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TLR4-dependent and -independent regulation of hepatic cytochrome P450 in mice with chemically induced inflammatory bowel disease

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ABSTRACT

The transcription and protein expression of many cytochrome P450 (P450) genes are down-regulated in animal models of inflammation and infection. We determined previously that hepatic P450 mRNAs are selectively regulated in a mouse model of enteropathogenic bacterial infection, and that this regulation was not dependent on the lipopolysaccharide (LPS) receptor protein toll-like receptor 4 (TLR4). In the dextran sulfate sodium (DSS) model of chemically induced inflammatory bowel disease (IBD), the reduction in activities of several hepatic P450 enzymes were concluded to be partially dependent on LPS from commensal bacteria [Masubuchi Y, Horie T. Endotoxin-mediated disturbance of hepatic cytochrome P450 function and development of endotoxin tolerance in the rat model of dextran sulfate sodium-induced experimental colitis. Drug Metab Dispos 2004;32:437–441]. In the present study, we sought to determine whether colitis induced by LPS regulates hepatic P450 mRNA and protein expression similarly to infectious colitis, and to determine the role of TLR4 in the response to DSS colitis. The role of LPS in the response to DSS was further examined by comparison with the effects of injected LPS. We demonstrate that administration of DSS results in the down-regulation of multiple P450 enzymes in mouse liver. However, there are discernable differences in the pattern of P450 expression in the two models. Some effects of DSS-induced colitis are TLR4-dependent, and others are not. In contrast, the effects of injected LPS on hepatic P450 mRNA expression are entirely TLR4-dependent. Thus, our results indicate that the pattern of hepatic P450 expression, and the mechanism of regulation, during inflammation of the bowel depend on the etiology of the disease.

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Abbreviations: P450, cytochrome P450; TLR4, toll-like receptor 4; DSS, dextran sulfate sodium; TNBS, trinitrobenzenesulfonic acid; IL, interleukin; TNFα, tumor necrosis factor alpha; LPS, lipopolysaccharide; PMN, polymorphonuclear lymphocytes; PPARα, peroxisome proliferator activated receptor alpha; AGP, α1-acid glycoprotein; FBG, fibrinogen alpha polypeptide; AGT, angiotensinogen; EPEC, enteropathogenic *Escherichia coli*; DSS, dextran sulfate sodium; IBD, inflammatory bowel disease; RT-PCR, reverse transcription-polymerase chain reaction; GAPDH, glyceraldehyde phosphate dehydrogenase; PCR, polymerase chain reaction.

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1. Introduction

Infectious and inflammatory disease states cause an impairment of hepatic drug metabolism in animals and humans. This is associated with the down-regulation of hepatic cytochrome P450 (P450) enzymes, Phase II drug metabolizing enzymes, and drug transporters [1,2]. Down-regulation of P450s in inflammatory states results in an impairment of associated drug clearance activities in humans [3–5]. However, the effects on individual P450 enzymes are dependent on the nature of the disease or inflammatory stimulus, and so the drugs affected clinically are likely to differ in different disease states. In support of this view, we recently demonstrated that infection with the murine pathogen *Citrobacter rodentium* causes a pattern of hepatic cytochrome P450s down-regulation that is more selective than occurs in the lipopolysaccharide (LPS) model of sepsis [6]; whereas, down-regulation of UDP-glucuronosyltransferases is similar in both models [7].

Infection by *C. rodentium* is an established model for human enteropathogenic *E. coli* infection, but it also causes intestinal pathology that is very similar to that occurring in human inflammatory bowel disease (IBD), which afflicts more than one million people in the North American continent alone [8]. The two forms of IBD that occur in humans, ulcerative colitis and Crohn's disease, are characterized by an exaggerated T-cell dependent inflammatory response in the intestinal mucosa, directed against luminal antigens or against commensal gastrointestinal bacteria [9,10].

Two well-characterized models of chemically induced IBD exist, and each has been used to study the regulation of hepatic P450 function or expression in humans or in animals. Masubuchi and Horie used the dextran sulfate sodium (DSS) model of chemically induced IBD to study the effect of IBD on hepatic P450 activities of rats. After 7 days of drinking 3% DSS, hepatic microsomal enzyme activities associated with CYP3A2, 2C11, 1A2 and 2E1, but not 2D2, were down-regulated [11]. It is unclear whether or not these changes in P450 activity reflected changes in gene expression. These effects on P450 activity were selectively prevented by treatment with polymyxin B (3A2 and 2E1) or metronidazole (3A2, 2C11 and 2E1), which led the authors to conclude that endotoxins of commensal bacteria are likely involved in some of the effects [11]. In these rats, portal blood endotoxin levels were elevated but liver weight and serum alanine aminotransferase levels were normal, suggesting lack of liver damage. Bacterial endotoxin, LPS, is a major component of the cell walls of Gram negative bacteria, and is responsible for the activation of the innate immune system by bacterial infection. The actions of LPS are achieved via the toll-like receptor 4 (TLR4) protein [12]. Several other proteins associate with TLR4 to produce an optimal cellular response, including CD14, MD-2 and LPS binding protein [13]. It was demonstrated more than 20 years ago that the down-regulation of total P450 and P450-associated enzyme activities in mouse livers caused by LPS injection was absent in the C3H/HeJ strain that lack a functional TLR4 [14]. We also used these mice to demonstrate that P450 down-regulation in the *C. rodentium* model of infection is TLR4-independent [6].

Weidenbach et al. [15] studied P450 activity in perfused livers from animals that had received a rectal instillation of an ethanolic solution of trinitrobenzenesulfonic acid (TNBS). The formation of monoethylgycylxylidide from lidocaine, a dealkylation reaction catalyzed by CYP3As in humans, was decreased by approximately 30% at 1 and 2 days after induction of colitis, and recovered by 7 days. More recently, the expression and activities of specific rat hepatic P450 enzymes in this model were studied [16], and it was found that hepatic CYP3A2 and 2C11 proteins were more susceptible to down-regulation than were CYP1A2 and 2E1. The effects of TNBS-induced colitis were also partially blocked by polymyxin B [16], again leading to the conclusion that the observed down-regulations are at least partially dependent on bacterial endotoxin.

As noted above, we have characterized the selective regulation of mouse liver P450s in the *C. rodentium* model of colonic infection. It is important to ask whether or not IBD of non-infectious etiology causes similar or different changes in hepatic P450 expression to those caused by enteropathogenic bacteria in the same species. To date, the only studies published on the effects of chemically induced IBD have been conducted in rats. A goal of the current research, therefore, was to characterize the regulation of mouse P450 expression in the DSS model of IBD, and to compare it to the known regulation during *C. rodentium* infection. Secondly, the idea that LPS is involved in the regulation of P450 expression during DSS-induced IBD in rats is in contrast to our finding that down-regulation of hepatic P450s in the *C. rodentium* model of infectious colitis was TLR4-independent because it was not attenuated in C3H/HeJ mice that lack functional TLR4 [6]. Therefore, a second goal of this study was to investigate the role of LPS in P450 down-regulation in the DSS model of IBD using the same genetic model of TLR4 deficiency.

2. Materials and methods

2.1. Chemicals, animals, and treatments

All protocols were reviewed and approved by the Institutional Animal Care and Use Committee of Emory University. Unless otherwise specified, all the reagents and chemicals were obtained from Sigma–Aldrich (St. Louis, MO). Female wildtype (C3H/HeOuJ; HeOu) and TLR4-mutant (C3H/HeJ; HeJ) mice were obtained from Jackson Laboratory (Bar Harbor, ME). The mice were housed in groups of six to a cage, and were acclimatized to the animal facility for at least 1 week before the beginning of an experiment. In the experiments involving DSS-induced IBD, 8–10-week-old mice were administered 3% DSS *ad libitum* as their sole source of drinking water. Control mice received normal drinking water. Six mice from each group were sacrificed 3, 5 and 7 days after the start of DSS administration. Livers were collected, rinsed in cold 1.15% potassium chloride, and stored at −80 °C for later RNA or microsome preparation. Blood was collected from the animals at sacrifice, and allowed to clot for 30 min at room temperature. Serum was separated by centrifugation and stored at −20 °C until analyzed.

For the experiments involving LPS administration, mice were injected i.p. with 1 mg/kg LPS (*E. coli* 0127:B8, chromatographically pure, Sigma–Aldrich; or *E. coli* 0111:B4, ultrapure,

Invivogen, San Diego, CA) dissolved in sterile saline, and were killed 12 or 24 h after injection. Control mice received an i.p. injection of sterile saline. Food was withheld from the animals after the injection. Livers and serum were collected and stored as stated above.

2.2. Microsome preparation

Liver microsomes were prepared by differential centrifugation and stored at −80 °C [17]. Microsomal protein concentrations were determined using a bicinchoninic acid protein assay kit (Thermo Fisher Scientific, Inc, Rockport, IL) using bovine serum albumin as the protein standard

2.3. Real-time Reverse Transcriptase-PCR

Total liver RNA was isolated using RNA-Bee reagent (Tel-Test, Friendswood, TX) using the protocol provided by the manufacturer. The total RNA concentration was estimated from its absorbance at 260 nm, and RNA purity and integrity were confirmed by formaldehyde–agarose gel electrophoresis with ethidium bromide staining. Purified total RNA was reverse-transcribed using the SuperScript First-Strand Synthesis System for the reverse transcriptase-polymerase chain reaction (RT-PCR) kit (Invitrogen, Carlsbad, CA), according to the manufacturer's protocol. The relative expression of mouse liver mRNAs was measured by real-time RT-PCR using the ABI PRISM 7000 Sequence Detection System and SyBr Green Master Mix reagent (Applied Biosystems, Bedford, MA) as described previously [18]. Results are expressed as relative levels of target mRNA, normalized to levels of the housekeeping gene GAPDH, as calculated by the ΔΔCt method [19], The expression level in control samples was arbitrarily set at 1. All primer sets yielded a single PCR product of expected size by agarose gel electrophoresis, and specificity was routinely monitored by checking product dissociation curves in each reaction well.

2.3.1. Primer sequences

All primers used in this study for real-time PCR are described in a previous publication previously [18]. Primers were custom-synthesized on a 50-nmol scale, desalted and lyophilized, by MWG Biotech, Inc. (High Point, NC). They were diluted to 100 mM in deionized water and stored at −80 °C.

2.4. Western blotting

P450 protein levels in mouse hepatic microsomes were measured by Western blotting and chemiluminescent detection as described previously [18]. P450 antibodies were generously provided by Dr. James Halpert (University of Texas Medical Branch, Galveston, TX; rat CYP2B and 3A), Dr. Gordon Gibson (University of Surrey, Guildford, UK; rat CYP4A), Dr. Magnus Ingelman-Sundberg (Karolinska Institute, Stockholm, Sweden; rat CYP2E1), and Dr. Masahiko Negishi (National Institute of Environmental Health Sciences, mouse CYP2D9). Polyclonal antibodies to rat CYP2B, 3A, 4A, 2D and 2E proteins were diluted 1:5000, whereas CYP2B and 2C antibodies were diluted 1:20,000. All assays were performed within a linear range, and the intensity of stained bands was measured by laser densitometry.

2.5. Statistical analysis

Differences between groups were determined by one-way ANOVA followed by Tukey's or Dunnett's test, or by Student's t-test, as appropriate. The level of significance was set at $P < 0.05$.

3. Results

3.1. DSS model of IBD

After 3 or 5 days of DSS treatment, no changes were noted in the behavior or appearance of the mice. After 7 days of drinking DSS, the mice were noticeably sluggish in their movements. Bloody stool was observed in the 5-day treated group: this was

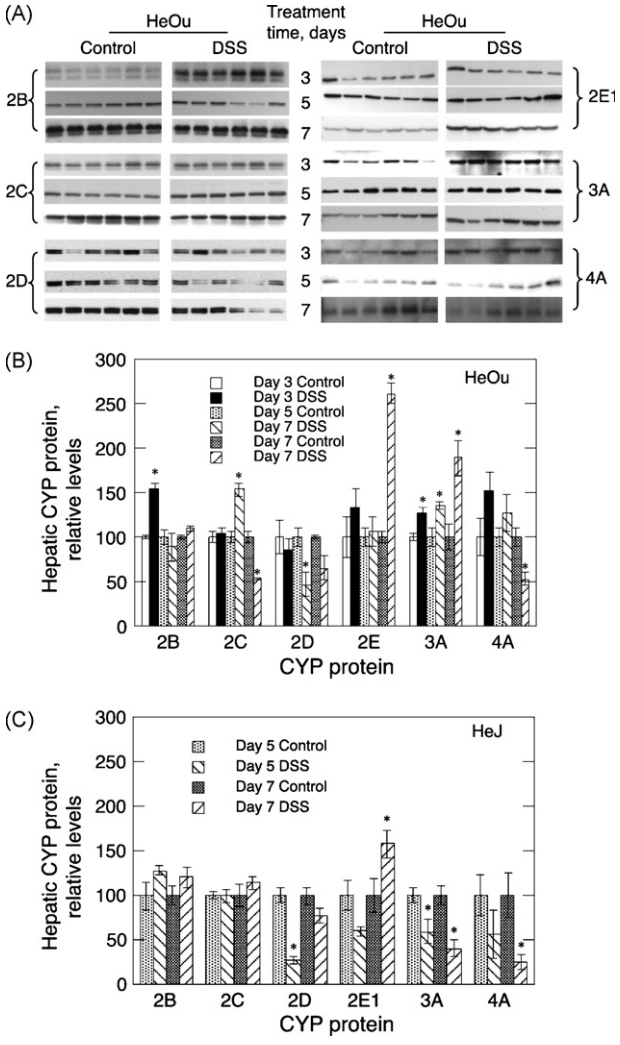

Fig. 1 – Effect of DSS administration on microsomal P450 proteins in mouse liver. HeOu or HeJ mice were treated with DSS, and livers were harvested at the indicated times for measurement of P450 protein levels by Western blotting as described in Section 2. (A) Western blots of samples from HeOu mice; (B) quantitative analysis of the data in panel A; (C) quantitative analysis of Western blots from HeJ mice. Values represent means ± S.E.M. of 6 mice per group. *P < 0.05 compared to controls, Student's t-test.

more severe in the 7-day treated mice, but negligible in the 3-day treated group. Drinking DSS had no effect on daily food consumption or body weight gain (Supplemental Fig. 1).

The effects of DSS-induced colitis on hepatic microsomal P450 protein levels in wildtype HeOu mice are shown in Fig. 1A and B. The five P450 protein subfamilies studied each exhibited a different pattern of regulation. CYP2B proteins were transiently increased by 55% after 3 days of DSS exposure and returned to control levels at 5 and 7 days. CYP2C proteins also exhibited a similar transient induction, but at 5 days, before descending to 54% of control on day 7. CYP2D proteins were significantly down-regulated at 5 days, and returned towards control levels at 7 days. CYP2E1 was significantly elevated to 261% of control on day 7 only, but CYP3A proteins were significantly elevated at all three time points. CYP4A proteins were significantly down-regulated to ~50% of control at 7 days of treatment. The effects of DSS on 2C proteins were TLR4-dependent, since they were not observed in TLR4-deficient HeJ mice (Fig. 1A and C). The induction of CYP2E1 on day 7 was still observed in the HeJ mice, although it was slightly attenuated. CYP2D and 4A

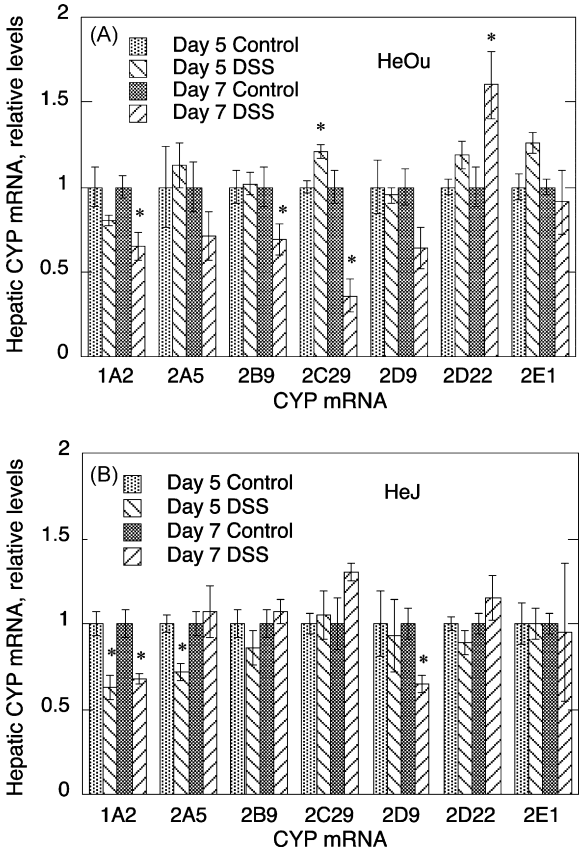

Fig. 2 – Effect of DSS administration on hepatic CYP1 and CYP2 family mRNAs in HeOu and HeJ mouse livers. HeOu or HeJ mice were allowed to drink water or 3% DSS solution, and livers were harvested at the indicated times for measurement of CYP1 and CYP2 mRNA expression by real-time RT-PCR as described in Section 2. (A) mRNA expression in HeOu mice; (B) mRNA expression in HeOu mice. Values represent means ± S.E.M. of 6 mice per group. *P < 0.05 compared to controls, Student's t-test.

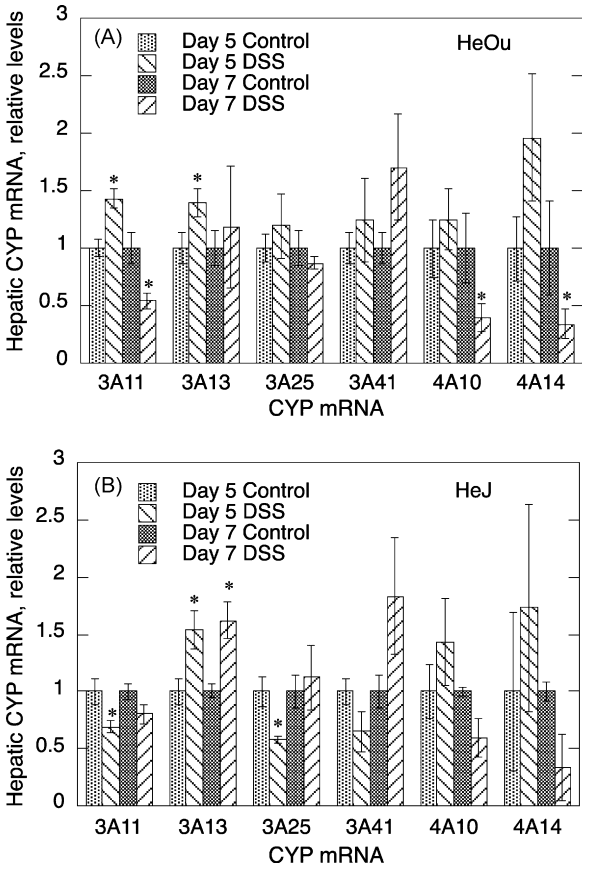

Fig. 3 – Effect of DSS administration on hepatic CYP3 and CYP4 family mRNAs in HeOu and HeJ mouse livers. HeOu or HeJ mice were allowed to drink water or 3% DSS solution, and livers were harvested at the indicated times for measurement of CYP3 and CYP4 mRNA expression by real-time RT-PCR as described in Section 2. (A) mRNA expression in HeOu mice; (B) mRNA expression in HeOu mice. Values represent means ± S.E.M. of 6 mice per group. *P < 0.05 compared to controls, Student's t-test.

protein down-regulations in this model of IBD were not different between genotypes. Notably, CYP3A proteins were down-regulated in the HeJ mice, whereas they were induced in the HeOu strain.

The regulation of P450 mRNAs belonging to families CYP1, 2, 3 and 4 in the livers of mice with DSS-induced IBD are shown in Figs. 2 and 3. In HeOu mice, CYP1A2, 2B9, 2C29, 3A11, 4A10 and 4A14 were significantly down-regulated only after 7 days of treatment (Figs 2A and 3A). Transient small inductions of CYP2C29 and 3A11 were observed at treatment day 5. CYP2D9, 2D22, 2E1, 3A25 and 3A41 were not significantly affected at any time point. CYP3A13 mRNA was induced at both 5 and 7 days of treatment. The down-regulation of CYP2B9, 2C29 and 3A11 at 7 days was absent in the HeJ mice (Figs 2B and 3B). In contrast CYP1A2 down-regulation was unaffected by inactivation of the TLR4 gene, and down-regulation of CYP2A5, 3A11 and 3A25 at day 5 was only observed in the HeJ mice. The influence of TLR4 on the down-regulation of CYP4A10 and 4A14 was less certain because of the relatively large variation in the HeJ mice. However, the

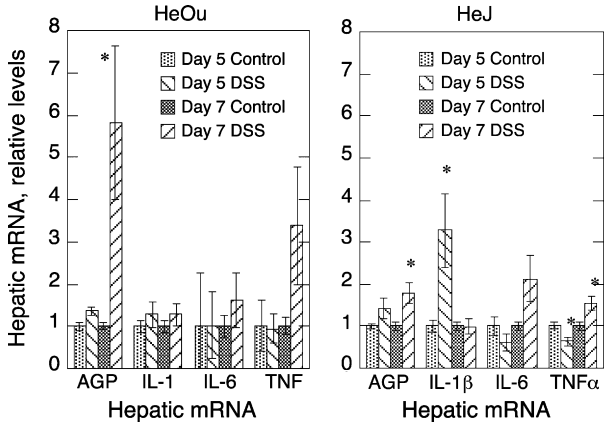

Fig. 4 – Hepatic cytokine and acute-phase mRNA expression mice consuming DSS. HeOu or HeJ mice were were allowed to drink water or 3% DSS solution, and livers were harvested at the indicated times for measurement of hepatic cytokine and acute-phase mRNA expression by real-time RT-PCR as described in Section 2. Values represent means ± S.E.M. of 6 mice per group. *P < 0.05 compared to controls, Student's t-test.

pattern of CYP4A mRNA expression appeared very similar in both strains.

DSS-induced colitis did not significantly affect the expression of proinflammatory cytokine mRNAs for IL-1β, IL-6 or TNFα in HeOu mouse livers (Fig. 4). In contrast, the mRNA of the acute phase protein α1-acid glycoprotein (AGP) was induced 6-fold. This effect was partially TLR4-dependent, as it was greatly attenuated in the HeJ mice. IL-1β and TNFα mRNAs were induced 3-fold and by 50%, respectively in the livers of HeJ mice only.

3.2. *LPS model of sepsis*

To account for potential differences in the purity of LPS preparations, we investigated the effects of two different commercial preparations on P450 mRNA expression in the livers of HeOu mice. As seen in Supplemental Fig. 2, the two LPS preparations elicited identical patterns of P450 regulation at 12 or 24 h after injection. The results for the two preparations were combined therefore, and are shown in Fig. 5. LPS treatment down-regulated the mRNAs of all the P450s studied, except CYP2D9, 3A13 and 4A14 (Fig. 5). In each case, the maximum effect occurred 12 h after injection, and in most cases the expression had recovered slightly 24 h after injection. CYP4A14 mRNA was unaffected by LPS treatment, whereas CYP2D9 and 3A13 mRNAs were induced. The 2-fold induction of CYP2D9 mRNA was relatively slow to develop, as it was only observed 24 h after injection. LPS injection of HeJ mice produced a 4-fold induction of CYP4A14 mRNA, but had no significant effect on any of the other P450 mRNAs measured (Fig. 6).

4. Discussion

The findings presented here demonstrate that, like colitis caused by the enteropathogenic bacterium *C. rodentium*, colitis elicited by the administration of DSS results in the down-regulation of multiple P450 enzymes in mouse liver. However, there are discernable differences in the pattern of P450 expression in the two models, as discussed in detail below. A comparison of the effects of DSS in HeOu and HeJ mice, a genetic model of TLR4 deficiency, revealed that some effects were TLR4-dependent, and others were not. Importantly, we also demonstrated that the effects of injected LPS on hepatic P450 expression were entirely TLR4-dependent.

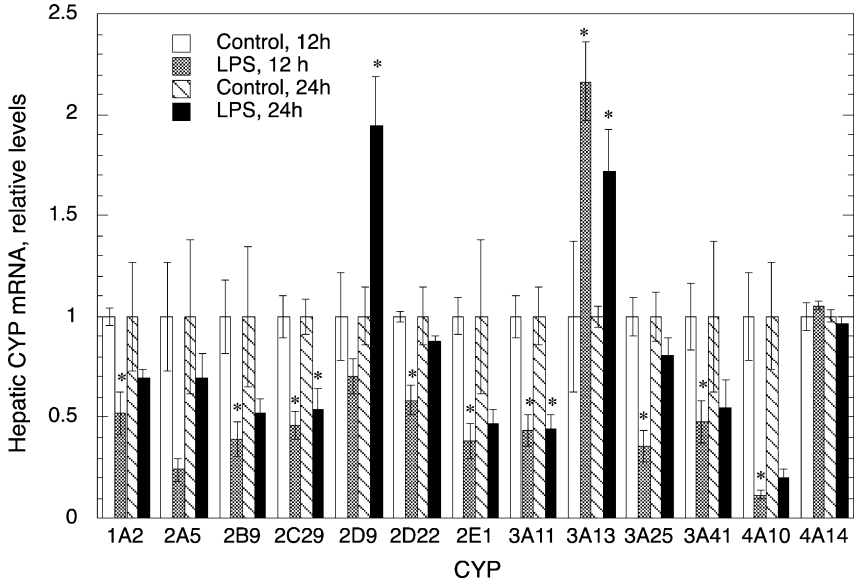

Fig. 5 – Effect of LPS injection on hepatic CYP mRNA expression in HeOu mice. HeOu mice were injected with 1 mg/kg LPS, and livers were harvested at the indicated times for measurement of hepatic CYP mRNA expression by real-time RT-PCR as described in Section 2. Control mice received the same volume of sterile saline. Values represent means ± S.E.M. of 3 mice in the control groups and 6 mice in the LPS-treated group. *P < 0.05 compared to controls, Student's t-test.

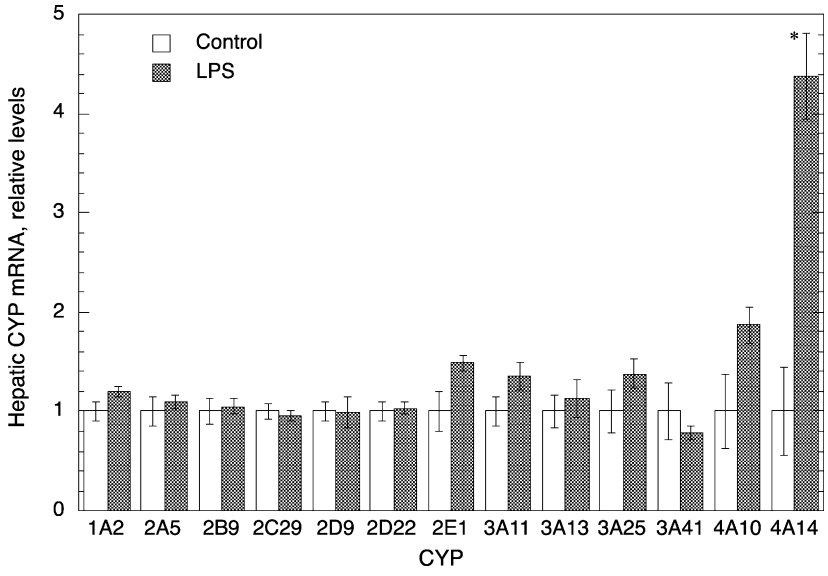

Fig. 6 – Lack of effect of LPS injection on hepatic CYP mRNA expression in HeJ mice. HeJ mice were injected with 1 mg/kg LPS (Invivogen), and livers were harvested 12 h later for measurement of hepatic CYP mRNA expression by real-time RT-PCR as described in Section 2. Control mice received the same volume of sterile saline. Values represent means ± S.E.M. of 6 mice per group. *P < 0.05 compared to controls, Student's t-test.

A comparison of the profile of P450 mRNA regulation in the DSS model of IBD (this study) with that in the same strain of mice (HeOu) infected with *C. rodentium* [6] reveals similarities and differences in the P450 regulation observed. The similarities included that CYP2B9, 2C29, 3A11, 4A10 and 4A14 mRNAs are each down-regulated in both models of inflammation, whereas CYP2E1 is unaffected. However, the magnitudes of down-regulation were substantially greater in the *C. rodentium*-infected mice for each of these P450s except 2C29. Moreover, the down-regulation of all P450 mRNAs tested in the *C. rodentium*-infected mice was independent of TLR4 expression [6], whereas in the DSS model the down-regulation of CYP2B9, 2C29 and 3A25 mRNAs was clearly blocked or attenuated in TLR4-deficient mice. This was supported by the fact that LPS injection down-regulated each of these P450s in a TLR4-dependent manner. These findings complement previous reports that the down-regulations of rat liver P450 proteins and P450-associated activities were attenuated by polymyxin B treatment in the DSS [11] and TNBS [16] models of IBD. Clearly, however, the down-regulations of CYP1A2, 2D9, 3A11, 4A10 and 4A14 mRNAs in DSS colitis operate via mechanisms that do not require TLR4 or LPS.

Significant differences between the infectious and chemical models of IBD included the following: CYP1A2 was down-regulated by DSS treatment, but not in the *C. rodentium* model; CYP2A5 was induced in the infected mice but not by DSS treatment, and CYP3A13 was induced in the DSS-treated mice but not in infected mice. We recently demonstrated that *C. rodentium* infection of C57BL/6 mice causes induction of CYP2D9 mRNA, and in the present study, we found that LPS treatment, but not DSS, also induces CYP2D9 mRNA. Therefore it appears that the induction of CYP2D9 during *C. rodentium* infection may not be due to colonic inflammation per se, but may be due to bacterial-specific factors or host responses to them. Alternatively, it could be due to a strain difference since we have not tested the effects of *C. rodentium* infection on CYP2D9 in HeOu mice.

The down-regulation of microsomal CYP2C, 2D and 4A proteins in the DSS model correlated well with the down-regulation of CYP2C29, 2D9, 4A10 and 4A14 mRNAs, respectively. CYP2C29 mRNA and CYP2C proteins were transiently induced at 5 days before being down-regulated at 7 days. Thus, CYP2C regulation in this model may reflect a balance between inductive and repressive mechanisms (both TLR4-mediated) whose relative strengths alter as the disease progresses. However, correlation of individual CYP mRNAs must be interpreted cautiously, because well-characterized specific antibodies are not generally available for most mice mouse P450s. That CYP2B protein levels did not correlate with the expression of CYP2B9 mRNA may suggest that the antibody used recognizes other CYP2B proteins in mouse liver. The induction of CYP3A proteins in DSS-HeOu-treated mice reflected increases in CYP3A11 and 3A13 mRNAs at 5 days of treatment. The induction of CYP3A protein at 7 days, despite no change or down-regulation of CYP3 mRNAs at this time point, may reflect a delay in the kinetics of protein down-regulation. Similarly, the elevation of CYP2E1 protein at 7 days of treatment in both strains of mice occurred in the absence of any effect on its mRNA. CYP2E1 protein stabilization is an important mode of regulation of the enzyme by various substrates and ligands [20]. Therefore, we speculate that the post-transcriptional regulation of CYP2E1 observed in this inflammation model is due to protein stabilization, although more work will be needed to elucidate the mechanism.

Given that the LPS model of inflammation has been used extensively to understand the regulation of drug metabolizing enzymes in inflammatory disease, and also to study the mechanism of this regulation, it is perhaps surprising that the

requirement of TLR4 for down-regulation of most hepatic P450 enzymes has not been reported previously in the LPS model. The exception is CYP3A11, whose down-regulation by LPS administered has been shown to be blocked in HeJ mice, whether the LPS was administered i.p. [21,22] or intracerebroventricularly [21]. Therefore, another important contribution of this study is to show that the regulation (up- and down-) of a broad panel of mouse hepatic P450s in the LPS model of inflammation is dependent on TLR4. We speculate that the induction of CYP4A14 in the TLR4 mutant animals, compared to no effect in the HeOu mice is due to the opposing effects of TLR4-dependent down-regulation and TLR4-dependent induction. It is not due to hypophagia [23] because none of the animals were allowed food after the LPS injection.

If LPS can down-regulate the great majority of P450s tested, why then is the down-regulation in the DSS model TLR4-dependent for some P450s but not others? This is unlikely to be due to off-target effects of injected LPS, since all of the effects of LPS were blocked in the HeJ mice. It could be due to the activation of different signaling pathways at different levels of TLR4 activation, so that at the relatively low LPS levels released in DSS-treated mice, only those pathways leading to CYP2B9, 2C29 and 3A25 down-regulation are activated. This could be addressed via a careful LPS dose–response study. An alternative explanation is that redundant pathways are activated to cause down-regulation of some P450 genes in the relatively chronic DSS model, such that removal of one pathway (e.g. TLR4) has no discernable effect.

In conclusion, the profile of hepatic P450 gene expression elicited by chemically induced IBD differs qualitatively and quantitatively from that evoked by a bacterial pathogen, and is caused by both TLR4-dependent and independent mechanisms. This provides further support of the concept that hepatic P450 regulation in inflammatory disease states depends on the etiology of the disease, and that there are multiple mediators and cellular mechanisms involved in this regulation.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bcp.2008.10.029.

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