrate the involvement of NO in the inhibition of CYP3A activity by *B. microti* infection in this study, we confirmed the induction of NOS2 mRNA in the infected liver. NO is regarded as one of the posttranslational regulators of hepatic CYPs during LPS-induced inflammation because of two reasons, 1) prevention of the inhibition of CYP activities with NOS inhibitor [22] and 2) observation of no decreases in some CYP activities in NOS2 knockout mice [22]. Hereafter, it will be necessary to examine whether the induction of NOS2 mRNA by *B. microti* infection actually leads to the inhibition of CYP3A activity.

Interestingly, there is a sex difference in disease susceptibility in the ICR mouse strain. Male ICR mice are susceptible to the diabetogenicity caused by infection with the D variant of encephalomyocarditis virus, while female mice are resistant [6]. This pattern of disease susceptibility and resistance can be reversed if males are treated with estrogen and females are treated with testosterone before virus infection. Since sex steroids can regulate immunity [24], a sex difference in this disease susceptibility reflects sexual dimorphism in immune response in the ICR strain. Considering that the induction of TNF-α mRNA in this study and the increase in serum IFN-γ previously reported [9] result from the host defense immune response against *B. microti* infection, it would be expected that the CYP response to *B. microti* infection in female ICR mice would be different from that in male ICR mice.

We demonstrated, for the first time, the downregulation of a major CYP, namely, CYP3A, in *Babesia*-infected animals at an early phase in recovery from the most severe anemia. Although the expression and activity of CYPs are also modulated in other infection and inflammation models, the changes varied with each model. For example, systemic IL-1 injection clearly decreased CYP2D [18], while meningitis failed to decrease hepatic CYP2D in spite of an apparent decrement of CYP1A, CYP2C11 and CYP3A [23]. Comprehensive comparisons of CYP profiles between experimental models will characterize drug metabolism during *B. microti* infection.

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