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Regulation of Hepatic Cytochrome P450 Expression in Mice with Intestinal or Systemic Infections of *Citrobacter rodentium*

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Abbreviations: HeOu, C3H/HeOuJ; HeJ, C3H/HeJ; P450, cytochrome P450; IL, interleukin; LPS, lipopolysaccharide; TLR4, toll-like receptor 4; EPEC, enteropathogenic *E. Coli*; IBD, inflammatory bowel disease; H&E, Hematoxylin and Eosin; RT-PCR, reverse transcription-polymerase chain reaction; GAPDH, glyceraldehyde phosphate dehydrogenase; TNFα, tumor necrosis factor-α; IFNγ, interferon-γ; GM-CSF, granulocyte macrophage colony stimulating factor; MIP-1α, macrophage inflammatory protein 1α; MCP-1, monocyte chemotactic protein-1; KC, chemokine CXCL1; RANTES, chemokine CCL5; p.i., post infection; AGP, α1-acid glycoprotein; FGA, fibrinogen alpha polypeptide.

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ABSTRACT

We reported previously that infection of C3H/HeOuJ (HeOu) mice with the murine intestinal pathogen *Citrobacter rodentium* caused a selective modulation of hepatic cytochrome P450 (P450) gene expression in the liver that was independent of the Toll-like receptor 4. However, HeOu mice are much more sensitive to the pathogenic effects of C. *rodentium* infection, and the P450 down-regulation was associated with significant morbidity in the animals. Here, we report that oral infection of C57BL/6 mice with *C. rodentium*, which produced only mild clinical signs and symptoms, produced very similar effects on hepatic P450 expression in this strain. As in HeOu mice, CYP4A mRNAs and proteins were among the most sensitive to down-regulation, whereas CYP4F18 was induced. CYP2D9 mRNA was also induced 8-9 fold in the C57BL/6 mice. The time course of P450 regulation followed that of colonic inflammation and bacterial colonization, peaking at 7-10 days post-infection and returning to normal at 15-24 days as the infection resolved. These changes also correlated with the time course of significant elevations in the serum of the proinflammatory cytokines interleukin-6 (IL-6) and tumor necrosis factor-α, as well as of interferon-γ and IL-2, with serum levels of IL-6 being markedly higher than the other cytokines. Intraperitoneal administration of *C. rodentium*, produced a rapid down-regulation of P450 enzymes that was quantitatively and qualitatively different from that of oral infection, although CYP2D9 was induced in both models, suggesting that the effects of oral infection on the liver are not due to bacterial translocation.

INTRODUCTION

Bacterial infection and/or inflammation down-regulate both hepatic and extrahepatic cytochrome P450 (P450) expression and other proteins involved in drug metabolism and transport (for reviews see (Hoshino et al., 1999; Renton, 2004; Aitken et al., 2006; Petrovic et al., 2007; Morgan et al., 2008), leading to altered drug responses which may result in significant variations in clinical drug therapy. The bacterial endotoxin, lipopolysaccharide (LPS) has been used extensively as a sterile model of sepsis to study the down-regulation of P450s during inflammation or infection and has been an invaluable tool in understanding the importance and mechanisms of hepatic P450 regulation in inflammation. LPS is the major constituent of the outer membrane of gram-negative bacteria that produces inflammation upon injection, and elicits is actions through Toll-like receptor 4 (TLR4) (Poltorak et al., 1998) in association with several proteins, including LPS binding protein, CD14, and MD-2 proteins (Beutler et al., 2001). However, it is hard to predict accurately from the LPS model how P450 enzymes will be regulated in other models of inflammation or in live bacterial infections. The fact that inflammatory cytokines differentially regulate P450 gene expression (Craig et al., 1990; Chen et al., 1995; Aitken and Morgan, 2007) led us to postulate that the LPS model and other chemical models of inflammation may not predict how P450 enzymes will be regulated in a live infection.

Citrobacter rodentium, a member of the family of enteropathogens, provides an excellent *in vivo* model to investigate pathogen-host interactions under physiological conditions with the ability to modify host/bacterial conditions. It is a natural murine pathogen which uses attaching and effacing lesions to colonize the host's gastrointestinal tract (Jurjus et al., 2004; Wales et al., 2005), and its infection in mice is equivalent to human enteropathogenic *Escherichia coli*

(EPEC) infection (Schauer and Falkow, 1993) and colitis. EPEC is a specific serotype of *E. coli* and the major cause of infantile diarrhea worldwide (Nataro and Kaper, 1998), associated with considerable mortality in developing countries. The colitis caused by *C. rodentium* infection is also characteristic of inflammatory bowel disease (IBD) in mice and humans, and so *C. rodentium* may be a live model for IBD (Higgins et al., 1999; Caradonna et al., 2000; Goncalves et al., 2001; Gobert et al., 2004).

Previously, we investigated hepatic P450 expression during *C. rodentium* infection in wild type C3H/HeOuJ (HeOu) mice and in C3H/HeJ **(**HeJ) mice deficient in functional TLR4, a critical component of LPS signaling. We found that *C. rodentium* infection produced effects on hepatic P450 expression that were more enzyme-selective than the effects produced by LPS injection, and were largely independent of TLR4 activation (Richardson et al., 2006). HeOu mice are much more sensitive to the pathogenic effects of *C. rodentium* infection than are most other mouse strains, and the P450 down-regulation was associated with significant morbidity in the animals. Since the host and pathogen factors that differentially regulate P450 expression during the course of infection are not known, there could be strain differences in the P450 responses to infection. Therefore, the goals of this study were a) to determine whether or not liver P450 expression is selectively modulated during *C. rodentium* infection in a mouse strain that exhibits minimal morbidity or mortality during infection; b) by characterizing the time course of P450 regulation, to determine if the effects of infection are reversible and to correlate the hepatic P450 regulation with serum cytokines and colonic inflammation; and c) to determine whether acute systemic infection with *C. rodentium*, produced by i.p. injection, produced similar changes in the liver to those caused by oral infection.

For this study, we selected the C57BL/6 strain, as it has been well characterized as a host for *C. rodentium,* displaying relatively low mortality upon infection (Vallance et al., 2003), and because it serves as the genetic background for many targeted genetic mutants. We report that the pattern of hepatic P450 gene regulation in these mice, that exhibited negligible clinical signs or symptoms, was very similar to that observed previously in the highly susceptible HeOu strain. The regulation of P450 expression in the liver was reversible and followed the time course of colonic bacterial colonization and pathology. However, the pattern of acute P450 regulation produced by parenteral injection of *C. rodentium* was more characteristic of the less selective effects reported previously for LPS injection.

MATERIALS AND METHODS

Bacteria. A wild-type strain of *C. rodentium* (#51116) was obtained from the American Type Culture Collection (Manassas, VA). Before infection, C. *rodentium* was grown in Luria broth without shaking overnight, and harvested by centrifugation. Bacteria were resuspended in sterile phosphate-buffered saline, and the nominal concentration was calculated spectrophotometrically. Actual concentrations were determined by retrospective plating on MacConkey agar, on which *C. rodentium* forms small pink colonies with white rims.

Chemicals, Animals, and Treatments. Unless otherwise specified, all the reagents and chemicals were obtained from Sigma-Aldrich (St. Louis, MO). Female C57BL/6 mice were obtained from Jackson Laboratory (Bar Harbor, ME). The mice were acclimatized to the animal facility for at least 1 week, and mice were 7-9 weeks of age at the time of infection. Mice were housed and sacrificed in a Biosafety Level-2 facility to prevent transmission of infection to other mouse colonies. Mice were housed in groups of six to a cage. Oral administration of *C. rodentium* was achieved by infection of the drinking water (with 20% sucrose) for 24 h at a nominal concentration calculated to result in a bacterial dose of 7×10^8 cells/mouse. The actual doses for each group were calculated from the volume consumed by each group of mice, times the concentration of *C. rodentium* determined by retrospective plating. Actual doses for each group were 8, 7, 6, and 6×10^8 bacteria/mouse for the 7 day, 10 day 15 day and 24 day groups, respectively. We included two control groups: normal control and pairfed control. The amount of food consumed by each cage of infected mice was measured and the amount of food consumed per day per mouse was calculated. The same amount of food was offered to the pairfed control group the next day. Both sets of control groups received 20% sucrose in their

drinking water on the first day. Six mice from each group were sacrificed at 7, 10, 15, and 24 days after administration of sucrose or bacteria. Livers and a piece of distal colon were collected. Livers were rinsed in cold 1.15% potassium chloride, and stored at -80 °C for later RNA or microsome preparation. Colon samples were washed for fecal content in saline solution and fixed in 10% neutral buffered formalin solution until they were processed for histology. They were stained with Hematoxylin and Eosin (H&E) for histopathological evaluation. Blood was also collected from all the animals, and allowed to clot for 30 minutes. Serum was separated immediately by centrifugation and stored at -20 °C until analyzed.

For the mice infected by i.p. administration of *C. rodentium,* groups of 6 *ad libitum*-fed, 10-week-old female C57BL/6 mice were injected with either saline (control group) or 10^4, 10^6, or 10^8 CFUs of *C. rodentium*, and were killed 24 h later. In the time course experiment, mice received either saline or 10^8 CFU and were killed at the indicated times. Livers, pieces of colon and serum were collected and stored as stated above. All the protocols were reviewed and approved by the Institutional Animal Care and Use Committee of Emory University.

Preparation of Total RNA. Total liver RNA was prepared using RNA-Bee isolation reagent according to the manufacturer's instructions (Tel-Test, Friendswood TX). Total RNA concentration was estimated spectrophotometrically at 260 nm, and RNA purity and integrity were confirmed by formaldehyde-agarose gel electrophoresis followed by visualization with ethidium bromide.

Preparation of post-mitochondrial and microsomal fractions. Post-mitochondrial supernatants were prepared by centrifuging liver homogenates at 13,000 g for 25 minutes. Liver microsomes were prepared from the supernatants by centrifugation at 250, 000g for 45 min. The microsomal pellets were resuspended in 10 mM Tris acetate buffer pH 7.4, containing 0.1 mM EDTA and 23% glycerol, and stored at -80 °C. Protein concentrations of the fractions were determined using a bicinchoninic acid protein assay kit (Thermo Fisher Scientific, Inc, Rockport, IL) with bovine serum albumin as the protein standard.

cDNA Synthesis. 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.

Primer Sequences. Primers for CYP3A25 and CYP3A41 were from Martignoni et al (2006), and for CYP2D9, CYP2D10 and CYP2D22 were from Blume et al (2000). For mouse CYP4F mRNAs, specific sequences for PCR primers and dual-labeled fluorescent probes were designed at the region of highest sequence specificity or intron/exon boundaries using Primer Express software (Applied Biosystems, Foster City, CA) and custom synthesized by Integrated DNA Technologies, Inc. (Coralville, IA). The isoform specificity of P450 primers and probes was confirmed through multiple alignments with the other members of the CYP4F subfamily as well as homology-searched to ensure that there was no cross reactivity with other genes using an National Center for Biotechnological Information BLAST search. The primer and probe sequences were: CYP4F18 forward 5' AGAGCCTGGTGCGAACCTT 3', reverse TGGAATATGCGGATGACTGG, probe 56Fam- TGCATGCTGCTGGTGGGTGG -Tamra;

CYP4F15 forward CATGACATGGCTGGGTCCTA, reverse GAGGCATTGAGAACAGATCGA, probe CCTATCATCACTCTGTGCCACCCTGACAT - 3BHQ1; cyclophilin forward CGATGACGAGCCCTTGG, reverse, TCTGCTGTCTTTGGAACTTTGTC, probe Fam- CGCGTCTCCTTCGAGCTGTTTGCA – Tamra.

All primers for other mouse P450s cytokines, acute phase proteins, and glyceraldehyde dehydrogenase (GAPDH) have been described and used by our laboratory previously (Richardson et al., 2006). All the primers were custom-synthesized on a 50-nmol scale by MWG Biotech, Inc. (High Point. NC), and obtained desalted and lyophilized. Primers were diluted to 100 μM in deionized water and stored at -80°C.

Quantitative Real-Time RT-PCR. With the exception of the CYP4Fs, real-time RT-PCR was performed using the ABI PRISM 7000 Sequence Detection System and SyBr Green to determine the expression of mRNAs of interest in mouse liver, as described previously (Richardson and Morgan, 2005). Briefly, duplicate reactions were performed in a total volume of 25 μl using SyBr Green Master Mix reagent (Applied Biosystems, Bedford, MA); 2 μl of cDNA/sample was used as template for the reaction, with 10 μM forward and reverse primers. Thermal cycling conditions included 2 min at 50°C and 10 min at 95°C, followed by 40 cycles of 95°C for 15 s and 1 min at the appropriate annealing temperature. Results are expressed as relative levels of target mRNA, normalized to levels of the housekeeping gene GAPDH, as calculated by the $\Delta\Delta C$t method (Livak and Schmittgen, 2001). The expression level in control samples was arbitrarily set at 1. All primer sets yielded a single PCR product of expected size as determined by agarose

gel electrophoresis. Specificity was routinely monitored by checking product dissociation curves in each reaction well. Analysis of mouse P450 4Fs was achieved via Taqman technology, as we described previously (Richardson et al., 2006). The relative amounts of each CYP4F mRNA in samples were determined by normalizing the values for copy number of the gene of interest to the copy number values of *m*-cyclophilin.

Western Immunoblotting. P450 protein levels in mouse hepatic microsomes or post-mitochondrial supernatants were measured by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting, as described previously by Richardson and Morgan (2005). Equal amounts of protein were loaded on the gels for each assay. 5-10 μg of microsomes or 30-75 μg of post-mitochondrial supernatants were used depending on the sensitivities of each antibody. Antibodies to rat CYP3A, 4A, and 2E were generously provided by Dr. James Halpert (University of Texas Medical Branch, Galveston, TX), Dr. Gordon Gibson (University of Surrey, Guildford, UK), and Dr. Magnus Ingelman-Sundberg (Karolinska Institute, Stockholm, Sweden), respectively. Polyclonal antibodies to rat CYP3A, 4A, and 2E proteins were diluted 1:5,000, whereas 2C antibody was diluted 1:20,000. Secondary antibodies were as follows: goat anti-rabbit, 3A, 2C, and 2E; rabbit anti-sheep, 4A; dilution for each was 1:2,500, with the exception of 2C, which was 1:10,000. All assays were performed within a linear range, and the intensity of stained bands was measured by laser densitometry.

Determination of Tissue Bacterial Loads To measure the CFU of *C. rodentium* in colon and liver, tissue samples weighing approximately 0.1 to 0.3g were homogenized at low speed with a Tissuemizer (Fisher Scientific) in 1ml of PBS. The lysate was plated on MacConkey agar plates

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at various dilutions, and *C. rodentium* colonies were recognized as pink with a white rim as previously described (Vallance et al., 2003), and were counted after 20 hours of incubation at 37^{o}C to determine the CFU per gram of tissue.

Colon and Liver Pathology. Paraffin-embedded colonic tissue sections stained with haematoxylin and eosin were examined by an observer blinded to the experimental condition. Tissues were scored (0 = normal, 1 = mild, 2 = moderate, 3 = severe) for the following observations: edema, hyperplasia, ragged epithelium, neutrophil infiltration, goblet cell depletion, and obvious bleeding. For crypt height measurements, three measurements of well-oriented crypts were taken in the distal colon for each mouse, using micrometry by a Zeiss (Thornwood, NY) 200M microscope with a 20X NA1.4 lens, and Slidebook (Intelligent Imaging Innovations, Denver CO). The livers of the mice were removed and placed in 10% neutral buffered formalin for two days for fixation. The livers were sectioned by a microtone at 5 μ and stained with hematoxylin and eosin. Paraffin-embedded liver tissue sections stained with haematoxylin and eosin were evaluated by a veterinary pathologist blinded to the experimental condition for the presence of inflammation containing neutrophils and macrophages, or degeneration and fibrosis and necrosis. The presence of liver changes was scored (0 = normal, 1 = minimal, 2 = mild, 3 = moderate, 4 = severe).

Cytokine Analysis. Serum samples were analyzed for cytokines using 25 μl of serum from each sample using xMAP technology (Luminex, Austin, TX), with a single anti-mouse Lincoplex™ kit (Linco Research, St. Charles, MO). The cytokines IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, tumor necrosis factor-α (TNFα), interferon-γ (IFNγ), granulocyte macrophage colony

stimulating factor (GM-CSF), macrophage inflammatory protein 1α (MIP-1α), monocyte chemotactic protein -1 (MCP-1), chemokine CXCL1 (KC), chemokine CCL5 (RANTES), IL-9 and IL-13 were analyzed by following the protocol supplied with the kit. Briefly, 96-well plate filter plates were washed with wash buffer, and samples (1:1 dilution, 50 µl final volume) were applied to each well. Antibody-coated beads were added to the wells, and the samples were incubated for 18 h at 4°C. After incubation, the plate was washed twice with buffer supplied with the kit. Biotinylated antibodies against the different cytokines and chemokines from the kit were added, and the mixture was incubated for 1 h at room temperature. Then the cytokine-antibody complexes were detected by addition of streptavidin coupled to phycoerythrin. For determination of the number of positive complexes, each sample was read in a Luminex XYP platform. Data were analyzed using MasterPlex 1.2 (Mai-Rabio) and the concentrations are expressed in picograms per milliliter.

Statistical Analysis. For data with continuous variables, differences between groups were determined by oneway ANOVA followed by Tukey's or Dunnett's test as appropriate. For discrete pathology data, a Wilcoxon Mann-Whitney Rank Sum test or a Kruskal-Wallis Test followed by Wilcoxon Mann-Whitney Rank Sum test with Bonferroni correction were employed as dictated by the experimental design. The level of significance was set at $P < 0.05$.

RESULTS

STUDIES ON ORAL INFECTION WITH C. *RODENTIUM* IN C57BL/6 MICE.

C57BL/6 mice have been well characterized as a host for *C. rodentium.* Colonic colonization peaks at ~6-10 days in mice of this strain (Vallance et al., 2003). Inflammatory cell infiltration of the colon is maximal at days 10-18, and this corresponds with the observed colonic pathology (Deng et al., 2004). Therefore, we studied hepatic P450 expression at 7, 10, 15 and 24 days of infection, to encompass the peak of colonization and colonic pathology, and the resolution phase. To control for the possible effects of reduced food intake on P450 expression, we included both untreated control groups and pairfed groups at each time point.

Pathology and bacterial loading of the liver and colon. Bacterial colonization of the colon was significant at 7 days post-infection (p.i.), reached its peak at 10 days (Suppl. Fig. 1A), and was negligible at days 15 and 24 p.i. Colon weights were not significantly increased until 10 days p.i., and the increase persisted at 15 days p.i. (Suppl. Table 1). Infection was associated with mucosal hyperplasia, ragged epithelia, edema and increased crypt length on day 7 p.i. (Suppl. Fig. 2, Suppl. Table 2), each of which persisted through day 15 and normalized by day 24 p.i. (Suppl. Table 2). PMN infiltration was significant by day 10, and persisted through day 24 p.i. A significant increase in goblet cells was only observed on day 10 p.i. (Suppl. Table 2).

C. rodentium cells were detected in the liver following infection, and the time course paralleled that of colonic colonization (Suppl. Fig 1B). However the bacterial load in the liver

was 50,000 times lower than that in the colon. Infected mice had increases in liver weight of 37, 16 and 25% at 7, 10 and 15 days p.i., respectively (Suppl. Table 1). Livers of infected mice at 7 days p.i. had minimal to mild lymphocytic inflammation (Suppl. Table 3, Suppl Fig, 3) characteristic of a bacterial infection. By day 10 p.i, inflammation was minimal (Suppl. Table 3).

Food intake and body weights. The food intakes of the infected mice were more variable from day to day than those of the control mice, but over the first 1-10 days p.i. they did not differ in a consistent manner from the untreated animals (Suppl. Fig 4A). Interestingly, the infected mice tended to eat more than the untreated animals in the resolution phase of the infection. Consistent with previous observations (Vallance et al., 2002), the body weights of the infected mice dropped significantly at day 10 p.i., and recovered shortly thereafter (Suppl. Fig. 4B). No such effect was observed on the body weights of the pairfed animals (Suppl. Fig. 4B).

Serum cytokines. Serum levels of IL-1β, IL-4, IL-5, IL-9, IL-10, IL-12 and GM-CSF were not significantly different from pairfed mice at any time point (data not shown). The time courses of serum cytokines that differed between infected and pairfed mice were highly similar, peaking between 7 and 10 days and returning to baseline at 15 days p.i. (Fig. 1). IL-6 was the most abundant cytokine in the plasma of infected mice, peaking at 380 pg/ml (Fig. 1). Levels of the chemokine KC (mouse IL-8 analog) were in the 200 pg/ml range, whereas MCP-1, MIP-1α, IL-13 and IFNγ peaked between 50 and 100 pg/ml. Peak levels of IL-2, RANTES and the proinflammatory cytokine TNFα were all <25 pg/ml.

Hepatic CYP4A expression. Our previous work found that enzymes of the CYP4Asubfamily exhibited the most profound changes in response to *C. rodentium* infection of C3H/HeOuJ or HeJ mice. As shown in Fig 2A, the mRNA for CYP4F18 was induced 8-fold in the infected but not the pairfed C57BL/6 mice at 7 and 10 days p.i. Its expression was still significantly induced by 2.75-fold at 15 days p.i., but had returned to only 1.4-fold of control by day 24. CYP4A12 mRNA was unaffected (Fig. 2A), whereas CYP4A10, 4A14, and 4F15 mRNAs were down-regulated at 7 and 10 days p.i., with each mRNA returning to control (or slightly above) within 15 days p.i (Fig. 2B). The down-regulation of hepatic microsomal CYP4A proteins followed a similar time course to that of the CYP4A mRNAs, except that the down-regulation of the proteins persisted at 15 days p.i. (Figs 2C, D). Unlike the CYP4A mRNAs, which were slightly induced at 7 days in the pairfed mice, CYP4A proteins were unaffected at 7 days of pairfeeding, and slightly suppressed at 15 days of infection (Figs 2B, C, D). Based on the observations that most of the parameters measured had returned to baseline or near baseline by day 15, we limited further measurements to the 7, 10 and 15 day p.i. samples only.

Expression of hepatic drug metabolizing P450 subfamilies 1, 2 and 3. Of the 11 members of the drug-metabolizing P450 families whose mRNAs we measured, four exhibited significant differences between the untreated and pairfed control groups at 7 and 10 days p.i. : 2A5 and 2E1 (Fig 3A); 3A25 and 3A41 (Fig 3B). Pairfeeding reduced CYP3A41 mRNA expression, but induced the 2A5, 2E1 and 3A25 mRNAs. All of the family 1, 2 and 3 P450 mRNAs examined, except CYP3A13 (Fig 3B) and 2D9 (Fig 3C), were down-regulated in *C. rodentium* -infected mouse livers (Fig 3). CYPs 1A2, 2B9, 2C29, 2D22, 3A11, 3A25 and 3A41 were suppressed whether compared to pairfed or untreated controls, whereas CYPs 2A5 and 2E1 were only

suppressed when compared to pairfed mice. Most of the down-regulated P450 mRNAs were suppressed to ~50% of untreated levels, except for CYP2B9 which was suppressed to 25% of the level in untreated mice (Fig 3). In each case, the degree of suppression was approximately equivalent in the 7 and 10 day p.i. groups and had returned to control levels by 15 days p.i. The exception was CYP1A2, which was not affected until 10 days p.i., and remained below control levels at 15 days. In contrast, CYP2D9 mRNA was induced 9.5 fold and 4.8-fold in the livers of infected mice at 7 and 10 days p.i., respectively (Fig 3C). CYP3A13 mRNA was unchanged in the day 7 and 15 p.i. mice, although it was significantly induced 1.4 fold at 10 days p.i. (Fig 3B).

C. rodentium infection caused a down-regulation of CYP 2B, 2C, 2E and 3A proteins relative to both untreated and pairfed controls at 7 days p.i (Fig. 4). Whereas CYP2B, 3A and 2E proteins were still down-regulated at 10 days p.i. and returned to control levels at 15 days p.i., CYP2C proteins were not different from controls at 10 days p.i. (Fig. 4). CYP2B proteins were significantly elevated after 7 days of pairfeeding compared to untreated mice. The CYP2D9 antibody recognized two proteins in mouse liver microsomes, and it appeared that both bands were affected similarly by infection (Fig. 4A). At 7 days p.i., CYP2D proteins were down-regulated in both pairfed and infected mice, whereas CYP2D protein expression progressively increased at later time points to become significantly induced at 15 days p.i. (Fig. 4), .

Hepatic cytokine and acute phase mRNAs. Hepatic expression of the acute phase protein α1-acid glycoprotein (AGP) was induced by 6- and 8-fold respectively at 7 and 10 days p.i., but returned to near baseline by 15 days (Fig. 3C). Hepatic mRNAs for IL-1, IL-6 and TNFα were each induced 3-fold, but only at day 10 p.i. (Fig. 3C).

STUDIES ON PARENTERAL INFECTION WITH *C. RODENTIUM* .

Since we detected low, but significant, levels of *C. rodentium* cells in the livers of infected mice, we next studied the effect of systemic infection with *C. rodentium* on hepatic cytochrome P450 expression. In the first experiment, mice were treated intraperitoneally with various doses of *C. rodentium* cells, and killed 24 h later for analysis.

Mice injected with the lowest dose of *C. rodentium* cells (10^4/mouse) did not show any behavioral differences compared to the control group, but at the highest dose of bacteria (10^8/mouse) the mice became very weak and sluggish. Only the highest dose of bacteria (10^8/mouse) caused a significant decrease in body weight of 15% (Suppl. Fig. 5A). The total bacterial contents of the livers of the infected animals were significantly elevated in the two highest dose groups (Suppl. Fig. 5B).

The bacterial dose dependencies of the effects of i.p. *C. rodentium* injection on hepatic P450 mRNA expression are shown in Fig. 5. The mRNAs of CYPs 1A2, 2A5, 2B9, 2C29, 3A11, 3A25 and 3A41 showed similar patterns of down-regulation, each reaching a nadir of 10-20% of control levels at the highest bacterial dose. (Fig. 5A). CYPs 1A2 and 3A25 mRNAs were significantly down-regulated at the lower doses as well (Fig. 5A). CYP4A14 and 2E1 mRNAs were unaffected at any bacterial dose (Fig. 5B), whereas CYP4A10 and 2D22 were down-regulated to 34% and 39% of control, respectively, at the highest dose. CYP 3A13 mRNA was significantly induced 1.5-fold at the 10^6 cell dose (Fig 5B), whereas CYP2D9 was induced

2.5 and 4-fold by the 10^6 and 10^8 cell doses, respectively (Fig. 5C). The mRNAs of the acute phase proteins AGP and fibrinogen-α (FGA) were induced with similar dose dependencies to CYP2D9, whereas angiotensinogen was unaffected (Fig. 5C). Of the hepatic cytokine mRNAs, TNFα was induced to a much greater extent (20-fold) than those of IL-1 and IL-6 (~3-fold) (Fig. 5D). TNFα was also significantly induced (2.8-fold) at the 10^6 cell dose.

The regulation of hepatic CYP proteins levels in the parenterally infected mice is shown in Fig 6. CYP2C and 3A proteins were down-regulated to 59% and 31% of control at the highest bacterial dose, correlating with the down-regulation of CYP2C29 and most of the CYP3A mRNAs studied. However, CYP2B, 2D and 4A proteins levels were unaffected. CYP2E1 protein was induced >2-fold at the two highest bacterial doses. Of cytokines measured in the serum 24h after parental infection, TNFα was moderately elevated at the highest bacterial dose, IL-1β was unaffected and IL-13 was reduced (Suppl. Fig. 6). IL-6 was not measured in these samples.

We next studied the time course of CYP regulation in mice injected with the maximally effective dose of *C. rodentium*. CYP 2A5 and 3A11 mRNAs were significantly down-regulated as early as 6h after injection, whereas CYP2B9 was not significantly affected until 24 h after injection (Fig. 7A). CYP 4A14 was not significantly affected at any time point, although there was a trend towards a decrease. The induction of CYP2D9 mRNA was first detected at 12 h after injection, and CYP3A13 was also significantly induced at that time point. TNFα was the predominant proinflammatory cytokine induced in the liver after parenteral injection, peaking at 6h (Fig. 7B). In contrast to CYP2B9 mRNA, CYP2B proteins were significantly down-

regulated to 50% of control levels within 12 h of injection (Suppl. Fig. 7). However, CYP2D9, 3A and 4A proteins were not affected at this time point (Suppl. Fig. 7).

DISCUSSION

These studies demonstrate that subclinical oral infection of mice with the enteropathogenic bacterium *C. rodentium* causes selective, and in some cases profound, down-regulation of hepatic cytochrome P450 mRNA and protein levels. Moreover, these effects are reversed after the organism has been cleared from the gastrointestinal tract. By contrast, acute systemic infection with the same organism causes rapid and less selective down-regulation of most P450s studied.

C. rodentium infection of C57BL/6 mice (this study) and HeOu mice (Richardson et al., 2006), shows a very similar profile of P450 regulation (Fig. 8) except for CYP2A5. This is remarkable because HeOu mice are highly sensitive to the infection and showed substantial morbidity, whereas C57BL/6 mice exhibited no behavioral changes, and the only clinical signs were loose stool, and perianal fecal staining. Reproduction in C57BL/6 mice of the findings from the sensitive HeOu strain will be crucial to future studies to elucidate the mechanisms of the observed down-regulation using genetically modified strains.

The small and variable effects of pairfeeding were more likely to be due to stress caused by competition for a limited food supply than to reduced caloric intake, because the food intake of mice was minimally affected by infection (Suppl. Fig 1). Since any changes in feeding behavior are part of the physiological response of the animal to infection, we conclude that untreated controls are more appropriate for these studies.

The time course of P450 regulation in the liver following oral infection with *C. rodentium* was correlated with the time course of bacterial colonization of the colon. Thus, expression of most P450s had returned to normal, or had begun to recover, by day 15 when the majority of the infection had been cleared from the colon. Residual down-regulation of some P450s at 15 days may be due to the residual inflammation seen in the colons (Suppl. Tables 1 and 2).

C. rodentium attach to cells of epithelial nature and modulate intracellular signaling processes (Vallance et al., 2003). Although *C. rodentium* cells were detected in the livers of our mice after oral infection, it is unlikely that the bacteria are attaching to hepatocytes to affect hepatocyte P450 expression. The peak bacterial content of the livers was 25,000 cells/g liver at 10 days of infection. With a conservative estimate of 3×10^7 hepatocytes/ g liver (Kim et al., 1997), this corresponds to about one bacterial cell per 1000 hepatocytes. Since we observed mild to moderate inflammation in the liver at 7 days p.i., it is possible that bacterial activation of Kupffer cells caused the P450 down-regulation. Arguing against this possibility is that we have already demonstrated that the P450 down-regulation is independent of bacterial LPS signaling via TLR4 (Richardson et al., 2006). Also, the down-regulation at 7 days occurred in the absence of increased expression of hepatic proinflammatory cytokine mRNAs, when the mild lymphocytic inflammation was at its peak, and at 10 days these cytokine mRNAs were minimally elevated.

Proinflammatory cytokines, as well as interferons, can selectively modulate P450 expression in cultured hepatocytes (Craig et al., 1990; Thal et al., 1994; Chen et al., 1995; Tinel

et al., 1995; Donato et al., 1997; Aitken and Morgan, 2007). In the present study, the time courses of serum elevations in IL-6, TNFα, IFNγ and IL-2 paralleled those of the down- or up-regulation of P450s in the liver. That IL-6 concentrations were the highest of the 16 cytokines measured is of particular interest, because this cytokine can down-regulate multiple P450s in cultured hepatocytes (Aitken and Morgan, 2007), and IL-6 null mice have impaired down-regulations of some hepatic P450s (Siewert et al., 2000). However, the importance of other cytokines or indeed of other blood-borne signals cannot be discounted. IL-13, MIP-1α, MCP-1 , RANTES and KC are chemokines involved in chemotaxis of immune cells, and their effects on P450 expression in hepatocytes are unknown.

In this study we observed an even greater induction of CYP4F18 (9-fold) in infected mice than we saw previously in the HeOu strain. Since CYP4Fs are the most efficient catalysts of leukotriene B_4 inactivation (Kalsotra and Strobel, 2006), it is tempting to speculate that induction of CYP4F18 contributes to the resolution of inflammation in the liver (and perhaps systemically) by removing this inflammatory eicosanoid.

The marked induction of CYP2D9 mRNA in mice infected with *C. rodentium* is a novel observation. Human CYP2D6 is an important enzyme for the clearance of a large number of therapeutic agents, but is one of the few drug-metabolizing hepatic P450s that appear not to be significantly induced by drugs or chemicals. Therefore will be important to determine whether CYP2D9 and/or2D6 are induced in other inflammatory models, and to elucidate the mechanism of this induction. Expression of CYP2D9 is sexually differentiated in mouse liver (Harada and Negishi, 1985), in response to the sex-specific growth hormone secretory pattern (Jarukamjorn et

al., 2006; Waxman and O'Connor, 2006). LPS causes down-regulation of the hepatic growth hormone receptor via proteolysis (Wang et al., 2008). Whether or not the up-regulation of CYP2D9 in the infected mice is secondary to changes in growth hormone secretion or growth hormone receptor expression remains to be determined. The down-regulation of rat hepatic CYP mRNAs by LPS was not due to changes in growth hormone secretion, since hypophysectomized rats infused with GH responded in the same manner as intact animals (Morgan, 1993).

While mice represent an attractive model to study mechanisms of P450 regulation, few tools are available to study the regulation of specific P450 proteins or their attendant activities. Thus, we were able to confirm that P450 proteins reacting with antibodies to rat liver 2B, CYP2C, 2E1, 3A and 4A are indeed down-regulated in the livers of infected mice, but our inability to identify specific immunoreactive proteins precludes making close associations between mRNA and protein regulation except for CYP2E1. CYP2E1 protein was down-regulated to a greater extent than its mRNA, relative to untreated controls at both 7 and 10 days p.i., suggesting that CYP2E1 protein degradation may be accelerated or its translation inhibited under these conditions.

The effects of i.p. injection of *C. rodentium* were rapid, and produced a more profound down-regulation of many of the P450s that are moderately down-regulated by oral infection. Strikingly, the CYP4As that are the most sensitive to down-regulation by oral infection were less sensitive to down-regulation by i.p. injection. These results suggest that the mechanisms of down-regulation are different in the two models, and therefore that bacterial translocation to the blood and liver is not responsible for the majority of the effects caused by oral infection.

However, i.p. injection of *C. rodentium* caused a rapid increase in CYP2D9 expression, similar in magnitude to that elicited by oral infection. It is tempting to speculate that CYP2D9 induction may be caused by bacterial LPS acting on the liver.

In conclusion, a gastrointestinal infection causing colonic inflammation, that produces minimal clinical signs or symptoms, exerts selective effects on hepatic P450 expression that resolve as the infection is cleared and the inflammation subsides. This selectivity is markedly different from that of systemic sepsis caused by the same bacterium, which has important implications for drug therapy in disease states and perhaps in different stages of the same infection. On the other hand, expression of CYP2D9 is markedly induced after either oral or systemic infection. A better understanding of the mechanisms involved will facilitate studies to determine whether or not this has importance for the metabolism of the many drugs that are substrates of CYP2D6 in humans.

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FOOTNOTES

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FIGURE LEGENDS

Fig. 1. Serum cytokine profile during *C. rodentium* infection in mice. Mice were infected with *C. rodentium* p.o. and blood was collected at sacrifice for measurement of serum cytokines as described in Materials and Methods. Values represent means ± S.E.M. of 6 mice per group except for the 15 day pairfed group (5 mice). * $P<0.05$ compared to pairfed (PF) control. Differences between groups were determined by Student's t- test.

Fig. 2. Effect of oral *C. rodentium* infection on CYP4A family mRNAs and proteins in mouse liver. Mice were infected with *C. rodentium* p.o. and livers were harvested at the indicated times for measurement of CYP4 mRNA and protein levels as described in Materials and Methods. A, CYP4F18 mRNA; B, CYP4A10, 4A14 and 4F15 mRNAs; C, CYP4 family proteins; D, Western blots from which the data in panel C were obtained. Values represent means ± S.E.M. of 6 mice per group except for the 15 day pairfed group (5 mice), and are calculated relative to the mean of the saline-injected control group, which was set at 1. * $P<0.05$ compared to untreated; # $P<0.05$ compared to pairfed. Differences among groups were determined by oneway ANOVA followed by Tukey's test.

Fig. 3. Effect of oral *C. rodentium* infection on P450, cytokine and acute phase protein mRNAs in mouse liver. Mice were infected with *C. rodentium* p.o. and livers were harvested at the indicated times for measurement of P450 mRNA levels as described in Materials and Methods. A, CYP2 family mRNAs; B, CYP1 and 3 family mRNAs; C, CYP2D9, cytokine and acute phase mRNAs. Values represent means ± S.E.M. of 6 mice per group except for the 15 day pairfed

group (5 mice), and are calculated relative to the mean of the saline-injected control group, which was set at 1.. * P<0.05 compared to untreated; # P<0.05 compared to pairfed. ^, not determined. Differences among groups were determined by oneway ANOVA followed by Tukey's test.

Fig. 4. Effect of oral *C. rodentium* infection on P450 family 2 and 3 proteins in mouse liver. Mice were infected with *C. rodentium* p.o. and livers were harvested at the indicated times for measurement of P450 protein levels by Western blotting as described in Materials and Methods. A, Western blots of samples from Day 7 infected and control liver microsomes; B quantitative analysis of the data in panel A. Values represent means ± S.E.M. of 6 mice per group except for the 15 day pairfed group (5 mice), and are calculated relative to the mean of the saline-injected control group, which was set at 1. * P<0.05 compared to untreated; # P<0.05 compared to pairfed. Differences among groups were determined by oneway ANOVA followed by Tukey's test.

Fig. 5. Dependence on bacterial dose of parenteral infection of mice with *C. rodentium* on hepatic P450, cytokine and acute phase mRNAs. Mice were injected i.p. with the indicated number of *C. rodentium* cells, and killed 24 h later. Livers were harvested for measurement of P450 mRNA levels by RT-real-time PCR. Values represent means ± S.E.M. of 6 mice per group, and are calculated relative to the mean of the saline-injected control group, which was set at 1 for each mRNA. * P<0.05 compared to control. Differences between treated groups and control were determined by oneway ANOVA followed by Dunnett's test.

Fig. 6. Effect of parenteral infection with *C. rodentium* on mouse hepatic P450 protein levels. Mice were injected i.p. with the indicated doses of C. rodentium, and killed 24 h later. Livers were harvested and post-mitochondrial supernatants were prepared for measurement of P450 protein levels by Western blot analyses. A. Images of the Western blots. B. Quantitative analysis of the Western blot data. Values represent means ± S.E.M. of 6 mice per group, and are calculated relative to the mean of the saline-injected control group. * $P<0.05$ compared to control. Differences between treated groups and control were determined by oneway ANOVA followed by Dunnett's test.

Fig 7. Time courses of hepatic P450 and cytokine mRNA regulation following parenteral infection of mice with *C. rodentium*. Mice were injected i.p. with 10^8 cells of *C. rodentium* or with the same volume of sterile saline, and killed 6, 12 or 24h later. A separate group of control animals was used for each time point. Livers were harvested for measurement of P450 mRNA levels by RT-real-time PCR. Values represent means ± S.E.M. of 6 mice per group, and are calculated relative to the mean of the respective saline-injected control groups, which was set at 1 for each mRNA. * $P<0.05$ compared to control. Differences between treated groups and control were determined by student's T-test. 24 h data are from the study in Fig 7.

Fig 8. Comparison of hepatic P450 mRNA regulation during C. rodentium infection in HeOu and C57BL/6 mice. Data for C57BL/6 mice are from this study (Figs 4 and 5), and data for HeOu mice are from Richardson et al. (Richardson et al., 2006). The levels of each P450 mRNA relative to pairfed controls are shown. * $P<0.05$ compared to pairfed.

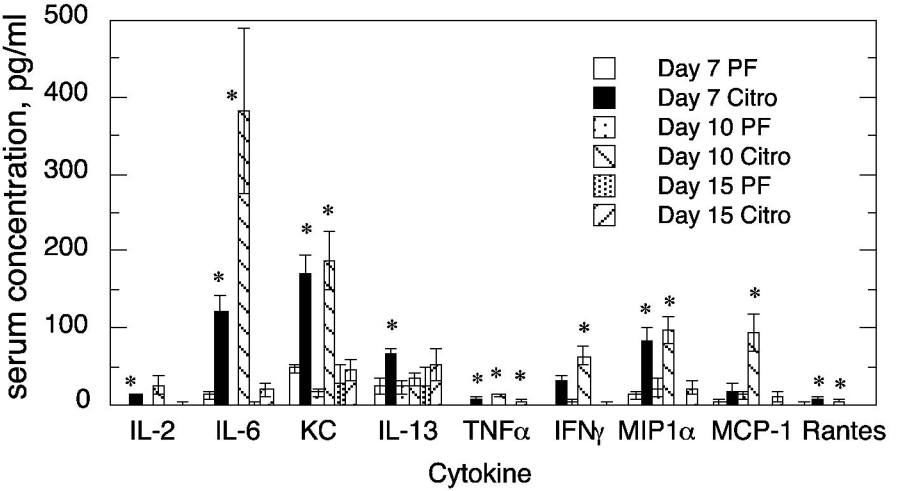

serum concentration, pg/ml
500
400
300
200
100
0
Day 7 PF
Day 7 Citro
Day 10 PF
Day 10 Citro
Day 15 PF
Day 15 Citro
IL-2
IL-6
KC
IL-13
TNFα
IFNγ
MIP1α
MCP-1
Rantes
Cytokine

Fig. 1

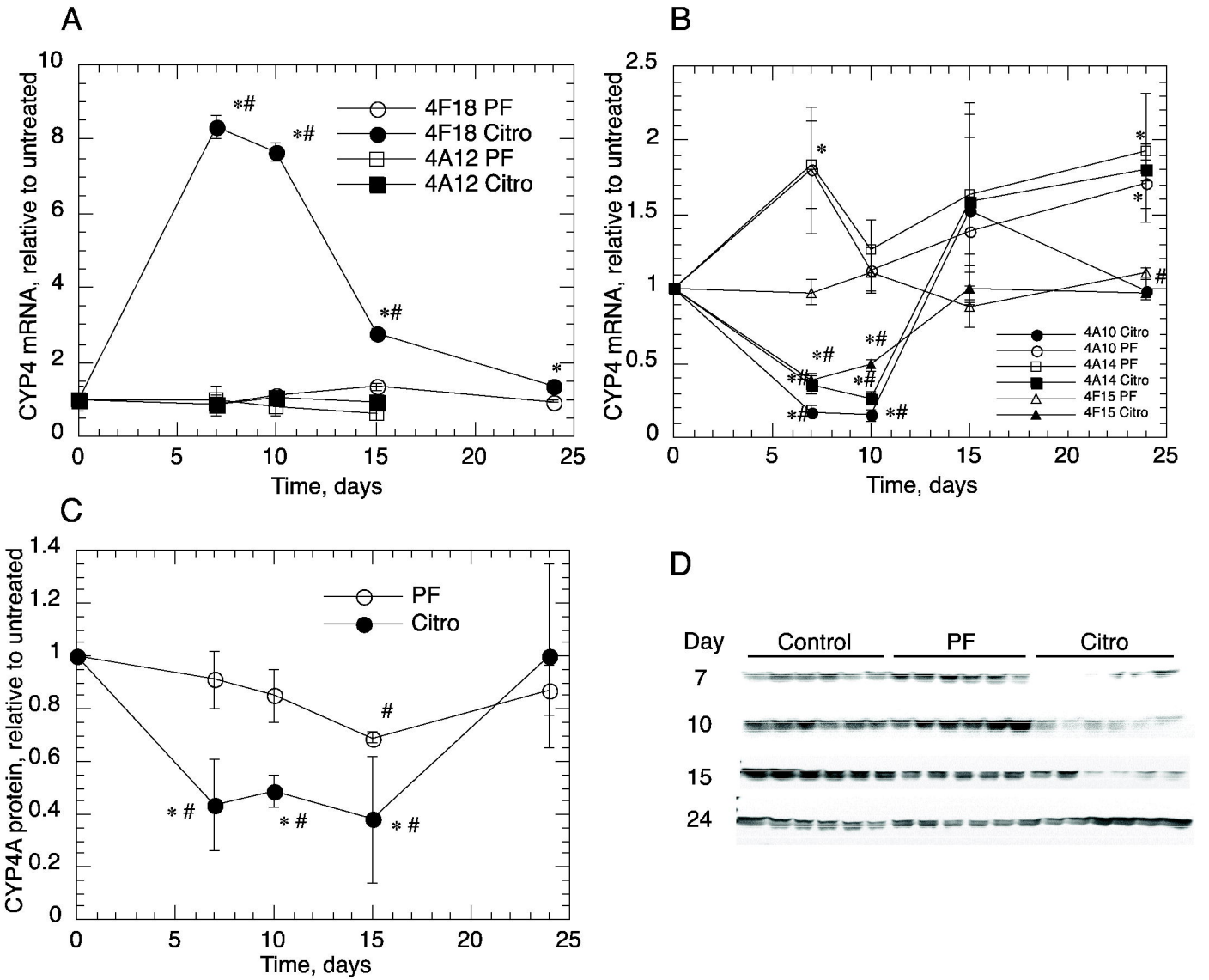

A
CYP4 mRNA, relative to untreated
Time, days
4F18 PF
4F18 Citro
4A12 PF
4A12 Citro
B
CYP4 mRNA, relative to untreated
Time, days
4A10 Citro
4A10 PF
4A14 PF
4A14 Citro
4F15 PF
4F15 Citro
C
CYP4A protein, relative to untreated
Time, days
PF
Citro
D
Day
Control
PF
Citro
7
10
15
24

Fig. 2

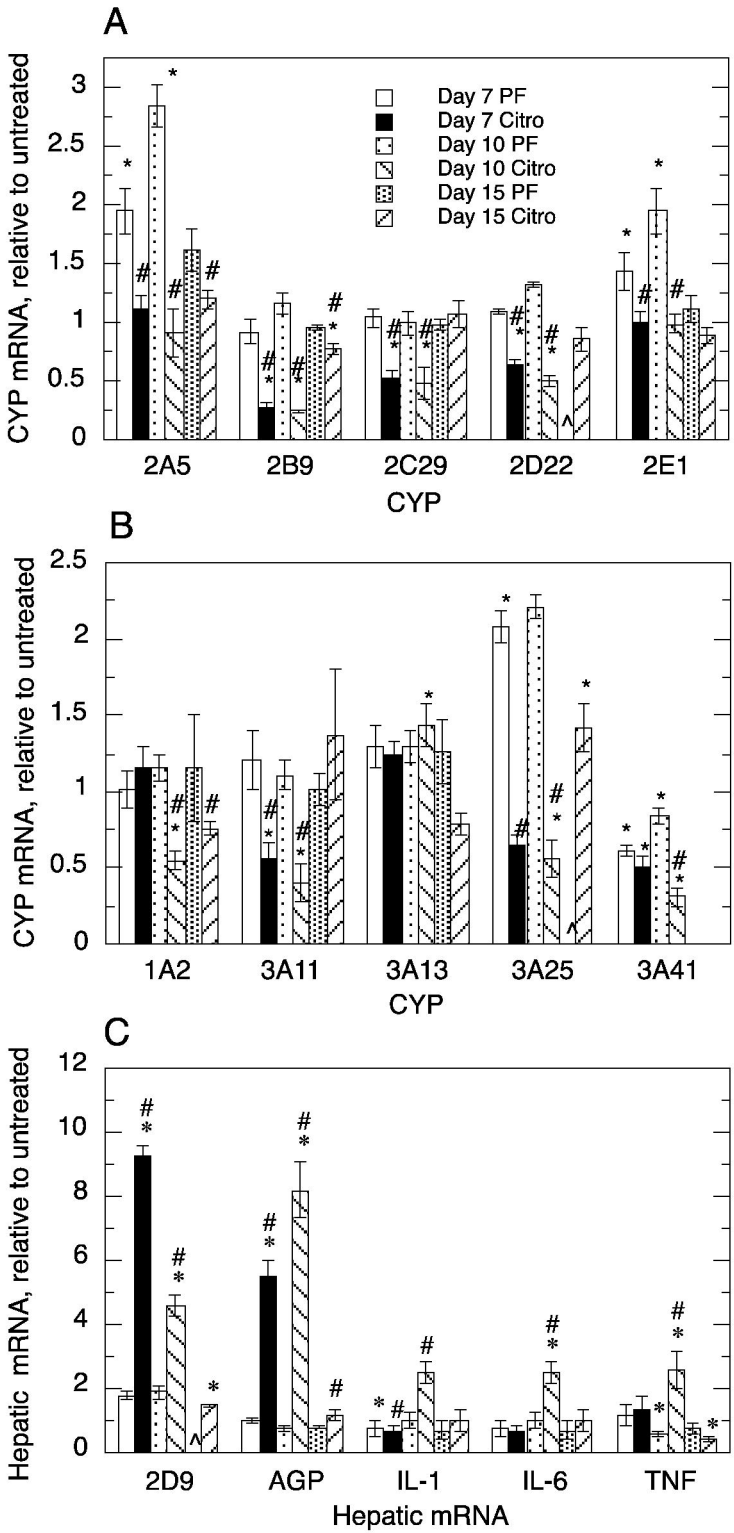

A
CYP mRNA, relative to untreated
Day 7 PF
Day 7 Citro
Day 10 PF
Day 10 Citro
Day 15 PF
Day 15 Citro
2A5
2B9
2C29
2D22
2E1
CYP
B
CYP mRNA, relative to untreated
1A2
3A11
3A13
3A25
3A41
CYP
C
Hepatic mRNA, relative to untreated
2D9
AGP
IL-1
IL-6
TNF
Hepatic mRNA

Fig. 3

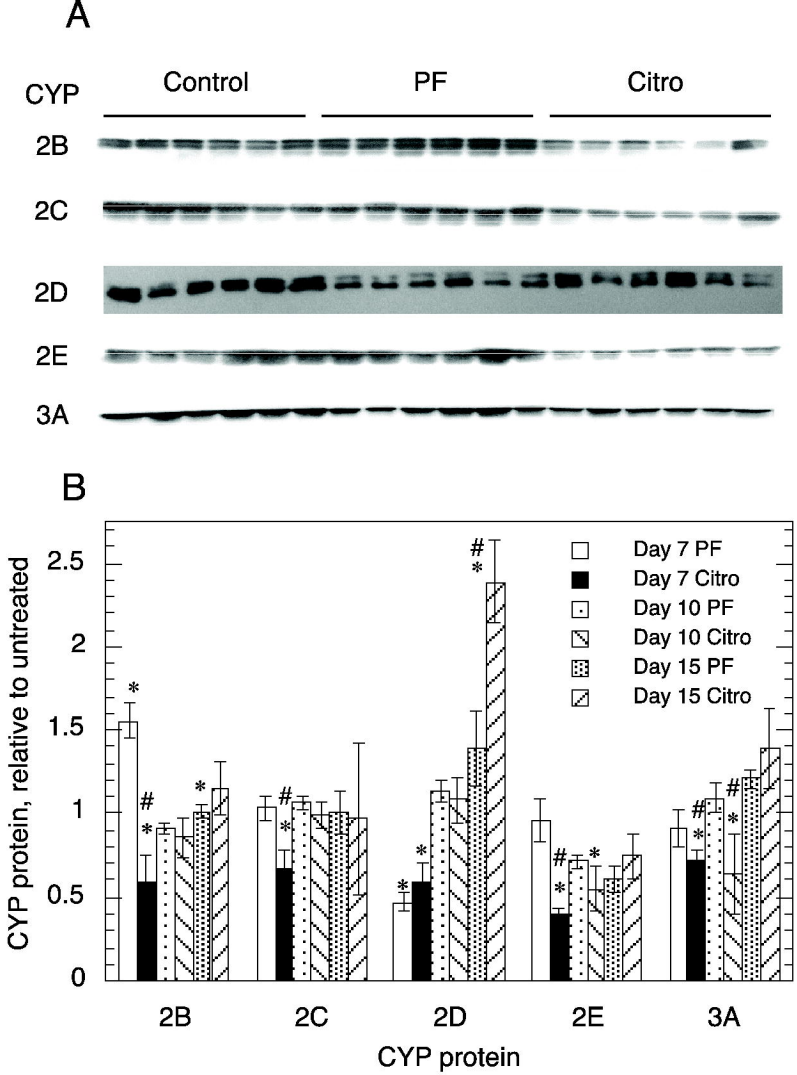

A
CYP
Control
PF
Citro
2B
2C
2D
2E
3A
B
CYP protein, relative to untreated
2.5
2
1.5
1
0.5
0
Day 7 PF
Day 7 Citro
Day 10 PF
Day 10 Citro
Day 15 PF
Day 15 Citro
2B
2C
2D
2E
3A
CYP protein

Fig. 4

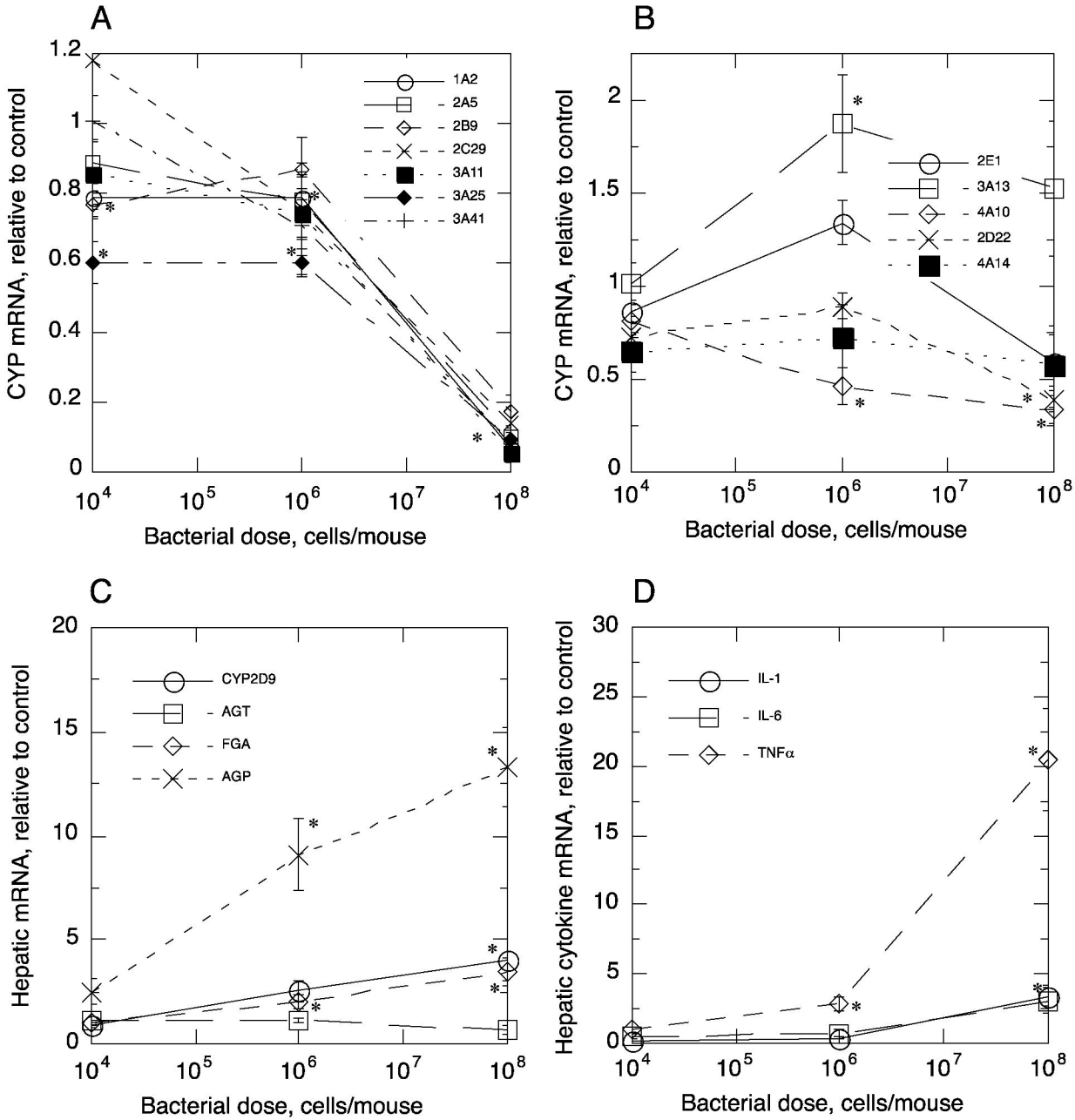
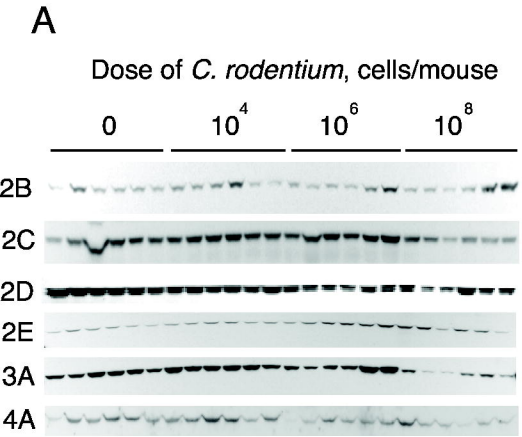

A
CYP mRNA, relative to control
Bacterial dose, cells/mouse
1A2
2A5
2B9
2C29
3A11
3A25
3A41
B
CYP mRNA, relative to control
Bacterial dose, cells/mouse
2E1
3A13
4A10
2D22
4A14
C
Hepatic mRNA, relative to control
Bacterial dose, cells/mouse
CYP2D9
AGT
FGA
AGP
D
Hepatic cytokine mRNA, relative to control
Bacterial dose, cells/mouse
IL-1
IL-6
TNFα

Fig. 5


A
Dose of C. rodentium, cells/mouse
0
10^4
10^6
10^8
2B
2C
2D
2E
3A
4A

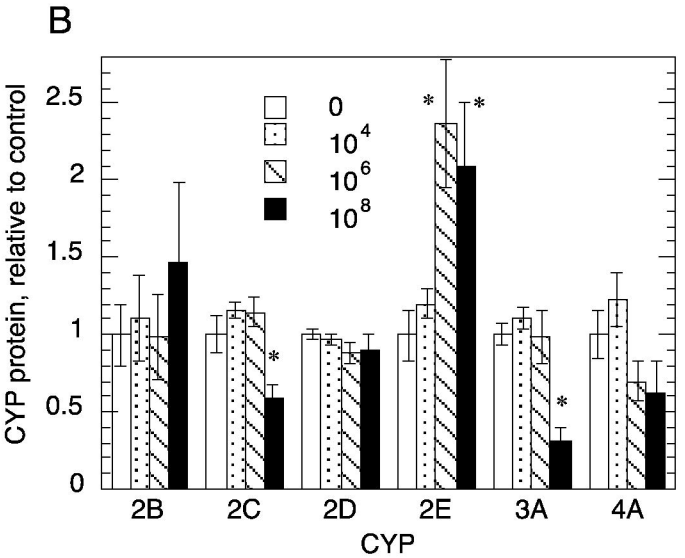

B
CYP protein, relative to control
2.5
2
1.5
1
0.5
0
0
10^4
10^6
10^8
*
2B
2C
2D
2E
3A
4A
CYP

Fig. 6

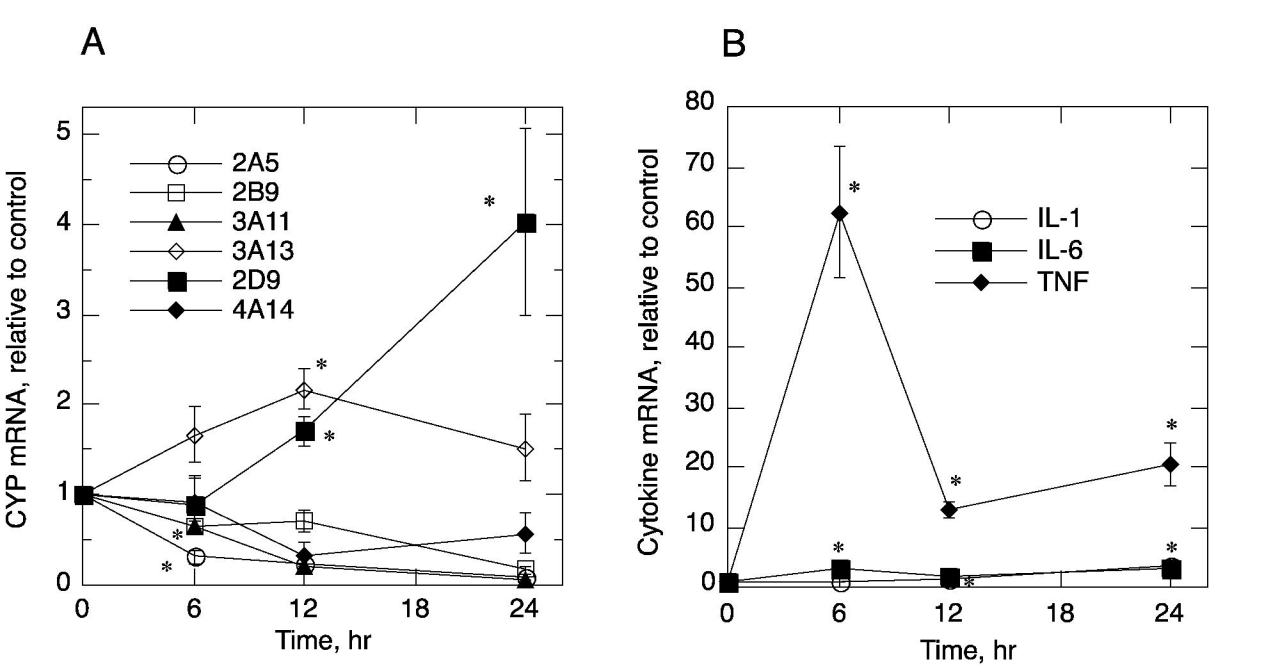

A
CYP mRNA, relative to control
2A5
2B9
3A11
3A13
2D9
4A14
Time, hr
B
Cytokine mRNA, relative to control
IL-1
IL-6
TNF
Time, hr

Fig. 7

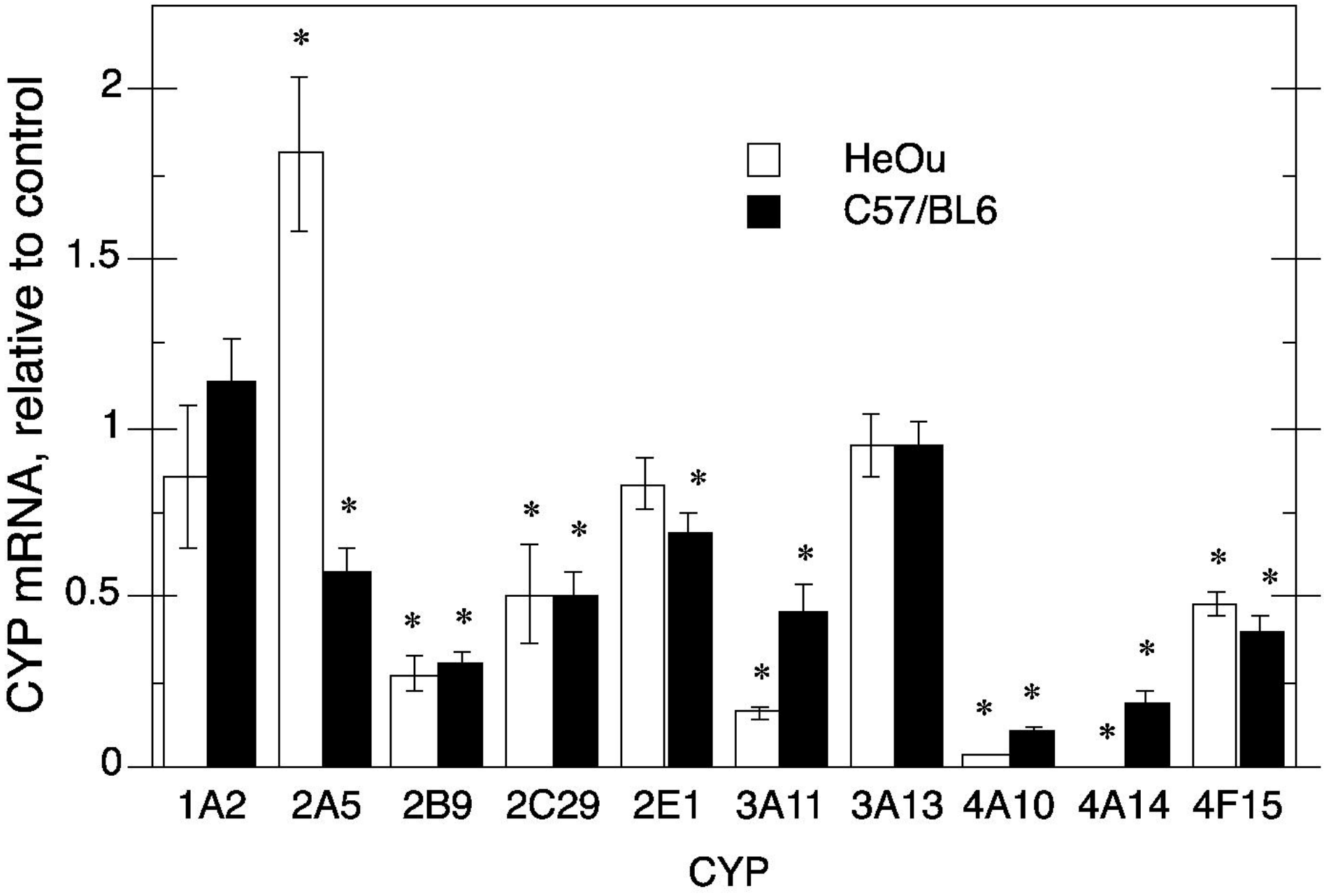

CYP mRNA, relative to control
2
1.5
1
0.5
0
HeOu
C57/BL6
1A2
2A5
2B9
2C29
2E1
3A11
3A13
4A10
4A14
4F15
CYP

Fig. 8