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Altered inflammatory responses to *Citrobacter rodentium* infection, but not bacterial lipopolysaccharide, in mice lacking the *Cyp4a10* or *Cyp4a14* genes

Beatrice A. Nyagode[1], **Ifor R. Williams**[2], and **Edward T. Morgan**[1,3]

[1]Department of Pharmacology, Emory University School of Medicine, Atlanta, GA 30322

[2]Department of Pathology and Laboratory Medicine, Emory University School of Medicine, Atlanta, GA 30322

Abstract

Murine hepatic Cyp4a mRNAs are markedly down-regulated during inflammation. Here we investigated the roles of Cyp4a10 and Cyp4a14 in the response to infection with *C. rodentium*. Absence of either *Cyp4a* gene attenuated or abrogated the changes in spleen weight, colon crypt length, hepatic cytokine and acute phase protein mRNAs, and serum acute phase proteins and cytokines caused by infection. *Cyp4a10*$^{-/-}$ mice on a low-salt diet had a similar hepatic acute phase response as those mice on a high salt diet, suggesting that hypertension associated with this genotype is not the cause of their altered inflammatory response. In contrast, wildtype, *Cyp4a10*$^{-/-}$ and *Cyp4a14*$^{-/-}$ mice showed similar responses to injected LPS. These results implicate Cyp4a10 and Cyp4a14 in the regulation of the host inflammatory response to enteropathogenic bacterial infection but not to acute aseptic inflammation. Understanding the mechanism of this role may lead to novel therapeutic approaches in some inflammatory diseases.

Keywords

Cyp4a; acute phase proteins; *C. rodentium*; LPS; fatty acids

INTRODUCTION

The CYP4A subfamily are cytochrome P450 (P450) fatty acid hydroxylases that catalyze the ω-hydroxylation of medium and long chain fatty acids and prostaglandins. Unlike the few other P450s that also carry out this oxidation, CYP4As preferentially target the terminal, primary C-H bonds rather than the adjacent and more easily oxidized secondary C-H bonds at the ω-1 and ω-2 carbons (1,2). This reaction initiates the degradation of long-chain fatty acids which are subsequently converted to dicarboxylic acids for use as fuel via mitochondrial or peroxisomal β-oxidation (3).

[3]To whom correspondence should be addressed at Department of Pharmacology, Emory University School of Medicine, 5119 Rollins Research Center, 1510 Clifton Road, Atlanta, GA 30322. edward.morgan@emory.edu.

Conflict of Interest. The authors declare that they have no conflict of interest.

The mouse has 5 functional Cyp4a genes, of which only Cyp4a10, Cyp4a12 and Cyp4a14 have been well characterized. Humans have two CYP4A genes, CYP4A11 and CYP4A22, however only CYP4A11 encodes a functional protein (4). CYP4A subfamily proteins have important roles in several physiological pathways. In addition to the above-mentioned roles in fatty acid metabolism, Cyp4a10 and Cyp4a12 in mouse and CYP4A11 and CYP4F2 in human catalyze the ω-hydroxylation of arachidonic acid to 20-hydroxyeicosatetranoic acid (HETE)(5-7). 20- HETE appears to have opposing roles in blood pressure regulation, either promoting hypertension by vasoconstriction or anti-hypertension by natriuresis (8-11). A polymorphism of either *CYP4A11* or *CYP2J2* genes in humans (4,12), the introduction into mice of human CYP4F2 (13) as well as the disruption of either *Cyp2j5, Cyp4a10* or *Cyp4a14* in mice result in hypertension (14-16).

We have previously published that upon infection with *Citrobacter rodentium*, a model of human enteropathogenic *Escherichia coli* (EPEC) infection that causes infectious colitis, Cyp4a10 and Cyp4a14 mRNAs and Cyp4a proteins are the most sensitive P450 isoforms to down-regulation in mouse liver and kidneys both in the C57BL/6 and C3H backgrounds (17-19). This led us to postulate that selective down-regulation might indicate a role of Cyp4a enzymes in the host's response to infection and (or) the bacterium's strategy for propagation and survival. If this indeed is the case, it is reasonable to hypothesize that genetic deletion of these enzymes might be expected to either protect the host from the infection and (or) its deleterious effects, or confer an advantage to the bacterium thereby sensitizing the host to infection. The objective of this study, therefore, was to examine the effect of the deletion of *Cyp4a10* or *Cyp4a14* in the regulation of hepatic P450s of the mouse liver during *C. rodentium* infection, and to compare this to the bacterial lipopolysaccharide (LPS) model of acute inflammation.

MATERIALS AND METHODS

Unless otherwise specified, all the reagents and chemicals were obtained from Sigma–Aldrich (St. Louis, MO).

Mice and treatments

Wild-type 129X1/SvJ and 129X1/SvEvTac animals, mice were purchased at 7-8 weeks of age from The Jackson Laboratory (Bar Harbor, ME) and Taconic Farms (Germantown, NY) respectively and were acclimatized to the animal facility for at least one week before the beginning of an experiment. *Cyp4a10*- deficient (*Cyp4a10*$^{-/-}$) and *Cyp4a14*- deficient (*Cyp4a14*$^{-/-}$) breeders were obtained from Dr. J. Capdevila, Vanderbilt University (15) and used to establish the respective colonies. All protocols were reviewed and approved by the Institutional Animal Care and Use Committee of Emory University.

All experiments were initiated when the mice were 9 weeks old. Mice were housed in groups of up to six to a cage. A wild-type strain of *C. rodentium* (#51116) was obtained from the American Type Culture Collection (Manassas, VA). Following overnight growth in Luria broth, without shaking at 37°C, the bacteria were serially diluted in sterile phosphate-buffered saline, and the nominal concentration calculated spectrophotometrically. Oral administration of *C. rodentium* was achieved by giving the animals an estimated 2×10^8

colony forming units (CFU) per mouse in 20% sucrose solution instead of drinking water for 24 h. To minimize differences between infections, whenever possible, all animals under treatment were infected with the same bacterial preparation. The bacterial concentrations given to each cage were determined by retrospective plating on MacConkey agar, from the volumes of liquid consumed. Infected mice were housed in a Biosafety Level-2 facility to prevent transmission of infection to other mouse colonies. Food consumption and changes in body weight were monitored daily and animals were sacrificed 7 days after administration of sucrose or bacteria. In the LPS experiment, mice in the treatment group were given an i.p. injection delivering 1mg/kg LPS (*E. coli* 0111:B4, ultrapure, Invivogen, San Diego, CA) dissolved in sterile saline while control mice received an i.p. injection of sterile saline alone. Food was withheld from the animals after the injection and the animals were sacrificed after 24h.

To investigate the effect of dietary sodium, breeders were switched from a standard breeder's diet containing 0.4% sodium to one with 0.24% sodium. The pups were weaned at 3-4 weeks of age and thereafter fed diets containing either 0.03 or 0.4% sodium (Lab Diet, St. Louis. MO). Infection was carried out as described above.

Tissue collection

Blood was collected from the animals at sacrifice, and allowed to clot for about 30 min. Serum was obtained by centrifugation for 10 min at 10,000*g* and stored at −80°C until analyzed. Liver and spleen were dissected and rinsed in cold 1.15% potassium chloride then weighed. The liver was then portioned, flash frozen then stored at −80°C for subsequent RNA or microsome preparation, or kept on ice for the determination of viable bacteria. The colon was removed and washed of fecal matter using cold 1.15% potassium chloride, sectioned, then kept on ice for the determination of viable bacteria or placed in histology cassettes and immersed in 10% formalin prior to histology analysis.

Histological analysis

Liver and distal colon sections were fixed in 10% formalin and embedded in paraffin. Sections (5 μm) were cut and stained with hematoxylin and eosin. The samples were coded and the observations made in a blinded fashion to avoid bias in the evaluation process. The heights of three well-oriented crypts were measured in the distal colon for each mouse, using a Zeiss 200M microscope (Thornwood, NY) with a 20X NA1.4 lens, and Slidebook (Intelligent Imaging Innovations, Denver CO. Stained liver tissue sections were scored under blinded conditions for the presence of inflammation, as indicated by the presence of neutrophils, macrophages, and hepatocyte necrosis using the following scale: 0, normal; 1, minimal; 2, mild; 3, moderate; 4, severe.

Determination of Tissue Bacterial Loads

The number of viable bacteria was determined from organ homogenates. Liver and colon were weighed and homogenized at low speed with a tissuemizer (IKA Works, Inc., Wilmington, NC) in 1ml of phosphate buffered saline. Liver homogenate, blood or serial dilutions of the colon homogenates were plated onto MacConkey's agar, on which *C.*

rodentium forms small pink colonies. The number of CFU were determined after overnight incubation at 37°C and the results reported per gram of tissue or 50μL of blood.

RNA extraction, cDNA synthesis and quantitative RT-PCR

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

To synthesize cDNA, purified total RNA was reverse-transcribed using the SuperScript First-Strand Synthesis System kit (Invitrogen, Carlsbad, CA), according to the manufacturer's protocol. PCR primers were custom-synthesized by MWGBiotech, Inc. (High Point, NC) or Operon Biotechnologies, Inc. (Huntsville, AL) and are listed in Supplemental Table 1. 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 (17,20). To normalize the inter-sample variation in quality inherently associated with RNA preparation, the transcription level of the housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), was quantified for all samples. The value obtained from each target gene was then normalized using the GAPDH value to calculate the relative target gene mRNA levels in comparison with the corresponding control groups by the $\Delta\Delta C_t$ method described by Livak and Schmittgen (21). The expression level in control samples was arbitrarily set at 1.

Serum Analytes

Using a customized Milliplex™ mouse cytokine/chemokine kit (Millipore Corporation, Billerica, MA) and following the manufacturer's protocol, serum samples were assayed for the cytokines interleukin (IL)-2 (IL2), IL3, IL4, IL6, IL7, IL9, IL10, IL17, interferon-γ (IFNγ) and tumor necrosis factor-α (TNFα) and the chemokines CC ligand 5 (CCL5), CXC motif ligand 1 (CXCL1), CXCL10 , macrophage inflammatory protein-1 alpha (MIP-1α), monocyte chemotactic protein-1 (MCP-1) and granulocyte colony-stimulating factor (G-CSF). The Milliplex™ mouse acute phase magnetic bead panel 2 kit (Millipore Corporation, Billerica, MA) was used to measure levels of α1-acid glycoprotein (AGP), C-reactive protein (CRP), haptoglobin (HAPT) and serum amyloid P component (SAP). Data was analyzed using MasterPlex software1.2 (Hitachi Software Engineering America, Ltd., San Francisco, CA) and the concentrations are expressed in picograms or nanograms per milliliter (pg/ml).

Statistical Analysis

All data are presented as mean values ± standard error of the mean (S.E.M.) unless otherwise designated. Data were analyzed by two-way ANOVA followed by Holm-Sidak's multiple comparison test, unless otherwise indicated. For statistical analysis to allow comparison of the size of the effect of infection among genotypes, each continuous variable was normalized to the uninfected group mean within each genotype. Liver pathology scores

are discontinuous variables, and were analyzed by a nonparametric Kruskal-Wallace test followed by Dunn's multiple comparison test.

RESULTS

Comparison of Wild-Type Animals

In the ensuing studies, we used 129X1/SvJ mice as the WT strain, as indicated in Holla et al (18) and Quigley et al. (22). However, we subsequently discovered that the correct 129 strain might in fact be 129X1/SvEvTac, which were the source of the embryonic stem cells used to construct the *Cyp4a14*$^{-/-}$ line (18). We therefore compared the effect of infection on wild-type female 129X1/SvJ (Jax) and 129X1/SvEvTac (Tac) mice. While there were no significant differences, the colony counts in the blood and liver but not the colon tended to be higher in Tac than in Jax animals (data not shown). As expected, infection down-regulated hepatic Cyp3a11, Cyp 3a25, Cyp4a10 and Fmo3 mRNAs while up-regulating Cyp 2d9, Cyp 4f18 and various cytokine mRNAs (Fig. 1). The effects of infection on hepatic mRNAs (Fig. 1A, B) and serum cytokines (Fig. 1C) were similar and therefore it was concluded that either 129X1/SvJ or 129X1/SvEvTac animals were appropriate WT controls for our study model.

Effects of *Cyp4a* genotype on responses to infection

Colony Counts—Female *Cyp4a10*$^{-/-}$ mice, and male *Cyp4a10*$^{-/-}$ and *Cyp4a14*$^{-/-}$ mice had significantly lower *C. rodentium* colonization of their colons than did WT mice (Supplemental Fig. 1). There was a similar trend in *Cyp4a14*$^{-/-}$ females, but it was not significant. Interestingly *Cyp4a10*$^{-/-}$ males or females trended towards lower bacterial levels in the blood also. There were no significant effects of genotype on levels of bacteria in the liver (Supplemental Fig. 1). The mean doses of bacteria ingested by the three groups were similar (Supplemental Table 2), although the exposure of the WT mice was slightly lower than that of the two Cyp4a-null genotypes.

Body weights, Organ weights, colon and liver pathology—In female animals, there were no significant differences in body weight gain among any of the groups (not shown). The body weights of male mice are shown in Fig. 2. After the initial loss of weight on day 1 (due to withdrawal of food), all groups quickly regained weight by day 2. Body weights of infected male mice were not significantly different from those of uninfected mice of the same genotype at any time point, except for infected WT mice which lost less weight than their uninfected counterparts on day 1, and infected *Cyp4a14*$^{-/-}$ mice which gained more weight than uninfected on day 2. In contrast to all of the other groups, however, infected WT did not gain weight on days 4-7, so that body weights were significantly higher in the infected *Cyp4a10*$^{-/-}$ and *Cyp4a14*$^{-/-}$ mice than in the infected WT animals.

In WT male mice infection resulted in a significantly increased spleen weight, which was attenuated in the *Cyp4a10*$^{-/-}$ or *Cyp4a14*$^{-/-}$ males (Table 1). In female mice, the splenomegaly observed in WT mice was not significant in the *Cyp4a10*$^{-/-}$ or *Cyp4a14*$^{-/-}$ mice, although the mean values for *Cyp4a14*$^{-/-}$ were not greatly less than in WT. There was no significant effect of infection on liver weight in males of any genotype. In females,

however, liver weight was increased by 24%, and this change was no longer observed in the Cyp4a-null strains (Table 1).

As expected, infection caused an increase in colonic crypt height (a measure of colonic hypertrophy) of the WT animals of both sexes (Table 1). In male mice, the magnitude of this increase was attenuated in both the *Cyp4a10*$^{-/-}$ or *Cyp4a14*$^{-/-}$ strains. In female mice, only the absence of *Cyp4a14* had this effect, whereas *Cyp4a10*$^{-/-}$ mice had the same degree of hypertrophy as WT (Table 1)

Infection of WT mice with *C. rodentium* induced a minimal to mild degree of liver inflammation, as judged by the density of inflammatory foci, and was more pronounced in WT males than in females (Fig. 3). No male *Cyp4a10*$^{-/-}$ mice exhibited histologically detectable liver inflammation ($p<0.05$), and minimal inflammation was detected in only one *Cyp4a14*$^{-/-}$ animal ($p=0.058$). Female mice showed the same trends, but the differences were not significant.

Hepatic Cytokine and Acute Phase Protein mRNAs—In male animals, the induction of hepatic cytokine and acute phase protein (APP) mRNAs caused by infection were either attenuated or abolished in *Cyp4a10*$^{-/-}$ and *Cyp4a14*$^{-/-}$ mice (Figure 4A and B). This phenotype appeared to be more robust in the *Cyp4a10*$^{-/-}$ than the *Cyp4a14*$^{-/-}$ mice. Thus, the mRNA levels of IL1β, IL6, TNFα, IFNγ, AGP, FBG, HAPT, SAA and SAP were all induced by infection in WT mice, whereas none were significantly affected by infection in the two *Cyp4a*-null strains. Basal expression of IL1β, AGP, FBG, HAPT, SAA and SAP was also significantly reduced in the *Cyp4a10*$^{-/-}$ and *Cyp4a14*$^{-/-}$ mice, with IL6, TNFα and IFNγ trending towards the same pattern (Supplemental Fig. 2).

For most cytokine and APP mRNAs, the pattern of regulation seen in female animals was similar to that in males, though several of the effects caused by infection in WT males failed to reach significance in female mice (Supplemental Fig. 3A, B). In notable contrast to males, however, the induction of SAA by infection was potentiated in the female *Cyp4a10*$^{-/-}$ and *Cyp4a14*$^{-/-}$ mice.

Hepatic P450 mRNAs—In both female and male animals, the relative constitutive levels of most P450 mRNAs were generally higher in uninfected wildtype, WT (Con), than in uninfected *Cyp4a10*$^{-/-}$ or *Cyp4a14*$^{-/-}$ mice (Supplemental Fig. 4) with some exceptions. Cyp3a41 mRNA expression was higher than WT levels in *Cyp4a10*$^{-/-}$ and *Cyp4a14*$^{-/-}$ females but not in males. Cyp4a14 mRNA expression was elevated 2-3 fold in both genders of the *Cyp4a10*$^{-/-}$ mice. Basal expression of Cyp4f18 (Supplemental Fig. 4), and of flavin monooxygenase 3 (Fmo3) and peroxisome proliferator-activated receptor-α(Pparα) (Supplemental Fig. 2) was not different among the genotypes.

In WT male mice, infection down-regulated Cyp2d22, Cyp3a11, Cyp3a41, Cyp4a10 and Cyp4a14 to differing degrees, and up-regulated Cyp4f18 (Fig. 4C). Cyp3a25 also trended towards down-regulation. Of these P450 mRNAs, none were significantly different from uninfected controls in the *Cyp4a10*$^{-/-}$ and *Cyp4a14*$^{-/-}$ strains, except for Cyp4a10, whose down-regulation by infection was not different from the WT mice (Fig. 4C). The mRNAs

for Cyp1a2, Cyp2a4/5, Cyp2b9, Cyp2c29, Cyp2d9, Cyp2e1, Cyp3a13, Cyp4a12, Fmo3 and Pparα were not significantly affected by infection in WT mice and are not shown.

As for the cytokine and APP mRNAs, the responses of WT female hepatic P450 mRNAs to infection were generally smaller than those seen the WT males (Supplemental Fig. 3C). Similarly, the impact of the *Cyp4a10* and *Cyp4a14* deletions on the responses to infection were more muted than observed in males. In female animals of both genotypes, infection did not have significant effects on mRNA levels of Cyp1a2, 2b9, 2e1, 3a13 and 4a12. Cyp3a25 and Fmo3 were down-regulated similarly in all three strains. Cyp4a10 and Cyp4a14 down-regulations were unaffected in the *Cyp4a14*$^{-/-}$ and *Cyp4a10*$^{-/-}$ mice, respectively. The changes caused by infection in WT female animals were attenuated (did not reach significance) in the *Cyp4a10*$^{-/-}$ and *Cyp4a14*$^{-/-}$ strains for Cyp3a11, 3a25, 2d9 and 4f18 (Supplemental Fig. 3C).

Serum Analytes—Serum cytokine, chemokine and APP profiles of the male animals are shown in Fig. 5. Serum levels of IL2, IL3, IL4, IL7, IL9, IL10, IL17, CXCL1, MIP-1α and CCL5 were not significantly changed by infection in any strain, and are not shown. Serum levels of IFNγ, TNFα, IL6, CXCL10 and G-CSF were all significantly increased by infection in WT male mice (Fig. 5A, B), and again there was a tendency for most of these responses to be attenuated or blocked in the *Cyp4a10*$^{-/-}$ and *Cyp4a14*$^{-/-}$ animals. Thus, no significant effect of infection was observed for any of these analytes (compared to uninfected controls of the same genotype) in the *Cyp4a10*$^{-/-}$ and *Cyp4a14*$^{-/-}$ mice. When the magnitudes of changes due to infection were compared via ANOVA (# symbols), *Cyp4a10*$^{-/-}$ mice were significantly different from WT for all six analytes whereas *Cyp4a14*$^{-/-}$ mice were significantly different from WT with respect to IL6, CXCL10 and G-CSF. Serum APPs showed a similar pattern (Fig. 5C). The increases in serum AGP and SAP were abolished in the *Cyp4a10*$^{-/-}$ and *Cyp4a14*$^{-/-}$ mice, whereas the increase in HAPT was significantly attenuated.

In contrast to the males, when the basal levels of cytokines and chemokines of uninfected female animals were compared, markedly higher serum levels of IFNγ, IL6, IL17, G-CSF, CRP and HAPT were observed in *Cyp4a14*$^{-/-}$ than in WT (Fig. 6). This was only true for CRP and HAPT in the *Cyp4a10*$^{-/-}$ mice. In females, the significant increases of serum TNFα, IL17, CXCL10, and G-CSF due to infection in WT animals were either attenuated or abrogated in the *Cyp4a10*$^{-/-}$ and *Cyp4a14*$^{-/-}$ animals (Fig. 6A, B). Infection did not cause significant elevations of any APPs in the WT females, but did significantly up-regulate AGP in *Cyp4a10*$^{-/-}$ mice, and HAPT in both *Cyp4a*-null strains.

Effect of dietary sodium, during infection, on Hepatic APP mRNA—Since *Cyp4a10*$^{-/-}$ mice exhibit salt-sensitive hypertension (16), we next asked whether the impact of the *Cyp4a10* gene deletion on the response to infection is also related to this phenotype. Therefore, we compared the responses in WT and *Cyp4a10*-deficient male mice fed diets containing either 0.03 or 0.4% sodium. In blood, liver and colon, there were no significant differences in bacterial counts among the different treatment groups (data not shown.

Among the animals fed the 0.4% Na diet, the up-regulation, by infection, of acute phase mRNAs observed in WT mice was either attenuated or abrogated in the KO animals (Fig. 7). Though some of the effects in WT mice were not significant ($p > 0.05$), the pattern of regulation in WT and Cyp4a-null animals fed 0.03% Na was similar to that seen at the 0.4% Na level with the exceptions that infection did not affect α-fibrinogen (FBG) in WT animals on the low salt diet (Fig. 7).

Effect of *Cyp4a* gene deletion on the response to acute, sterile inflammation— The effect of acute, sterile inflammation caused by injection of LPS on hepatic P450 mRNA levels was investigated in male mice. Unlike the selective effects of infection, LPS administration resulted in the decrease of almost all P450 mRNAs 24 h after a 1mg/kg i.p. injection (Fig. 8A). Similarly, all APP mRNAs measured were induced (Fig. 8B). There were no significant differences among the genotypes in these responses to LPS.

Essentially the same results were found in female animals (not shown), except that Cyp2d9 mRNA was up-regulated in female mice (Supplemental Fig. 5). This induction occurred to the same extent in all three genotypes as well.

DISCUSSION

In agreement with our hypothesis that *Cyp4a10* or *Cyp4a14* ablation would affect the host response to *C. rodentium* infection, many of the hepatic and humoral inflammatory responses of both *Cyp4a*-deficient strains to *C. rodentium* infection were attenuated or abolished compared to WT mice. These effects of *Cyp4a* deletion were particularly pronounced for hepatic APP and cytokine mRNAs. APPs are synthesized primarily by hepatocytes and are induced and secreted as part of the acute phase response to inflammation or infection. APPs are involved in homeostasis as well as restraint of microbial growth before an acquired immunity is established (23). APP concentrations may be correlated with the severity of a disorder and thus may be valuable biomarkers of the inflammatory response (24). In this study, infection induced the expression of most hepatic APP and cytokine mRNAs. The absence of *Cyp4a10* or *Cyp4a14*, especially in male animals greatly attenuated or obviated this induction (Fig. 4). Liver inflammation observed histologically was significantly attenuated in *Cyp4a10*$^{-/-}$ males and similar trends were seen in the *Cyp4a10*$^{-/-}$ males as well as the females of each genotype. This suggests that both *Cyp4a10* and *Cyp4a14* are involved in the innate response of the host liver to the infection. In WT mice, the effects of infection on the liver were more pronounced in males than in females. Thus, the impact of the *Cyp4a10*$^{-/-}$ and *Cyp4a14*$^{-/-}$ genotypes were more clearly observed in males although females followed the same pattern.

The regulation of most P450 enzymes followed the same trends in both male and female animals; that is, their down-regulation by infection was attenuated in *Cyp4a14*$^{-/-}$ mice. This is consistent with the reduced hepatic inflammatory response inferred from the hepatic APP and cytokine mRNAs as well as from histological analysis. Down-regulation of Cyp2d22, Cyp3a11 and the female-dominant Cyp3a41 were abolished in male but not female *Cyp4a10*$^{-/-}$ and *Cyp4a14*$^{-/-}$ mice, consistent with the APP and cytokine data above. Conversely, the absence of Cyp4a10, and to a lesser extent Cyp4a14 attenuated the up-

regulation of the male dominant Cyp2d9 in females whereas it had no effect on Cyp2d9 in males of either strain. Interestingly, the down-regulation of Cyp4a10 was not attenuated in *Cyp4a14*$^{-/-}$mice, nor was the down-regulation of Cyp4a14 attenuated in *Cyp4a10*$^{-/-}$, supporting evidence from our previous work that these enzymes are regulated by different mechanisms than the drug-metabolizing P450s in this disease model (18,25).

Consistent with the hepatic cytokine and APP mRNAs, serum cytokine and APP responses to infection in male mice were also attenuated in the *Cyp4a10*$^{-/-}$ and *Cyp4a14*$^{-/-}$ animals. However, uninfected female *Cyp4a14*$^{-/-}$ (but not males or *Cyp4a10*$^{-/-}$ mice) had very high serum levels of IFNγ, IL1, IL3, IL4, IL6, IL9, IL10, IL17, as well as chemokines CXCL1, MIP-1α, CCL5 and G-CSF and the APPs CRP and HAPT (Fig. 6 and data not shown). This suggests that female *Cyp4a14*$^{-/-}$ have constitutive inflammation. However, hepatic cytokine or APP mRNAs tended to be reduced in uninfected *Cyp4a14*$^{-/-}$ mice. This suggests that the elevated APPs in sera of female *Cyp4a14*$^{-/-}$ mice may be of extrahepatic origin. In any case, the blunted responses of these analytes to infection could be due to the already high basal levels in *Cyp4a14*$^{-/-}$ females.

Colon crypt lengths are a measure of mucosal inflammation and hypertrophy caused by infection. The magnitude of the increase in crypt length caused by infection was significantly reduced in *Cyp4a14*$^{-/-}$ males and females (Table 1), whereas in the *Cyp4a10*$^{-/-}$ mice this phenotype was only observed in the females. Conversely, the bacterial counts in colons of *Cyp4a10*$^{-/-}$ and *Cyp4a14*$^{-/-}$ trended towards being lower than WT animals, an effect that was more pronounced in males than in female *Cyp4a14*$^{-/-}$ mice. Attenuated inflammatory responses are also indicated by the fact that *Cyp4a10*$^{-/-}$ and *Cyp4a14*$^{-/-}$ mice were resistant to hepatomegaly and splenomegaly in response to the infection, again most apparent in male mice.

The above findings are consistent with the hypothesis that Cyp4a14 down-regulation during infection could be a homeostatic response to help moderate or terminate inflammation before it becomes deleterious. Alternatively, the reduced inflammation might help the invading organism to thrive. Since colonic *C. rodentium* counts were, if anything, lower in the *Cyp4a* – null strains than in WT mice, the former explanation seems more likely. It should also be considered that, since expression of other P450 enzymes in the liver was generally lower in the uninfected Cyp4a-null mice compared to WT, this altered expression may have also contributed to the reduced inflammatory responses observed in the *Cyp4a10*$^{-/-}$ and *Cyp4a14*$^{-/-}$ mice.

With some exceptions noted above, the impacts of the *Cyp4a10*$^{-/-}$ and *Cyp4a14*$^{-/-}$ genotypes on the responses to *C. rodentium* infection were remarkably similar. This indicates that the functions of these enzymes in regulating the inflammatory response are not redundant. This could be because the enzymes catalyze different steps in a common pathway. Alternatively, the substrates they metabolize, or the products they produce, may be different. By analogy, both *Cyp4a10*$^{-/-}$ and *Cyp4a14*$^{-/-}$ mice are hypertensive, but the mechanisms by which hypertension is elicited are quite different. The *Cyp4a14*$^{-/-}$ hypertensive phenotype is gender (androgen)-dependent and insensitive to dietary sodium (15). It is related to elevated Cyp4a12 expression and higher rates of 20-

hydroxyeicosatetraenoic acid (20-HETE) formation in the kidneys. Disruption of the *Cyp4a10* gene results in salt-sensitive hypertension that is not gender-dependent (16). Thus, *Cyp4a10*$^{-/-}$ mice fed on diets containing 0.3 or 8% sodium were severely hypertensive, and became normotensive when fed 0.05% sodium diets (16).

The hypertensive phenotype per se does not appear to be the cause of the attenuated inflammation in the Cyp4a-null animals. As shown in Fig. 7, reduced inflammation as assessed by APP regulation was no different in animals consuming high-salt or low-salt diets. While we did not measure the blood pressure, it has been reported that in WT animals of the same strain, even 8% NaCl had no significant effects on the blood pressure, while Cyp4a10$^{-/-}$ mice on 0.3% or 8% but not 0.05% sodium were severely hypertensive (16)). Although both hypertension and the diminished responses to inflammation are both more prevalent in male than female *Cyp4a14*$^{-/-}$ mice, the tendency to observe a reduced impact of Cyp4a gene deletion on inflammation in females appears to be mainly because WT females have a smaller response to infection compared to males. The female Cyp4a-null strains themselves showed little if any gender differences in the response to infection.

The mechanisms by which *Cyp4a10* and *Cyp4a14* deletions cause resistance to inflammation can only be speculated upon. If 20-HETE were involved in the effects observed here, one would expect it to be pro-inflammatory (since eliminating Cyp4a enzymes that produce it is anti-inflammatory). To the contrary, a synthetic agonistic analogue of 20-HETE was reported to reduce renal activation of NF-κB and elevation of serum TNF in LPS-induced septic shock in rats (26). 20-HETE is also a potent agonist of PPAR (27), which can exert anti-inflammatory effects via inhibition of NF-κB (28). Also, recombinant Cyp4a12a and Cyp4a12b are about 100 times more active than Cyp4a10 in 20-HETE formation from arachidonic acid, whereas Cyp4a14 is essentially inactive (29). Accordingly, Cyp4a12 appears to be the important 20-hydroxylase in the mouse kidney (15). Thus, the roles of Cyp4a10 and Cyp4a14 in this disease model may be to regulate the levels of fatty acids or other substrates that are precursors of anti-inflammatory molecules. Alternatively, this phenomenon could be related to the fact that CYP4A enzymes are catalytically uncoupled (30), resulting in production of reactive oxygen species. Oxidative stress caused by induction of Cyp4a10 has been implicated in hepatic neoplasia (31).

Christmas et al (32) identified Cyp4f18 as the functional orthologue of human CYP4F3A, a critical protein in the inactivation of leukotriene B4 (LTB4), a potent mediator of inflammation and chemoattractant for polymorphonuclear leukocytes. The abolition of Cyp4f18 induction in response to infection in *Cyp4a10*$^{-/-}$ and *Cyp4a14*$^{-/-}$ mice seems to belie the hypothesis that the absence these P450s is beneficial for the resolution of the infection, at least in terms of LTB4 action. It would be interesting to find out if these animals have defective LTB4 synthesis and therefore do not need to increase their production of the LTB4- ω-hydroxylase Cyp4f18.

Rodent CYP4As are induced via activation of the nuclear receptor PPARα in fasting and starvation commensurate with their roles in fatty acid metabolism (33). The hepatic expression of Cyp4a10 and 4a14 were higher in wildtype compared to PPARα-null mice suggesting a role of the nuclear receptor in the basal expression of Cyp4a enzymes (34). The

down regulation of Cyp4as by *C. rodentium infection* however is not due to changes in PPARα mRNA levels as these are not significantly different when control and infected animals are compared (Supplemental Fig. 2A and data not shown).

P450 mRNAs are selectively modulated during *Citrobacter rodentium* infection in a pattern that is different from the LPS model. Infection down-regulates Cyp4a10 and Cyp4a14 in the liver and kidney of female mice (17-19). LPS treatment induces these enzymes in the kidney (20) as well as liver of CD-1 mice (35) but down-regulates Cyp4a10 in the liver of 129/SvJ and C57BL/6J mice (18,34). In contrast to the sub-chronic inflammation caused by *C. rodentium* infection, LPS injection is a model of sterile acute inflammation. Here we report that LPS caused the down-regulation of P450s and up-regulation of APPs in a pattern that was similar in all three genotypes of both sexes, hence there was no evidence of the involvement of Cyp4a10 or Cyp4a14 in the LPS response. These results indicate that the presence or absence of Cyp4a10 and Cyp4a14 affects hepatic and humoral inflammatory responses to enteral *C. rodentium* infection but not to acute exposure to endotoxin. Unresolved questions include mechanisms by which Cyp4a10 and Cyp4a14 affect the inflammatory response to infection, and whether it is applicable to other bacterial, viral or parasitic infections.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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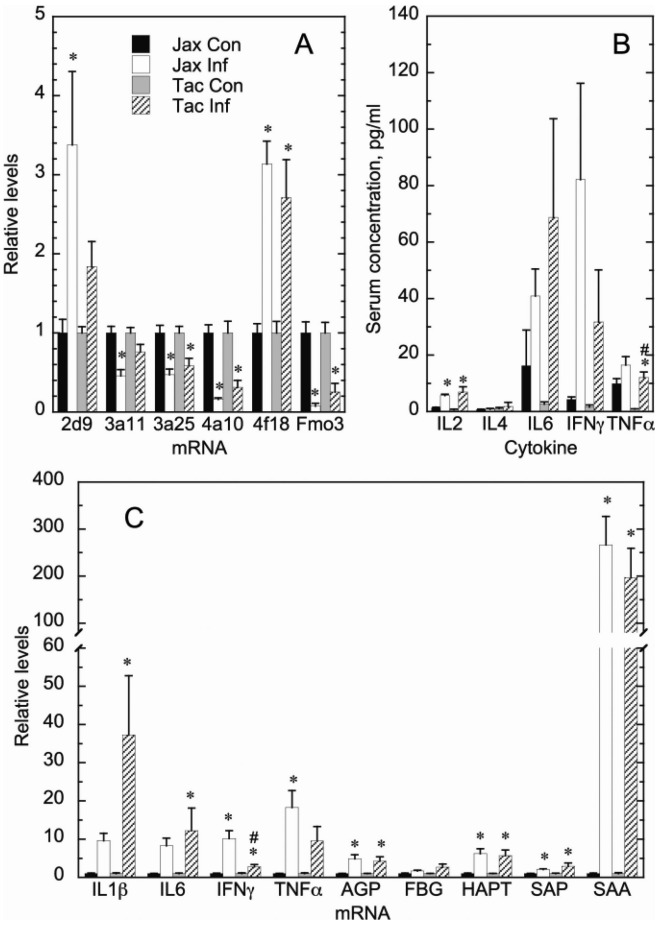

Figure 1.

Comparison of the effect of oral *C. rodentium* infection in wildtype 129X1/SvJ (Jax) and 129X1/SvEvTac (Tac) mice. Over 24 h, female mice were allowed to drink either sucrose solution (Control) or *C. rodentium* in sucrose (Infected) then sacrificed after 7 days. Livers were harvested for measurement of mRNAs by real-time RT-PCR, and serum was prepared for analysis of serum cytokines A) Hepatic P450 and Fmo3 mRNAs. B) Hepatic cytokine and acute phase protein mRNAs. C) Serum cytokines. mRNA levels are expressed as relative levels of mRNA expression after normalization to GAPDH, with sucrose-treated (Control) group set to 1. Values represent mean ± S.E.M, (n = 6). *, significantly different from control mice of same genotype; #, significantly different from Jax LPS-treated mice, $p < 0.05$.

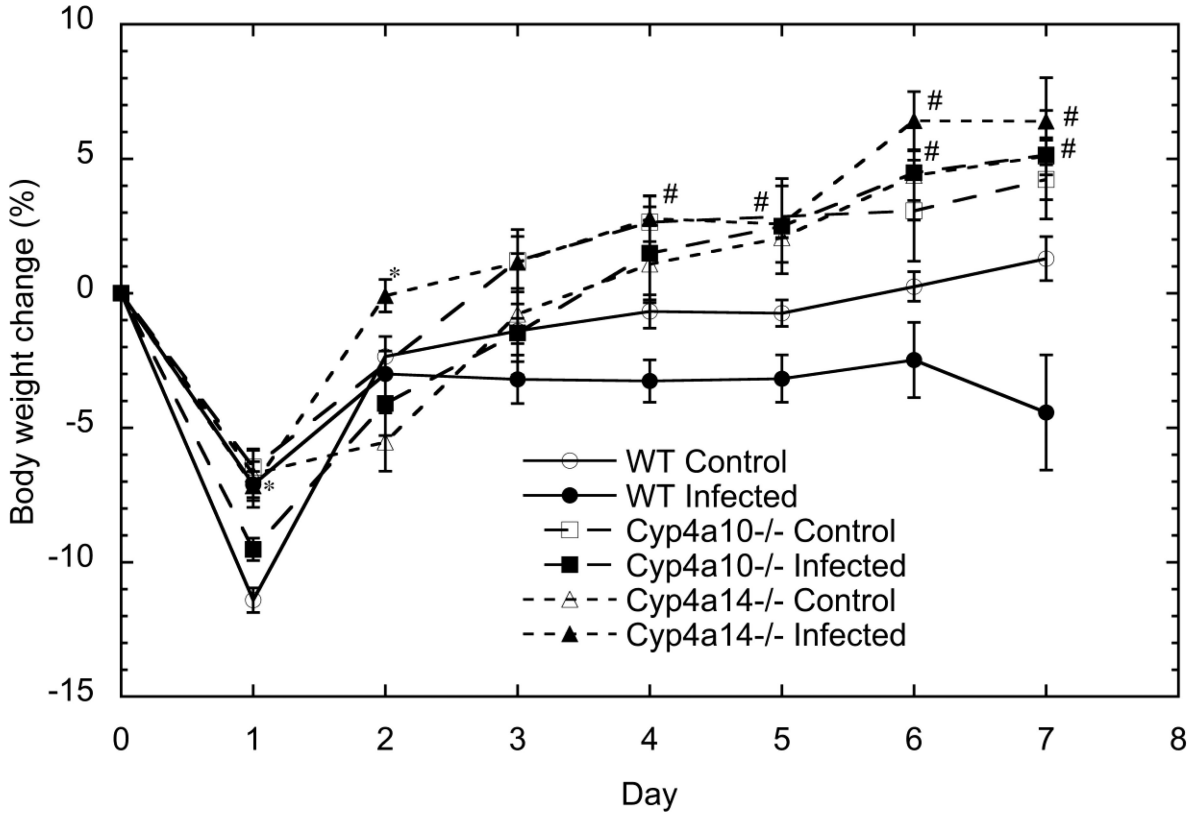

Figure 2.

Body weight changes in control and infected mice. Over 24 h, male mice were allowed to drink either sucrose solution (Control) or *C. rodentium* in sucrose (Infected). Body weights were monitored, and expressed as a percentage of the original body weight for each animal. Most groups had n= 5 or 6 animals, but data are missing for some animals on days 2-6. Values represent mean ± S.E.M. *, significantly different from control mice of same genotype; #, significantly different from WT infected mice, $p < 0.05$.

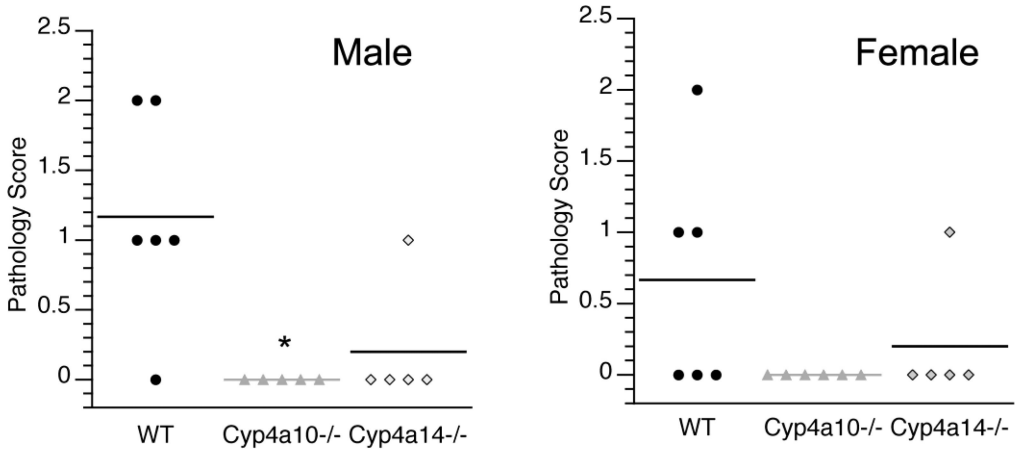

Figure 3.

Liver pathology during *C. rodentium* infection wildtype (WT), *Cyp4a10*-deficient (*Cyp4a10*$^{-/-}$) and *Cyp4a14*-deficient (*Cyp4a14*$^{-/-}$) mice. Liver sections were stained with hematoxylin and eosin and scored for the presence of inflammation using the following scale: 0, normal; 1, minimal; 2, mild; 3, moderate; 4, severe. Points on the graph are livers from individual mice. All livers for uninfected mice had a score of zero, and the data are not shown. *, significantly different from WT group, $p<0.05$ by Kruskal-Wallace test followed by Dunn's multiple comparison test.

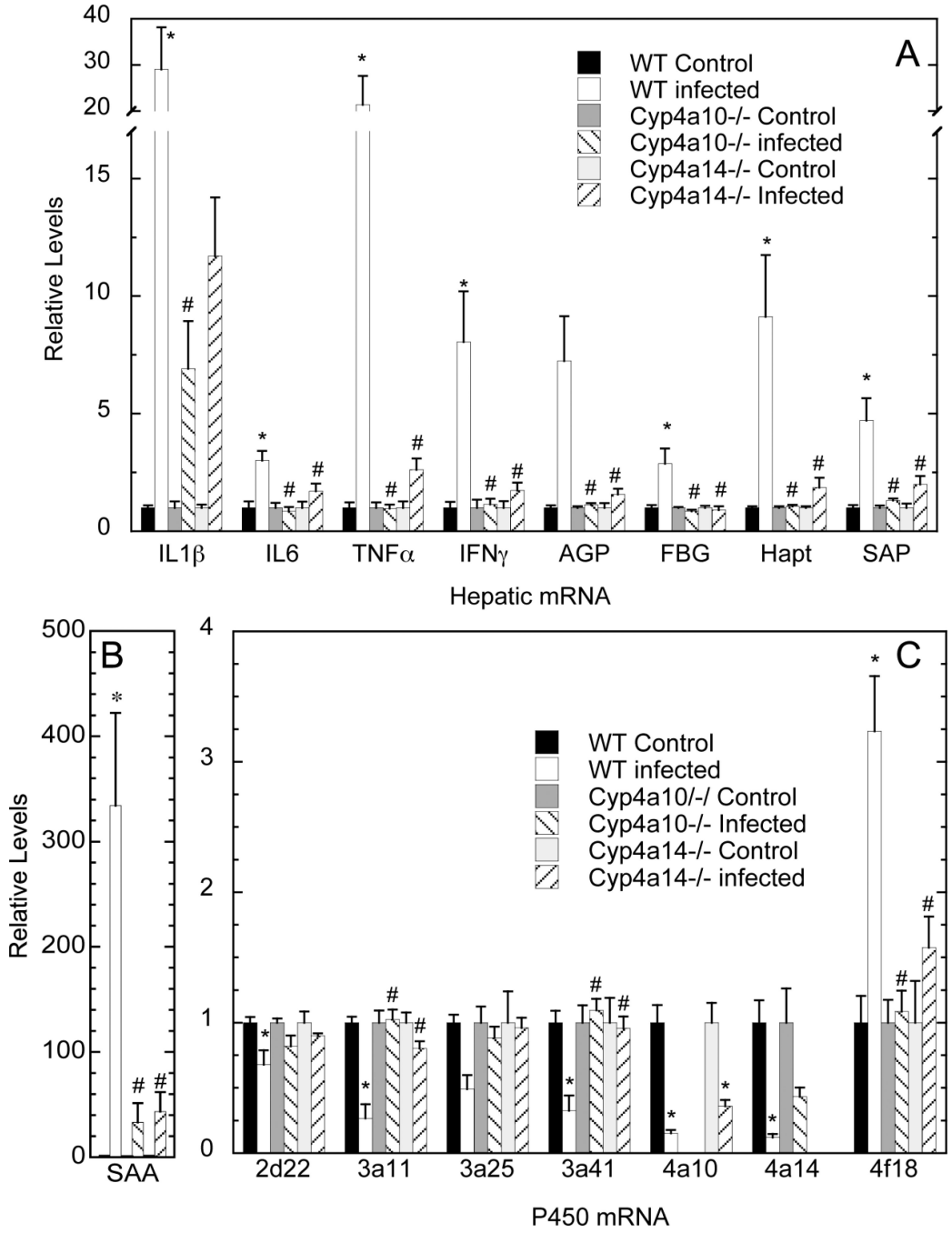

Figure 4.

Effect of oral *C. rodentium* infection on hepatic mRNA expression in wildtype (WT), *Cyp4a10*-deficient (*Cyp4a10*$^{-/-}$) and *Cyp4a14*-deficient (*Cyp4a14*$^{-/-}$) mice. Over 24 h, male mice were allowed to drink either sucrose solution (Control) or *C. rodentium* in sucrose (Infected) then sacrificed after 7 days and livers harvested for measurement of mRNA by real-time RT-PCR. Cytokines and acute phase proteins (A and B); P450s (C). Values are expressed as relative levels of mRNA expression after normalization to GAPDH, with sucrose-treated (Control) group set to 1. Values represent mean ± S.E.M, (n = 5 or 6).

*, significantly different from control mice of same genotype; #, significantly different from WT infected mice, $p < 0.05$.

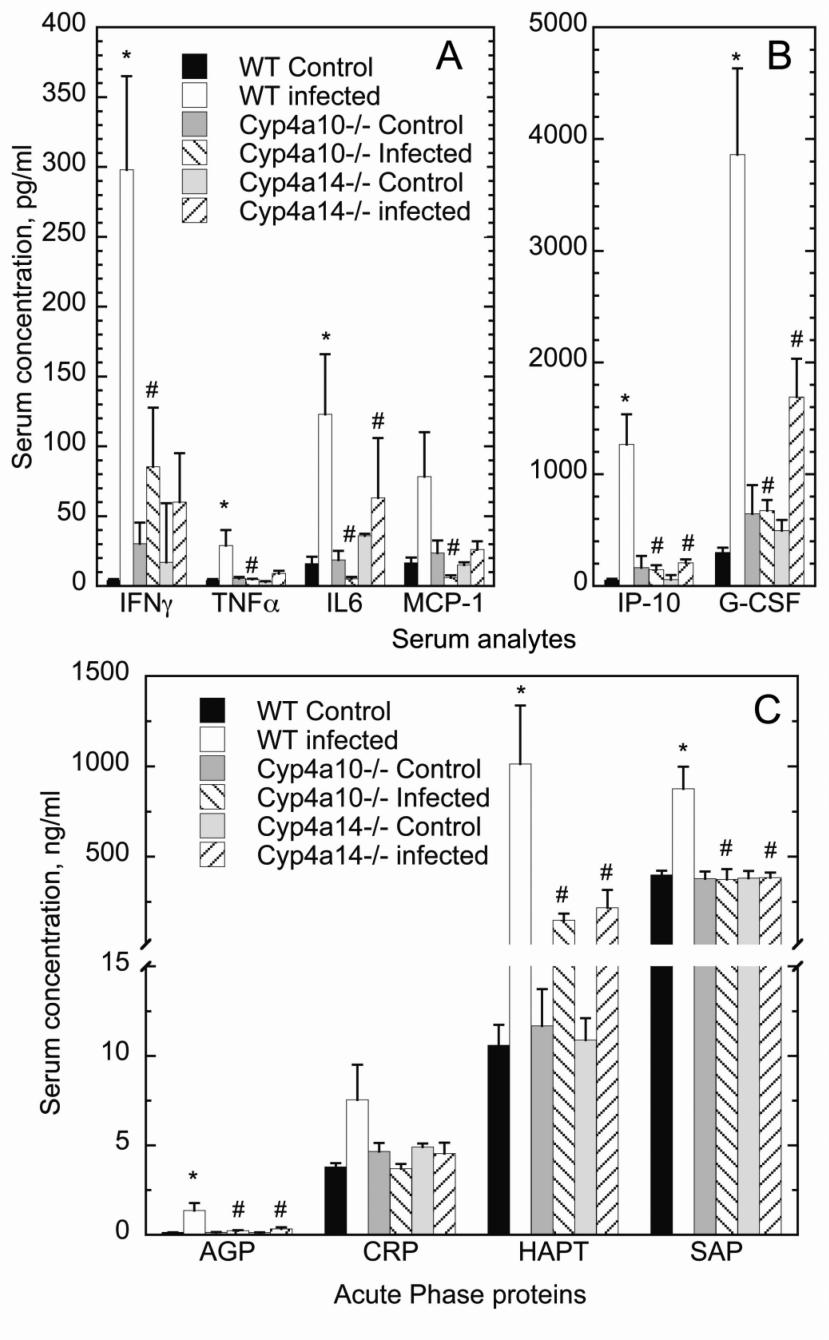

Figure 5.

Effect of oral *C. rodentium* infection on serum cytokine and acute phase protein concentrations in male wildtype (WT), *Cyp4a10*-deficient (*Cyp4a10*$^{-/-}$) and *Cyp4a14*-deficient (*Cyp4a14*$^{-/-}$) mice. Mice were treated as described in Fig. 2 then sacrificed after 7 days, at which time blood was collected for measurement of serum cytokines, chemokines and APPs. IL2, IL3, IL4, IL7, IL9, IL10, IL17, CCL5, CXCL1, MIP1α did not change with infection and are not shown. Values represent mean ± S.E.M, (n = 5 or 6). *, significantly

different from control mice of same genotype; #, significantly different magnitude of response from that of WT infected mice, $p < 0.05$.

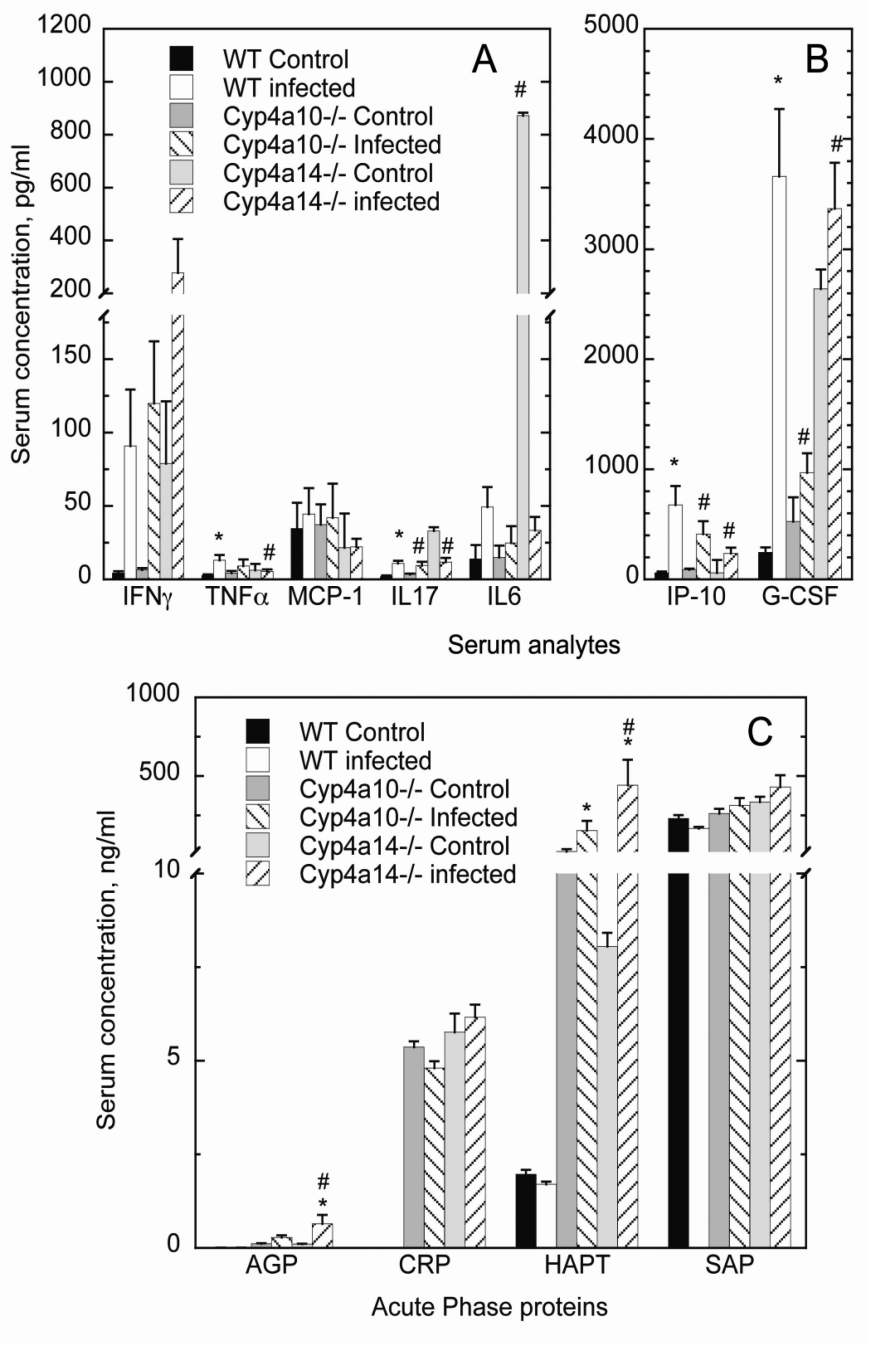

Figure 6.

Effect of oral *C. rodentium* infection on serum cytokine and acute phase protein concentrations in female wildtype (WT), *Cyp4a10*-deficient (*Cyp4a10*$^{-/-}$) and *Cyp4a14*-deficient (*Cyp4a14*$^{-/-}$) mice. Mice were treated as described in Fig. 2 then sacrificed after 7 days, at which time blood was collected for measurement of serum cytokines, chemokines and APPs. IL2, IL3, IL4, IL7, IL9, IL10, IL17, CCL5, CXCL1, MIP1α did not change with infection and are not shown. Values represent mean ± S.E.M, (n = 5 or 6). *, significantly

different from control mice of same genotype; #, significantly different magnitude of response from that of WT infected mice, $p < 0.05$.

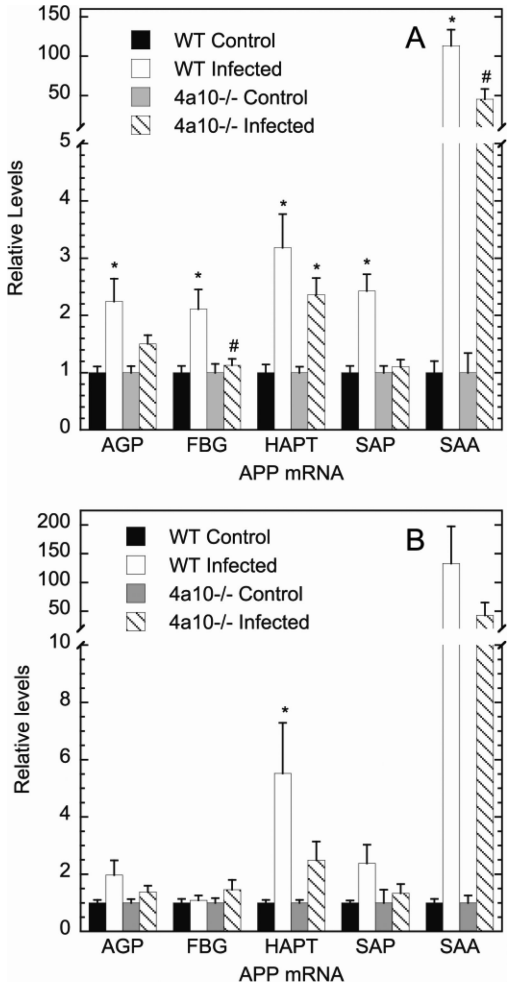

Figure 7.

Effect of dietary sodium on hepatic acute phase response to oral *C. rodentium* infection in wildtype (WT) and *Cyp4a10*-deficient (*Cyp4a10*$^{-/-}$) mice. Over 24 h, male mice on diets containing either 0.03 or 0.4% sodium were allowed to drink either sucrose solution (Control) or *C. rodentium* in sucrose (Infected) then sacrificed after 7 days and livers harvested for measurement of mRNA by real-time RT-PCR. Values are expressed as relative levels of mRNA expression after normalization to GAPDH, with sucrose-treated (Control) group set to 1. Values represent mean ± S.E.M, (n = 5 or 6). *, significantly different from control mice of same genotype; #, significantly different from WT infected mice, $p < 0.05$.

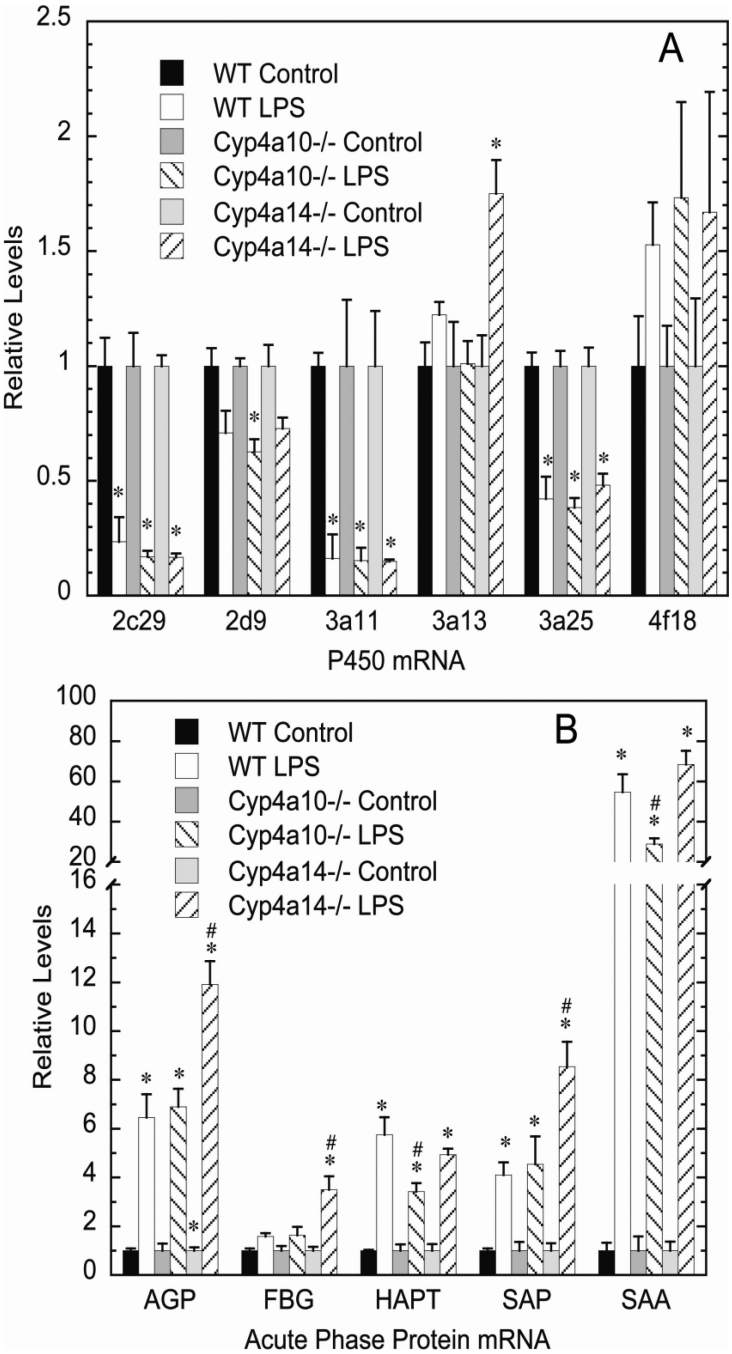

Figure 8.

Effect of LPS injection on hepatic P450 and acute phase protein mRNAs in wildtype (WT), *Cyp4a10*-deficient (*Cyp4a10*$^{-/-}$) and *Cyp4a14*-deficient (*Cyp4a14*$^{-/-}$) mice. Male mice were injected i.p. with either saline (Control) or 1 mg/kg LPS (LPS), and livers harvested after 24 h for measurement of mRNA expression by real-time RT-PCR. Values are expressed as relative levels of mRNA expression after normalization to GAPDH with saline-treated group set to 1. Values represent mean ± S.E.M, (n = 5 or 6). *, significantly different

from control mice of same genotype; #, significantly different from WT infected mice, $p < 0.05$.

Table 1

Crypt heights, liver and spleen weights of control and infected mice.

	Liver (% of body weight)		Spleen (% of body weight)		Crypt heights (μm)	
	Control	**Infected**	**Control**	**Infected**	**Control**	**Infected**
WT (M)	4.17 ± 0.076 (6)	4.73 ± 0.258 (6)	0.309 ± 0.017 (6)	0.786 ± 0.115 (6)*	164 ± 5.10 (17)	282 ± 9.01 (16)*
Cyp4a10$^{-/-}$ (M)	3.80 ± 0.147 (5)	3.85 ± 0.188 (5)	0.352 ± 0.062 (5)	0.442 ± 0.028 (5)#	219 ± 6.68 (13)	305 ± 18.4 (12)*#
Cyp4a14$^{-/-}$ (M)	3.52 ± 0.123 (6)	3.96 ± 0.195 (6)	0.308 ± 0.065 (6)	0.501 ± 0.109 (6)	210 ± 7.65 (14)	266 ± 7.17 (15)*#
WT (F)	3.48 ± 0.049 (6)	4.31 ± 0.235 (6)*	0.370 ± 0.025 (6)	0.882 ± 0.177 (6)*	205 ± 7.75 (17)	287 ± 11.8 (17)*
Cyp4a10$^{-/-}$ (F)	3.40 ± 0.223 (5)	3.67 ± 0.143 (6)	0.313 ± 0.022 (5)	0.472 ± 0.041 (6)	218 ± 9.37 (15)	301 ± 12.2 (17)*
Cyp4a14$^{-/-}$ (F)	3.37 ± 0.193 (5)	3.44 ± 0.152 (5)	0.349 ± 0.073 (5)	0.716 ± 0.071 (5)	208 ± 11.32 (15)	236 ± 5.49 (12)#

Values are the mean ± S.E.M. The number of animals is given in parentheses.

*$p < 0.05$ compared to uninfected mice of same genotype

#$p < 0.05$ compared to infected WT mice.

Table 2

Liver and spleen weights of control and infected mice.

	Liver (% of body weight)		Spleen (% of body weight)	
	Control	**Infected**	**Control**	**Infected**
Jax	3.89 ± 0.178 (6)	4.78 ± 0.133 (6)*	0.336 ± 0.024 (6)	0.852 ± 0.098 (6)*
Tac	3.90 ± 0.184 (6)	4.28 ± 0.238 (6)	0.319 ± 0.040 (6)	0.667 ± 0.108 (6)*

Wildtype female 129X1/SvJ (Jax) and 129X1/SvEvTac (Tac) mice were allowed to drink either sucrose solution or *C. rodentium* in sucrose then sacrificed after 7 days. Values are the mean ± S.E.M. The number of animals is given in parentheses.

* $p < 0.05$, compared to control, *t* test