Hepatic Cytochrome P450s, Phase II Enzymes and Nuclear Receptors Are Downregulated in a Th2 Environment during *Schistosoma mansoni* Infection[§]

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ABSTRACT

Inflammation and infection downregulate the activity and expression of cytochrome P450s (P450s) and other drug metabolizing enzymes (DMEs) involved in hepatic drug clearance. *Schistosoma mansoni* infection was reported to cause a downregulation of hepatic P450-dependent activities in mouse liver, but little is known about the specific enzymes affected or whether phase II DMEs are also affected. Here we describe the effect of murine schistosomiasis on the expression of hepatic P450s, NADPH-cytochrome P450 reductase (Cpr), phase II drug metabolizing enzymes, and nuclear receptors at 30 and 45 days postinfection (dpi). Although the hepatic expression of some of these genes was altered at 30 dpi, we observed substantial changes in the expression of the majority of P450 mRNAs and proteins measured, Cpr protein, as well as

many of the UDP-glucuronosyltransferases and sulfotransferases at 45 dpi. *S. mansoni* infection also altered nuclear receptor expression, inducing mRNA levels at 30 dpi and depressing levels at 45 dpi. *S. mansoni* evoked a T helper 2 (Th2) inflammatory response at 45 dpi, as indicated by the induction of hepatic Th2 cytokine mRNAs [interleukins 4, 5, and 13], whereas the hepatic proinflammatory response was relatively weak. Thus, chronic schistosomiasis markedly and selectively alters the expression of multiple DMEs, which may be associated with Th2 cytokine release. This would represent a novel mechanism of DME regulation in disease states. These findings have important implications for drug testing in infected mice, whereas the relevance to humans with schistosomiasis needs to be determined.

Introduction

Schistosomiasis is a tropical waterborne disease that affects more than 200 million people in Africa, Asia, and South America (King, 2010). It is caused by flatworms (trematode worms) of the genus *Schistosoma*. There are many species of schistosomes, of which three main species cause disease in humans: *S. mansoni*, *S. hematobium*, and *S. japonicum*. The life cycle involves both a sexual reproduction stage in humans or other vertebrate hosts and an asexual stage in freshwater snails (Gryseels et al., 2006). The acute phase of human

schistosomiasis is asymptomatic, whereas the chronic phase is more severe due to the inflammatory response to parasite eggs retained in the liver and intestines. The egg-laying period begins approximately 30 days after infection; as the infection continues, granulomas are formed at the sites of maximal accumulation of schistosome eggs in the liver, resulting in hepatosplenomegaly, hepatocellular fibrosis, and portal vein hypertension (Wilson et al., 2009; Gotardo et al., 2011). Given that schistosomiasis is prevalent in countries where coinfection for diseases such as HIV or malaria is common, it is important to ask how schistosomiasis affects the enzymes responsible for the metabolism of drugs given to treat not only schistosomiasis but also these coinfections.

Cytochrome P450s (P450s) are a superfamily of drug metabolizing enzymes (DMEs) that catalyze the oxidation of a variety of exogenous and endogenous compounds. It has been well documented that infection and inflammation cause an impairment of hepatic DMEs in animals and humans. This is associated with the downregulation of hepatic P450s, phase II DMEs [including UDP-glucuronosyltransferase (UGTs) and sulfotransferase (SULTs)], and drug transporters, leading to alterations in pharmacokinetics and drug clearance (Morgan, 2001; Renton, 2005; Aitken et al., 2006; Morgan et al., 2008).

The downregulation of DME during infection and inflammation has been studied intensely in disease models of type I inflammation,

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ABBREVIATIONS: CAR, constitutive androstane receptor; Cpr, NADPH-cytochrome P450 reductase; DME, drug metabolizing enzyme; dpi, days postinfection; Fmo3, flavin monooxygenase 3; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IFN γ , interferon- γ ; IL, interleukin; LPS, lipopolysaccharide; NOS2, nitric oxide synthase 2; NOx, nitrate plus nitrite; P450, cytochrome P450; PPAR α , peroxisome proliferator-activated receptor- α ; PXR, pregnane X receptor; RXR α , retinoid X receptor- α ; RT-qPCR, reverse-transcriptase quantitative real-time polymerase chain reaction; SULT, sulfotransferase; Th1, T helper 1; Th2, T helper 2; TNF α , tumor necrosis factor- α ; UGT, UDP-glucuronosyltransferase.

including administration of lipopolysaccharide (LPS) or infection with *Citrobacter rodentium* (Bleau et al., 2003; Renton, 2004; Aitken and Morgan, 2007; Teng and Piquette-Miller, 2008; Nyagode et al., 2010). We and others have attributed the changes in P450 expression in the LPS and *C. rodentium* models to the production of proinflammatory cytokines associated with the innate immune response [interleukin (IL)-1 β , IL-6, interferon- γ (IFN γ), and tumor necrosis factor- α (TNF α)] (Siewert et al., 2000; Warren et al., 2001; Aitken et al., 2006; Xu et al., 2006; Aitken and Morgan, 2007; Nyagode et al., 2010). There is some evidence that hepatic P450 downregulation during inflammation may be at least partly mediated by modulation of nuclear receptors including peroxisome proliferator-activated receptor- α (PPAR α), pregnane X receptor (PXR), constitutive androstane receptor (CAR), retinoid X receptor- α (RXR α), and farnesoid X receptor (FXR) (Beigneux et al., 2002; Teng and Piquette-Miller, 2005; Kosters et al., 2009; Gerbal-Chaloin et al., 2013). This can occur via downregulation of receptor expression, inhibition of receptor activation, or both (Gerbal-Chaloin et al., 2013).

Although there have been several studies that reported changes in P450-dependent hepatic drug metabolizing activities during schistosomal infections, much less is known about what happens to specific P450 gene products. Gotardo et al. (2011) reported that hepatic Cyp1a2, Cyp2e1, and Cyp3a11 mRNAs were all downregulated in both male and female Swiss Webster mice at 55 days postinfection (dpi), but were downregulated at 30 dpi in female mice only. Even fewer data exist on phase II DME regulation during *S. mansoni* infection.

Schistosomal eggs, once trapped in the liver, induce a strong T helper 2 (Th2)-biased response in the liver by virtue of egg glycoprotein antigens including IPSE (IL-4 inducing principle of *S. mansoni* eggs)/ α -1 (Schramm et al., 2006) and the secreted T2 ribonuclease omega-1 (Steinfelder et al., 2009). Other carbohydrate-mediated interactions are also involved in the Th2 biasing (Prasanphanich et al., 2013). Such findings led us to ask whether the Th2 immune response in the chronic stage of infection regulates P450s and phase II DMEs. In addition, little is known as to whether infection with a pathogen-driven Th2 inflammatory response affects the expression of nuclear receptors that regulate DMEs in the liver. Thus, we investigated the effect of acute and chronic schistosomiasis on the expression levels of hepatic P450s and phase II DMEs, acute phase proteins, proinflammatory and Th2 inflammatory cytokines, and nuclear receptors associated with xenobiotic metabolism. Our results show that acute and chronic schistosomiasis are associated with significant changes in hepatic cytochrome P450s and phase II DMEs.

Materials and Methods

Animals and Treatments. Female Swiss Webster mice (aged 4–6 weeks) were purchased from Taconic Inc. (Hudson, NY) and infected at the National Institutes of Health National Institute of Allergy and Infectious Diseases Schistosomiasis Resource Center (Rockville, MD) according to established protocols (Lewis, 2001). Briefly, mice were placed in plastic restraining tubes and infected by tail immersion for 1 hour in water containing 200–250 *S. mansoni* cercaria, within less than 5 hours of their liberation from *Biomphalaria glabrata* snails. Penetration of an average of 201 cercaria per mouse was confirmed by counting the organisms remaining in each tube after infection. Mice were housed in groups of five to a cage and shipped to the Emory University animal facility 5 days after infection, and were fed a normal diet with water ad libitum. Uninfected control mice were received directly from Taconic Inc., and were housed under the same conditions and fed the same diets as the infected mice for 8 and 23 days before euthanasia for the 30 dpi and 45 dpi groups, respectively. Mice were monitored for general health, abdominal distention, and other signs of stress that might necessitate euthanasia; all mice survived until the time points used in this study. All protocols were reviewed and approved by the Institutional Animal Care and Use Committee of Emory

University. The infected mice were euthanized by decapitation under isoflurane anesthesia at 30 and 45 dpi and assayed with the corresponding control group. The control and infected groups contained six mice and five mice, respectively.

Tissue Collection. Blood was collected from the animals at euthanasia and allowed to clot for approximately 30 minutes at room temperature. Serum was separated by centrifugation for 10 minutes at 10,000g and stored at -80°C until analyzed. Liver and spleen were dissected out of the abdominal cavity, rinsed in cold 1.15% potassium chloride, and then weighed. The liver was portioned, flash-frozen, and stored at -80°C for subsequent RNA, S9 fraction, or microsomal preparation.

Liver Microsomes and S9 Fraction Preparation. Liver portions were weighed and homogenized in 0.1 M potassium phosphate buffer (pH 7.4) containing 0.125 M potassium chloride, 1.0 mM EDTA, and a protease inhibitor mixture (Sigma-Aldrich, St. Louis, MO) and centrifuged at 13,000g for 25 minutes at 4°C to yield the postmitochondrial supernatant (S9 fraction). The S9 fraction was centrifuged for 45 minutes at 250,000g to obtain microsomes. The resulting 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 were determined with a bicinchoninic acid protein assay kit (Thermo Fisher Scientific, Inc., Rockport, IL) with bovine serum albumin as the protein standard.

Western Blotting. Relative levels of P450 proteins in mouse hepatic S9 fractions were measured by SDS-polyacrylamide gel electrophoresis, Western blotting, and chemiluminescent detection as previously described (Chaluvadi et al., 2009). Equal amounts of protein (32 μg) were loaded on the gels for control and 30 dpi samples, whereas 128 μg protein was loaded for 45 dpi samples in an attempt to detect quantifiable amounts of P450s because of the loss of signal at that time point. All of the primary antibodies used for Western blotting were polyclonal, from rabbits. Antibodies to human CYP2A13 (Sc-33214) and mouse nitric oxide synthase 2 (NOS2) (Sc-650) were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), and were diluted 1:1000 and 1:2000, respectively. Antibodies to rat CYP2B1 and CYP3A1 were gifts from Dr. James Halpert (University of Texas Medical Branch, Galveston, TX), and were diluted 1:5,000. Dr. Masahiko Negishi (National Institute of Environmental Health Sciences, Research Triangle Park, NC) provided antibodies to mouse Cyp2d9, which were used at a dilution of 1:5000. Antibodies to NADPH-cytochrome P450 reductase (Cpr) were donated by Dr. Bettie Sue Masters (University of Texas, San Antonio, TX). Antibodies (Ab-140635) to Cyp4as were from Abcam (Cambridge, MA), and were diluted 1:2000. Antibodies to CYP2C11 were prepared in our own laboratory, and were diluted 1:10,000 for blotting (Chaluvadi et al., 2009). The secondary antibody in each case was a horseradish peroxidase-conjugated goat anti-rabbit IgG diluted 1:5000, except in the case of Cyp2c (1:20,000). Proteins were detected by using a SuperSignal West Pico chemiluminescent substrate kit (Thermo Fisher Scientific, Waltham, MA). The intensities of chemiluminescent protein bands were visualized by fluorography using X-ray film and the relative intensities of the protein bands were quantified by densitometry. Densitometric analysis was performed within a linear range and the intensity of stained bands was measured using Image Laboratory software (Bio-Rad, Hercules, CA).

The relative contents of specific P450s in the livers were calculated by averaging the densitometric values on three different blots, relative to the control group mean on each blot. These values were multiplied by the yields of S9 protein per gram of liver for the individual samples, and this value was multiplied by the liver weight for each animal.

RNA Extraction, cDNA Synthesis, and mRNA Measurement. Mouse livers were weighed and RNA was prepared using RNA-Bee isolation reagent (Tel-Test Inc., Friendswood, TX), according to the manufacturer's instructions. RNA concentration was determined by spectrophotometry and absorbance was measured at 260 nm. RNA purity and integrity were confirmed by formaldehyde-agarose gel electrophoresis followed by visualization with ethidium bromide. Relative levels of specific mRNAs in the samples were measured by reverse-transcription quantitative real-time polymerase chain reaction (RT-qPCR). Purified RNA was reverse-transcribed using a high-capacity cDNA reverse transcription kit (Invitrogen, Carlsbad, CA), according to the manufacturer's protocol for cDNA synthesis. Mouse primers were designed as described below. The relative expression of mouse liver mRNAs was measured using the ABI PRISM 7000 Sequence Detection System and SYBR Green Master Mix reagent (Applied Biosystems, Foster City, CA) as described previously (Richardson and

Morgan, 2005; Chaluvadi et al., 2009). The value obtained from each target gene was then normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH). PCR data were analyzed using the $\Delta\Delta C_t$ method (Livak and Schmittgen, 2001). The expression level was arbitrarily set to 1 in control samples.

Primers used in this study are listed in Supplemental Table 1.

Nitrate and Nitrite Level Detection in Blood Serum. Serum samples were deproteinized by diluting them 5-fold with water and filtering through Ultrafree-MC filter units (Millipore Corporation, Billerica, MA) at 10,000g and 4°C for 60 minutes. The plasma nitrate plus nitrite (NOx) level in mouse serum was measured by a colorimetric method based on the Griess reaction, as described previously (Sewer and Morgan, 1997).

Statistical Analysis. All data are presented as mean values $\pm$ S.E.M. Statistical significance between the control and infected groups was analyzed by a two-tailed *t* test. Levene's test was used to determine the equivalence of variance between compared groups, and log transformation of the data was performed if the variances were not equivalent. The Mann-Whitney *U* test was used for data whose variances were still not equivalent after transformation. The level of significance was set at $P < 0.05$.

Results

***S. mansoni* Infection Induces Hepatomegaly Associated with a Th2 Response.** Schistosomal infection did not significantly change the liver weight of mice at 30 dpi, whereas the livers of infected mice increased to 258% of control at 45 dpi. Spleen weights were significantly increased at both 30 and 45 dpi (211% and 655%, respectively) (Fig. 1).

Given that infection with *S. mansoni* initially induces a T helper 1 (Th1) (proinflammatory) T cell response and then a Th2 inflammatory response at the chronic stage of this infection (Pearce et al., 1991; Pearce and MacDonald, 2002), we decided to explore the status of both proinflammatory Th1 and Th2 cytokine mRNAs. The Th2 cytokines were upregulated at both 30 and 45 dpi, including IL-4 (15- and 20-fold), IL-5 (4- and 53-fold), and IL-13 (10.1- and 18.3-fold), respectively, with the greater increases occurring at 45 dpi (Fig. 2A). Immunoregulatory anti-inflammatory cytokines were also marginally upregulated at 45 dpi, namely IL-10 (3.67-fold), TGF β 1 (2.7-fold), and TGF β 2 (3.6-fold) (Fig. 2A). Compared with the Th2 cytokines, proinflammatory cytokines were much less affected. Only TNF α (2-fold) was significantly induced at 30 dpi. At 45 dpi, IL-1 β (6.4-fold), IL-6 (3.4-fold), IFN γ (3.2-fold), and TNF α (3-fold) were significantly induced (Fig. 2A).

Fig. 1. Liver and spleen weight of control and infected mice. Mice were euthanized 30 and 45 dpi, and livers and spleens were harvested and weighed. Values are the mean $\pm$ S.E.M. ($n = 6$ control mice; $n = 5$ infected mice). * $P < 0.05$, significantly different from control. Con, control; Inf, infected.

Fig. 2. Effect of *S. mansoni* infection on hepatic inflammatory indices. (A) Th2 and proinflammatory cytokines. (B) Acute phase proteins. (C) NO production and hepatic NOS2 mRNA expression. Mice were euthanized at 30 and 45 dpi and livers were harvested for measurement of mRNAs by RT-qPCR. Values are expressed as relative levels of mRNA after normalization to GAPDH, with the control group set to 1. Serum NOx (NO $_2$ and NO $_3$) concentrations were measured by the Griess reaction. Values are the mean $\pm$ S.E.M. ($n = 6$ control mice; $n = 5$ infected mice). * $P < 0.05$, significantly different from control. Con, control; Inf, infected.

Next, we studied the expression of hepatic genes that are well characterized for their regulation by proinflammatory cytokines. Hepatic mRNA expression of the acute phase proteins was minimally affected by *S. mansoni* infection. α -fibrinogen and serum amyloid P were not significantly affected at either time point. At 30 dpi, serum amyloid A and haptoglobin mRNAs were significantly increased (Fig. 2B). At 45 dpi only, α_1 -acid glycoprotein was significantly induced to 175% (Fig. 2B). Hepatic NOS2 mRNA expression was not significantly changed either (Fig. 2C), and NOS2 protein expression was not detected in any of the livers (data not shown). Serum NOx was also unchanged (Fig. 2C).

Hepatic P450 mRNA and Protein Expression Are Down-regulated at 45 dpi. Measuring hepatic mRNA protein levels during schistosomal infection presents some challenges because of the presence of adult worms in the liver, as well as eggs and host immune cells that form the granulomas that result in the hepatomegaly seen in Fig. 1. The contribution of these cells to measured “control” mRNAs and proteins makes the use of such controls problematic. Using RT-qPCR, we tested the relative levels of the mRNAs for four commonly used control genes in infected livers: GAPDH, cyclophilin, β -actin, and 18S rRNA. Infection downregulated each of these mRNAs similarly, both at 30 and 45 dpi (68% and 88% for GAPDH; data not shown). We decided to use GAPDH as our normalizing mRNA, reasoning that the almost identical decreases in these four mammalian housekeeping gene mRNAs are likely due to overall dilution of the total RNA pool by schistosomal RNAs. We recognize that the calculated decreases seen in the various DMEs might be conservative estimates of the magnitudes of the actual effects.

For P450 protein measurements, we tested guanine nucleotide binding protein β , GAPDH, and connexin 32 as microsomal or S9 fraction loading controls, but all were significantly upregulated at 45 dpi. We decided, therefore, to minimize loading and transfer errors by performing and averaging three independent Western blot assays of the samples without using any control protein for normalization. Since the total amount of each enzyme in the liver (as opposed to the content per gram of protein) is the most important predictor of hepatic drug clearance, we calculated the results of Western blot assays as relative expression per whole liver.

At 30 dpi, the levels of P450 mRNAs of all of the genes studied were either slightly upregulated or remained unaffected (Fig. 3). Cyp1a2, Cyp2c29, Cyp2e1, Cyp2j5, Cyp3a11, Cyp4f13, and Cyp4f18 were significantly upregulated by 42%, 29%, 36%, 25%, 56%, 32%, and 67%, respectively ($P < 0.05$) (Fig. 3). Interestingly, at 45 dpi, most P450 mRNAs investigated were markedly downregulated between 30% and 96% relative to control mice, whether they were primarily xenobiotic metabolizing enzymes (Fig. 3A) or endobiotic metabolizing enzymes (Fig. 3B). In contrast, Cyp4a12, Cyp4f16, and Cyp4f18 mRNAs increased approximately 2- to 3.5-fold compared with their controls (Fig. 3) at 45 dpi.

To investigate the relationship between P450 mRNA expression and protein level, Western blotting was performed on liver S9 fractions. As observed with the mRNAs, the protein levels of all P450s and Cpr remained unaffected at 30 dpi except for Cyp2a, which was increased 3.8-fold compared with its control and Cyp2b, which was slightly downregulated. At 45 dpi, all of the P450s and Cpr were drastically depressed, although Cyp2d proteins were less affected than the others (Fig. 4). Note that in the blot shown, 4 times as much

protein was loaded for the 45 dpi infected samples as for the other groups (except for the Cpr blot, which had the same amount of protein as controls).

Expression of Flavin Monooxygenase 3 and Phase II DMEs. Flavin monooxygenase 3 (Fmo3) plays a significant role in the metabolism of xenobiotics as well as of the waste product trimethylamine (Motika et al., 2007; Ferreira et al., 2013). Fmo3 mRNA expression was downregulated by 99% compared with controls at 45 dpi (Fig. 5A). At 30 dpi, Sult1a mRNA was upregulated by 52% (Fig. 5A), whereas other Sult mRNAs and Ugt mRNAs were not significantly affected ($P < 0.05$) (Fig. 5). However, at 45 dpi, mRNA levels of many of these genes were drastically downregulated [Ugt1a1 (82%), Ugt1a9 (80%), Ugt2b5 (67%), Sult1b1 (50%), Sult1d1 (59%), and Sult2a1/2 (87%)]; Sult3a1 and Sult3a2 showed trends toward downregulation, although they were not significant. In contrast, Ugt1a2 and Sult2b1 mRNAs were induced 2-fold and 5-fold, respectively. Ugt1a6 and Sult1 AMRNA expression remained unchanged (Fig. 5).

Expression of Nuclear Receptors RXR α , CAR, PPAR α , and PXR. To investigate whether schistosomal infection affects the expression of nuclear receptors that regulate many of the DME genes, we conducted RT-qPCR analyses for RXR α , CAR, PPAR α , and PXR mRNA expression. All four receptors tended to be induced at 30 dpi and downregulated by 50%–65% at 45 dpi, although not all showed statistically significant changes. The mRNAs for RXR α and CAR were significantly increased 1.7-fold at 30 dpi; however, the mRNAs

Fig. 3. Effect of *S. mansoni* infection on mRNA expression of hepatic P450s in female Swiss Webster mice. Mice were euthanized at 30 and 45 dpi and livers were harvested for measurement of mRNA by RT-qPCR. Values are expressed as relative levels of mRNA expression after normalization to GAPDH, with the control group set to 1. Values are the mean $\pm$ S.E.M. ($n = 6$ control mice; $n = 5$ infected mice). * $P < 0.05$, significantly different from control. Con, control; Inf, infected.

Fig. 4. Effect of *S. mansoni* infection on protein expression of hepatic P450s and Cpr in female Swiss Webster mice. Mice were infected with *S. mansoni* and euthanized at 30 and 45 dpi. Livers were harvested and S9 fractions were prepared. (A) Western blot of samples from 30 and 45 dpi. Each lane contains 32 μ g protein except the 45 dpi group of infected mice, which has 128 μ g in the lanes of the P450 blots and 32 μ g in the Cpr blot. (B) Quantitative analysis of the data. Values are the relative contents of P450 or Cpr protein per liver, and were averaged from three identical blots. Values are the mean $\pm$ S.E.M. ($n = 6$ control mice; $n = 5$ infected mice). * $P < 0.05$, significantly different from control. Con, control; Inf, infected.

Fig. 5. Effect of *S. mansoni* infection on mRNA expression of Fmo3 and phase II DMEs. (A) Fmo3 and UGTs. (B) SULTs. Mice were euthanized after 30 and 45 dpi and livers were harvested for measurement of mRNA by RT-qPCR. Values are expressed as relative levels of mRNA expression after normalization to GAPDH, with the control group set to 1. Values are the mean $\pm$ S.E.M. ($n = 6$ control mice; $n = 5$ infected mice). * $P < 0.05$, significantly different from control. Con, control; Inf, infected.

for CAR (51%), RXR α (50%), and PXR (70%) were significantly downregulated at 45 dpi (Fig. 6).

Discussion

Inflammation associated with infection or other diseases can downregulate hepatic P450 and transporters (Renton, 2004; Teng and Piquette-Miller, 2005, 2008; Morgan, 2009; Cressman et al., 2012). However, there are discernible differences in the pattern of P450 expression depending on the disease and the type of immune response. Few studies have reported expression of P450 mRNAs or proteins during schistosomiasis (Sheweita et al., 2002; Gotardo et al., 2011), and almost nothing is known about phase II enzyme expression in this disease. Our data demonstrate that the granulomatous Th2 cytokine-dominated stage of *S. mansoni* infection is associated with a marked downregulation of most P450, Ugt, and Sult mRNAs studied, as well as Fmo3. Remarkably, Cyp2a, Cyp2b, Cyp2c, Cyp3a, and Cyp4a, as well as Cpr, proteins were almost undetectable in the granulomatous livers at 45 dpi, which would predict a severe impairment of drug clearance at this stage of the disease.

Much knowledge about the effects of schistosomiasis on P450-dependent drug metabolism has been gathered from studies on microsomal activities (Cha and Edwards, 1976; Cha et al., 1980a,b; Sheweita et al., 1998, 2002; Manhães-Rocha et al., 2005; Conte et al., 2007; Gotardo et al., 2011). However, the contributions of mouse P450s to the metabolism of most drugs have not been rigorously established, and it is critical to know what happens to P450 gene products in this model. We show a significant downregulation of Cyp1a2, Cyp2a5, Cyp2b10, Cyp2c29, Cyp2d10, Cyp2d22, Cyp3a11,

Fig. 6. Effect of *S. mansoni* infection on hepatic nuclear receptor mRNA expression. Mice were euthanized at 30 and 45 dpi and livers were harvested for measurement of mRNA by RT-qPCR. Values are expressed as relative levels of mRNA after normalization to GAPDH, with the control group set to 1. Values are the mean $\pm$ S.E.M. ($n = 6$ control mice; $n = 5$ infected mice). * $P < 0.05$, significantly different from control. Con, control; Inf, infected.

Cyp3a25, Cyp4a10, Cyp4a14, Cyp4f14, and Cyp4f15 mRNAs at 45 dpi, whereas P450 mRNA expression was unchanged or slightly induced at 30 dpi. These findings extend the data of Gotardo et al. (2011), who reported a similar magnitude of downregulation of Cyp3a11 at 55 dpi. As noted above, the mRNA downregulations that we observed may actually be of greater magnitude because our “control” mRNA GAPDH is decreased in the granulomatous livers. Correspondingly, the apparent induction of some DME mRNAs at 30 dpi could be due to the downregulation of GAPDH. Nevertheless, the induction of some mRNAs at 30 dpi is consistent with previous reports of increases in some P450-dependent activities before eggs are laid (El-Bassiouni et al., 1984; Manhães-Rocha et al., 2005). Induction of Cyp4f18 and Cyp4f16 mRNAs at 45 dpi is not surprising considering the roles of Cyp4f enzymes in eicosanoid metabolism (Kalsotra and Strobel, 2006).

Data regarding the effects of *S. mansoni* infection on phase II DMEs are scarce. The activity of glutathione-*S*-transferase in the hepatic S9 fraction of Balb/C mice was increased at 33 dpi in animals infected with 60–180 cercariae and decreased in those exposed to 300–600 cercariae (Sheweita et al., 1998). No alterations of UGT activities were found during the early phase of schistosomiasis (Manhães-Rocha et al., 2005). Here we showed a significant depression of Ugt1a1, Ugt1a9, and Ugt2b5 mRNAs (67%–80%), and the same for Sult1b1, Sult1d1, and Sult2a1/2 (50%–87%). Sult3a1 and Sult3a2 also trended toward a significant downregulation. Thus, the clearance of drugs and toxicants cleared by phase II enzymes is also likely to be significantly impaired under chronic *S. mansoni* infection. The profound downregulation of Fmo3 mRNA seen in this study is similar to that during LPS-induced inflammation and *C. rodentium* infection (Nyagode et al., 2012).

Proinflammatory cytokines (IL-1 β , IL-6, IFN γ , and TNF α) have been implicated in DME regulation in other models (Morgan et al., 2008), being capable of downregulating P450 enzymes in hepatocyte cultures as well as in vivo (Siewert et al., 2000; Jover et al., 2002; Ashino et al., 2004; Aitken et al., 2006; Lee et al., 2009). However, the inductions in liver of these cytokines and the acute phase proteins they regulate at 45 dpi are smaller than the cytokine and acute phase

responses in the livers of *C. rodentium*-infected mice (Nyagode et al., 2010) or mice injected with LPS (Richardson and Morgan, 2005). In addition, we found no significant increases in hepatic NOS2 mRNA or protein and no elevation of NOx in the sera of *S. mansoni*-infected animals. Again, this is reflective of the fact that the immune system switches from a proinflammatory to a Th2-dominated response at 5–6 weeks of infection (Hams et al., 2013). These findings suggest that factors other than IL-1 β , IL-6, IFN γ , and TNF α may contribute to DME regulation in *S. mansoni* infection, and also demonstrate that DME downregulation in this model is not NO dependent.

Schistosoma eggs or soluble egg antigens are the major stimuli for Th2 responses during *S. mansoni* infection (Pearce et al., 1991; Pearce and MacDonald, 2002; Steinfelder et al., 2009). It is not known whether a pathogen-driven Th2 inflammatory response in the liver could contribute to regulation of DMEs. The Th2 cytokines produced during infection with *S. mansoni* include IL-4, IL-5, and IL-13 (Fairfax et al., 2012; Hams et al., 2013). IL-4 and IL-13 are involved in granuloma formation, whereas IL-10 and TGF β act as regulatory cytokines (Burke et al., 2009). Our data confirm that the Th2 response dominates in the liver at 45 days when eggs and granulomas are present, and that this response is correlated with DME downregulation. The idea that Th2 cytokines are important for P450 regulation in this model is supported by studies in athymic nude mice and mice infected with unisexual *S. mansoni* parasites, showing that the downregulation of P450-associated activities during murine schistosomiasis is dependent on the presence of a robust adaptive immune response to parasite eggs (Cha et al., 1980a,b). Moreover, as shown here and by others studying P450-dependent enzyme activities, the dramatic effects of infection are only observed in the later phase when the immune reaction to parasite eggs and the granulomas around the eggs are maximal (Sheweita et al., 2002; Manhães-Rocha et al., 2005; Conte et al., 2007; Gotardo et al., 2011).

Western blotting showed a dramatic downregulation of Cyp2a, Cyp2b, Cyp2c, Cyp3a, and Cyp4a proteins such that they were essentially undetectable even when the gels were overloaded. Similar effects have been described for Cyp1a, Cyp2b, Cyp2c, and Cyp4a proteins with single microsomal samples (Sheweita et al., 2002). Cyp2d proteins were less dramatically affected (20% of control levels, Fig. 4), consistent with the relative insensitivity of Cyp2d mRNAs to downregulation. Correlation of individual P450 mRNAs with protein levels must be interpreted cautiously, because well characterized specific antibodies for each mouse P450 isoform are not yet available. Together, all of these studies (Sheweita et al., 1998, 2002; Manhães-Rocha et al., 2005; Conte et al., 2007; Gotardo et al., 2011), including our own, clearly show that infection with *S. mansoni* at a chronic stage substantially depresses the majority of P450 mRNAs, proteins, and activities. This is the first report of a dramatic reduction in Cpr expression in schistosomiasis, which in itself would be predicted to greatly reduce P450-catalyzed drug clearance.

In contrast to many P450s that are downregulated during infections, Cyp2a5 was induced in malarial, helminthic, viral, and bacterial infections as well as by hepatotoxic compounds (Kirby et al., 1994; Sipowicz et al., 1997; Montero et al., 1999; De-Oliveira et al., 2010; Abu-Bakar et al., 2013). On the contrary, our results showed a downregulation of Cyp2a4/5 at both mRNA and protein levels, a trend that was also observed during bacterial infection with *C. rodentium* (Chaluvadi et al., 2009). The differences in Cyp2a regulation could be explained by a change in liver homeostasis created by the pathogen, the mouse strain used, or even by the infection time point.

Many P450s and phase II enzymes are regulated by nuclear receptors such as CAR, PXR, PPAR α , and RXR (Aleksunes and Klaassen, 2012; Gerbal-Chaloin et al., 2013). We describe here for the

first time significant downregulations of CAR, PXR, and PPAR α mRNAs expression in *S. mansoni* infected mice at 45 dpi. It has been suggested that downregulation of Cyp2b and Cyp3a11 after LPS administration in mice and CYP3A4 in human hepatocytes treated with IL-6 was due to the downregulation of PXR and CAR (Beigneux et al., 2002; Teng and Piquette-Miller, 2005; Yang et al., 2010). In addition, RXR α mRNA expression was affected by LPS-induced inflammation, endotoxin exposure, and IL-1 β treatment (Fang et al., 2004; Kusters et al., 2009). However PXR-null mice undergo the same P450 downregulations as wild-type mice when injected with LPS (Richardson and Morgan, 2005). It is possible that the regulation of the nuclear receptors could contribute to the suppression of DMEs in schistosomal infection, but this remains to be determined.

In conclusion, our results show that chronic murine schistosomiasis downregulates many P450s, including Cyp1a2, Cyp2a4/5, Cyp2b10, Cyp2c29, Cyp3a11, Cyp3a13, Cyp4a14, Cyp4a10, Cyp4f13, Cyp4f14, and Cyp4f15, while increasing Cyp4a12, Cyp4f16, and Cyp4f18. Cpr and the majority of phase II DMEs studied are also downregulated. These downregulations are associated with greater induction of Th2 inflammatory cytokines than proinflammatory cytokines, suggesting that Th2 cytokines may be involved in the downregulation of P450s during chronic murine schistosomiasis. This study suggests that the capacity of the liver to eliminate drugs via both P450 and phase II pathways is severely impaired during chronic murine schistosomiasis, and this should be considered in the design of drug testing in infected mice. The worm loads typically achieved in mouse studies are much higher than occurs in humans (Cheever, 1969). However, egg production in mice (which determines granuloma formation) increases linearly from the onset of egg-laying to 25 weeks of infection (Cheever, 1969). Thus, the egg burden in our study at 45 dpi is closer to the human burden than in mice at a steady state of infection. Moreover, humans are infected for much longer periods than can be modeled in mice. Together, these factors make extrapolation of findings from mouse models to humans difficult (Cheever, 1969; Abdul-Ghani and Hassan, 2010). Until the relevance of these findings for more chronic, lower-level infection in humans can be determined, vigilance should be exerted when treating infected individuals with drugs metabolized by P450s or phase II enzymes.

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Authorship Contributions

Participated in research design: Mimche, Nyagode, Merrell, Lee, Prasanphanich, Cummings, Morgan.

Conducted experiments: Mimche, Nyagode, Lee, Prasanphanich.

Contributed new reagents or analytic tools: Prasanphanich, Cummings.

Performed data analysis: Mimche, Nyagode, Merrell.

Wrote or contributed to the writing of the manuscript: Mimche, Nyagode, Merrell, Lee, Prasanphanich, Morgan.

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