Repression of Hepatic Cytochrome P450 2D Expression in Mice during Babesia microti Infection

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ABSTRACT. To examine the effect of Babesia infection on the level of the drug-metabolizing enzyme hepatic cytochrome P450 (CYP) 2D, we intraperitoneally inoculated Babesia microti into male ICR mice. CYP2D protein and CYP2D9 mRNA were significantly decreased at 12 days after infection with B. microti. The activity of buniitolol 4-hydroxylase, which is catalyzed by CYP2D, was also significantly decreased. The mRNA levels of transcriptional regulators of CYP2D9, hepatocyte nuclear factor 4α and signal transducer and activator of transcription 5b, were markedly suppressed. These results suggest that Babesia infection represses CYP2D expression in the mouse liver. The decline in CYP2D-dependent drug metabolism might be involved in the incidence of adverse drug reactions in patients with babesiosis.

KEY WORDS: Babesia microti, CYP2D, cytochrome P450

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Babesiosis is a parasitic infection caused by hemotropic protozoa of the genus Babesia. It is a well-recognized disease of veterinary importance in both wild and domestic animals and has gained attention as an emerging zoonotic disease. Infection with Babesia leads to a host-mediated pathology and hemolysis, resulting in anemia, hyperbilirubinuria, hemoglobinuria and possibly organ failure [11]. Chemotherapy against babesiosis is indicated in only moderately to severely ill cases, and side effects associated with the drug used for treating babesiosis in humans have been observed in clinical studies [8, 14]. As most drugs are partially or completely biotransformed by hepatic metabolism prior to their elimination from the body, it is of clinical importance to evaluate alterations in drug metabolism during Babesia infection.

Hepatic cytochromes P450 (CYPs) are heme proteins, existing in several isoforms, that function as monoxygenases, which play a key role in the metabolism of clinically used drugs, chemical compounds and steroids, leading to detoxification or activation of the parent compounds [20]. We previously reported that the levels and activities of hepatic CYP3A were down-regulated during anemia caused by Babesia infection [22]. However, it remains unclear whether Babesia infection has any effect on other CYPs in the mouse liver. Furthermore, to identify the mechanism underlying CYP2D regulation during Babesia infection, the mRNA levels of hepatocyte nuclear factor 4α (HNF4α) and signal transducer and activator of transcription 5b (STAT5b) were also examined as these act transcriptional regulators of CYP expression, including that of the CYP2D9 gene in the male mouse liver [13].

Male ICR mice (aged 6 weeks, Charles River, Yokohama, Japan) were intraperitoneally inoculated with red blood cells (RBCs) containing $1 \times 10^9$ 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 parasitic RBCs in tail blood smears stained with Giemsa. Hematocrit in the blood was measured using Celltac α (NIHON KODEN, Tokyo, Japan). In our preliminary experiment, in which we examined parameters at 3 time points (10, 12 and 21 days after the infection, corresponding to most severe anemia phase, early phase in recovery from anemia and recovery phase, respectively), the only significant change in CYP2D activity was observed at 12 days after infection. Therefore, mice were sacrificed by cervical dislocation 12 days after infection. Liver samples and hepatic
microsomes were prepared, and protein concentrations were determined as previously described [15, 19]. All the animal experiments were conducted in a biosafety level 2 containment laboratory with clearance from the Animal Ethic Committee of Kitasato University.

Extraction of total RNA and reverse transcription quantitative polymerase chain reaction (RT-qPCR) were performed as previously reported [22]. 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 RT-qPCR reaction, the absence of genomic DNA was confirmed by a reverse transcription negative control. To normalize expression data, β-actin (ACTB) was used as an internal control. The thermal cycling program consisted of 2 min at 95°C for enzyme activation and 40 cycles of denaturation for 20 sec at 95°C, and annealing for 30 sec at 60°C.

The activity of bunitrolol 4-hydroxylase (BTL), which is catalyzed by CYP2D, was measured according to the method of Ishizuka et al. [12]. Western blot analysis was performed as previously described [22]. In brief, liver microsomal proteins (15.6 µg) were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a polyvinylidene fluoride membrane. After immunoreaction using a polyclonal rabbit antibody against human CYP2D6 (Daichi Pure Chemical Co., Tokyo, Japan), as the primary antibody, followed by horseradish peroxidase conjugated anti-rabbit goat antibody (Cell Signaling Technology, Danvers, MA, U.S.A.), the bands of CYP2D protein were detected using an ECL Plus Western Blotting Detection System (GE Healthcare Japan, Tokyo, Japan). An image of the bands was captured using Lumicube (Liponics, Tokyo, Japan). Band intensity was analyzed using ImageJ [1].

All the data are shown as the mean ± SD. Statistical comparisons were made by Student’s t-test. A P-value<0.05 was regarded as statistically significant.

*Bernol* infection caused parasitemia and anemia. Parasitemia reached a peak (83 ± 4.0%) at 10 days after infection and then began to decline (25 ± 5.5%) at 12 days (Fig. 1A). Conversely, the hematocrit values were lowest (13 ± 2.4%) at 10 days after infection and then recovered slightly (19 ± 2.8%) at 12 days (Fig. 1B).

CYP2D9 mRNA was significantly decreased (Fig. 2A). Western blot analyses and Bunitorol metabolism revealed that the CYP2D protein and activity levels were also decreased at the same point in time (Fig. 2B and 2C). The decreases in CYP2D9 mRNA, CYP2D protein and CYP2D activity were 16.3, 64.0 and 58.2% of the control, respectively. These results suggest that *Babesia* infection has an inhibitory effect on the expression and activity of hepatic CYP2D in mice.

We previously reported an increase in tumor necrosis factor α (TNFα) mRNA in the mouse liver after *Babesia* infection [22]. Pro-inflammatory cytokines, such as interleukin (IL)-1, TNFα and interferon gamma, are increased in mouse serum during babesiosis [2, 24]. These cytokines act to decrease the expression and activity of hepatic CYP2D in rodents [17]. Therefore, we speculate that the downregulation of CYP2D caused by *Babesia* infection may be caused through the action of pro-inflammatory cytokines.

A concomitant decrease in the level of BTL activity, which is catalyzed by CYP2D, was observed with that of CYP2D protein, although the extent of the decrease in CYP2D9 mRNA was greater than that of the decrease in the activity and protein level of CYP2D in this study. Recently, a global mass spectrometry-based proteomics approach has demonstrated the expression of four CYP2D proteins, CYP2D9, 2D10, 2D22 and 2D26, in the male mouse liver [9]. BTL activity is mostly catalyzed by CYP2D in mouse liver microsomes [16]. In addition, polyclonal anti-CYP2D6 antibody used in this study has the potential to cross-react with these CYP2D proteins in mice as CYP2D6 displays high amino acid sequence homology with CYP2D9 (71%).

| Table 1. Primer pairs used in RT-qPCR |
|-------------------------------------|
| **NCBI Accession No.** | **Forward primer (5′- 3′)** | **exon (s)** | **Reverse primer (5′- 3′)** | **exon (s)** | **Product size (bp)** |
| CYP2D9 | NM_010006.2 | TGCTCATGGTGTTGCGTGACCT | 6 | CTTGTGGACTCTGGCCTGACA | 6/7 | 117 |
| HNF4α | NM_008261.2 | TTGCCGCGATGGAAGATGGCCGA | 1 | AGATGGGCCGACCCGCTGACCC | 1/2 | 110 |
| STAT5b | NM_014849.3 | TCAGGCGGACCTGCTCCTAAACGCT | 4 | TCAGGCGGACCTGCTCCTAAACGCT | 5 | 101 |
| ACTB | NM_007393.3 | CACCCCGCGACACGGCTTCTTT | 1 | TTGTCGACGACCACGCGGCT | 1 | 99 |

Fig. 1. Timecourses for parasitemia (A) and hematocrit (B) after *Babesia microti* infection. Representative data for two sets of experiments are shown. Data are shown as the mean ± SD of 4 mice from the infected group.
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2D10 (70%), 2D22 (76%) and 2D26 (71%) (protein blast; http://blast.ncbi.nlm.nih.gov/Blast.cgi). Therefore, the disparity in the decreases in level between CYP2D9 mRNA and the protein level and activity of CYP2D may be explained by the participation of CYP2D proteins other than CYP2D9 in BTL activity and immunoreactivity.

We observed that the level of HNF4α mRNA was significantly decreased in the infected mouse liver (Fig. 3). Since HNF4α acts as a positive regulator of the liver-specific transcription of CYP genes [13], the decrease in expression of this regulator may lead to a down-regulation of CYP2D9 as a result of Babesia infection. TNFα decreases the amount of HNF4α protein in HepG2 cells through the nuclear factor κB and MEK1/2 pathways [21]. In addition, a TNFα-induced reduction in sex hormone-binding globulin expression is mediated by the downregulation of HNF4α [23]. Therefore, a quantitative alteration in HNF4α could be involved in the repression of CYP2D9 expression resulting from Babesia infection. In addition, suppression of the DNA-binding ability of HNF4α by nitric oxide (NO) contributes to a downregulation of CYP2D6 during inflammation or cytokine stimulation [7]. Considering the induction of NO synthase (NOS) 2 in the mouse liver during Babesia infection [22], we speculate that the functional inhibition of HNF4α by NO might also contribute to the down-regulation of CYP2D9.

We also observed the repression of STAT5b mRNA in the infected mouse liver (Fig. 3). The expression of CYP2D9 requires the growth hormone (GH) pulse-activated transcription factor STAT5b [13]. The decrease in STAT5b mRNA could contribute to the repression of CYP2D9 during B. microti infection. Moreover, endotoxin lowers the levels of nuclear phosphorylated STAT5b and reduces the binding of phosphorylated STAT5b to DNA along with a marked increase in TNFα and IL-6 mRNA in the liver [4]. IL-6 medi-
ates hepatic GH resistance by the time-dependent inhibition of GH-inducible promoter activity which is associated with reductions in STAT5 DNA binding [3]. The down-regulation of CYP2D9 might be caused by the inhibition of the DNA binding of STAT5b through the action of inflammatory cytokines.

We previously reported that hepatic CYP3A as well as mRNA levels of nuclear receptors, that participate in CYP3A expression, pregnane X receptor (PXR), constitutive androstane receptor (CAR) and retinoid X receptor α (RXRα), were down-regulated after B. microti infection [22]. The down-regulation of CYP2D and CYP3A and the reduction in the mRNA levels of their transcriptional regulators appear to be due to insufficient liver function after B. microti infection, but we also observed that the hepatic expression of CYP2E1 mRNA was up-regulated (267% of control, P<0.05) at 12 days after infection (unpublished data). These results suggest that the down-regulation of CYP2D and CYP3A does not arise from hepatic dysfunction after infection, indicating that hepatic CYPs could be differentially regulated during B. microti infection.

The results of this study demonstrated the repression of CYP2D expression during anemia resulting from Babesia infection. The decline in CYP2D-dependent metabolism can lead to an increase in the concentration of substrate drugs, such as antihypertensive agents and antidepressants, in the blood, leading to an increase in the incidence of adverse drug reactions. Therefore, careful selection of drug dosage is needed in the treatment of hypertensive or depressive patients with babesiosis.

Unfortunately, we could not clarify the mechanism underlying the down-regulation of hepatic CYP2D and CYP3A caused by B. microti infection in this study. We now put forward TNFα as a major candidate for the mediator of the decrease in CYP2D and CYP3A after B. microti infection. TNFα is released from macrophages or Kupffer cells after B. microti infection [10]. Nuclear factor κB (NFκB) in hepatocytes, after its activation by TNFα, then lowers the DNA binding of transcriptional regulators of CYP2D and CYP3A, such as PXR-RXRα complex and HNF4α [6, 21]. A NO-mediated pathway also exists in the NFκB-induced reduction in the binding of HNF4α to CYP2D regulatory sites in the DNA [7]. TNFα could reduce the binding of phosphorylated STAT5b to DNA [4]. Therefore, we hypothesized that the TNFα-NFκB axis plays a pivotal role in the down-regulation of CYP2D and CYP3A during B. microti infection (Fig. 4). A further study is needed to elucidate the mechanism underlying the alteration in CYPs in Babesia infection.

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Fig. 4. Proposed mechanism of down-regulation of hepatic CYP2D and CYP3A in mice infected with Babesia microti. TNFα; tumor necrosis factor α, NFκB; nuclear factor κB, NOS2; nitric oxide synthase 2, NO; nitric oxide, PXR; pregnane X receptor, CAR; constitutive androstane receptor, RXRα; retinoid X receptor α, HNF4α; hepatocyte nuclear factor 4α, STAT5b; signal transducer and activator of transcription 5b.

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