REGULATION OF CONSTITUTIVE MOUSE HEPATIC

CYTOCHROMES P450 AND GROWTH HORMONE SIGNALING

COMPONENTS BY 3-METHYLCOLANTHRENE

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Abbreviations used are: AHR, aryl hydrocarbon receptor; CIS, cytokine-inducible SH2 domain-containing protein; dNTP, 2'-deoxynucleoside 5'-triphosphate; DRE, dioxin-responsive element; EROD, 7-ethoxyresorufin O-deethylation; GH, growth hormone; GHR, growth hormone receptor; HAH, halogenated aromatic hydrocarbon; JAK2, Janus kinase 2; MC, 3-methylcholanthrene; MUP2, major urinary protein 2; PAH, polycyclic aromatic hydrocarbon; P450, cytochrome P450; RT-PCR, reverse transcriptase-polymerase chain reaction; SOCS, suppressor of cytokine signaling; STAT5, signal transducer and activator of transcription 5; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; WAP, whey acidic protein
ABSTRACT

3-Methylcholanthrene (MC) activates the aryl hydrocarbon receptor and increases expression of cytochrome P450 (P450) enzymes such as CYP1A1. MC also decreases expression of CYP2C11, the major hepatic P450 in male rats that is regulated by pulsatile growth hormone (GH) secretion via a pathway partially dependent on signal transducer and activator of transcription 5b (STAT5b). If disruption of this GH signaling pathway is important for MC’s ability to suppress CYP2C11 transcription, we hypothesize that MC suppresses other male-specific genes (e.g. mouse Cyp2d9) regulated by pulsatile GH with STAT5b-dependence. We examined the time-course of MC’s effects on hepatic P450s and GH signaling components in male C57BL/6 mice. P450 content, heme content, and NADPH P450 oxidoreductase activity were induced 2.3-, 1.8-, and 1.3-fold, respectively, by MC. MC dramatically induced CYP1A1 mRNA, protein, and catalytic activity. MC caused a 42% decrease in CYP2D9 protein, a 28% decrease in CYP2D9 mRNA, and a 27% decrease in testosterone 16α-hydroxylation activity. MC caused a pronounced decrease in CYP3A protein; however, there was no apparent change in testosterone 6β-hydroxylation activity and changes in mRNA levels for CYP3A forms were relatively small. Expression of GH receptor and major urinary protein 2, a gene regulated by GH with STAT5b-dependence, was decreased by MC at the mRNA level. These results show that MC suppresses mouse Cyp2d9, a pulsatile GH- and STAT5b-dependent male-specific gene, via a pre-translational mechanism that may involve disrupted GH signaling. Mouse CYP3A protein levels are dramatically decreased by MC via a mechanism that is not yet understood.
Polycyclic aromatic hydrocarbons (PAHs) and halogenated aromatic hydrocarbons (HAHs) are important classes of environmental contaminants that pose threats to the health of humans and wildlife species. 3-Methylcholanthrene (MC) is a laboratory chemical that serves as a model PAH, and the prototypical HAH is the potent environmental toxicant 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) (Riddick et al., 1994). Many biological responses to both PAHs and HAHs are mediated by the aryl hydrocarbon receptor (AHR) and the most fully characterized AHR-mediated response is the induction of cytochrome P450 (P450) 1A and 1B subfamily members (Riddick et al., 1994). Expression of several other genes, including many with important roles in the control of cell growth and differentiation, is suppressed following PAH or HAH exposure (Riddick et al., 2003).

Expression of CYP2C11, the predominant constitutive hepatic P450 in male rats, is down-regulated by aromatic hydrocarbons via a transcriptional mechanism both \textit{in vivo} (Jones and Riddick, 1996; Lee and Riddick, 2000) and in cultured primary rat hepatocytes (Safa et al., 1997; Bhathena et al., 2002). Although the AHR appears to be involved in this response (Safa et al., 1997) and TCDD-activated AHR binds to a putative dioxin-responsive element (DRE) in the \textit{CYP2C11} 5′-flanking region (Bhathena et al., 2002), use of \textit{in vitro} DNase footprinting and luciferase reporter assays has not yet elucidated the definitive molecular mechanism involved. The primary physiological regulator of hepatic \textit{CYP2C11} expression is the pulsatile pattern of pituitary growth hormone (GH) secretion, and signal transducer and activator of transcription 5b (STAT5b) appears to be a key intracellular messenger that is at least partially responsible for this process (Park and Waxman, 2001). MC interferes with the ability of GH to stimulate hepatic
CYP2C11 expression in the liver of hypophysectomized male rats (Timsit and Riddick, 2000); however, our in vivo and cell culture work in the rat system has not uncovered an effect of MC on the GH-stimulated STAT5b signaling pathway (Timsit and Riddick, 2002). More recently, treatment of mice with MC was shown to decrease hepatic levels of mRNA encoding GH receptor (GHR), Janus kinase 2 (JAK2) and two STAT5 target gene products, cytokine-inducible SH2 domain-containing protein (CIS) and major urinary protein 2 (MUP2), in an AHR-dependent manner (Nukaya et al., 2004).

To examine if disruption of GH-stimulated STAT5b signaling is involved in the transcriptional down-regulation of gene expression by aromatic hydrocarbons, this study focused on CYP2D9 and other P450s expressed constitutively in the liver of male mice. The mouse Cyp2d9 gene, which encodes the male-specific steroid 16α-hydroxylase (Harada and Negishi, 1984), is clearly regulated by pulsatile GH in a STAT5b-dependent manner (Udy et al., 1997; Davey et al., 1999). Along with our primary focus on Cyp2d9 regulation, we report an extensive analysis of the effects of treatment of male mice with MC on the expression of the major hepatic P450s involved in testosterone hydroxylation, components of the GHR-JAK2-STAT5 signaling pathway, and representative STAT5 target genes.
MATERIALS AND METHODS

Animals and treatment. Male (23 to 28 g) and female (18 to 21 g) C57BL/6 mice, 8 to 9 weeks of age, were purchased from Charles River Canada (St. Constant, Quebec). Mice were fed standard chow and water ad libitum, and were housed under controlled conditions (two mice per cage; 22°C; 12-h light/12-h dark cycle, with lights on at 7:00 am) in the Division of Comparative Medicine, University of Toronto. Mice were cared for in accordance with the principles of the Canadian Council on Animal Care and all animal experimentation was approved the University of Toronto Animal Care Committee. Male mice received a single intraperitoneal injection of either MC (80 mg/kg; Aldrich Chemical Company, Milwaukee WI; 98% purity) or an equivalent volume of vehicle (sterile Mazola corn oil). Untreated female mice were used for comparisons in studies of sex-differentiated gene expression. Groups of eight to ten vehicle- and MC-treated mice were euthanized by cervical dislocation at 1, 2, 3, 4 or 7 days following injection. Small pieces (~0.1 g) of individual livers were frozen in liquid nitrogen and stored at −70°C for subsequent RNA isolation. Livers from two or three individual mice were pooled and hepatic microsomes were prepared in 1.15% KCl buffered with potassium phosphate (pH 7.4) by standard differential centrifugation techniques. Microsomes were suspended in storage buffer (10 mM Tris, pH 7.4/20% glycerol/1 mM EDTA), frozen in liquid nitrogen and stored at −70°C until use. Depending on the specific assay, microsomal protein concentrations were determined by the method of Lowry et al. (1951) or Bradford (1976).

Microsomal P450 and heme content. Total P450 and heme contents were determined by the reduced-carbon monoxide difference spectrum and the pyridine hemochromagen method, respectively (Omura and Sato, 1964).
NADPH P450 oxidoreductase activity. NADPH P450 oxidoreductase activity was assayed at 30°C in 1-ml incubation mixtures containing 300 mM potassium phosphate (pH 7.7), 70 nmol cytochrome c and 30 µg microsomal protein. Reactions were initiated by the addition of 1 mM NADPH and the rate of cytochrome c reduction was determined spectrophotometrically at 550 nm based on $\epsilon = 21 \text{ mM}^{-1}\text{cm}^{-1}$ (Strobel and Dignam, 1978). Product formation was linear with respect to protein concentration and incubation time. Cytochrome c and NADPH were purchased from Sigma Chemical Company (St. Louis, MO).

7-Ethoxyresorufin O-deethylation (EROD) activity. EROD activity was assayed at 37°C in 1.25-ml incubation mixtures containing 0.1 M Hepes (pH 7.8)/60 µM EDTA/5 mM MgSO$_4$, 1.875 nmol 7-ethoxyresorufin and either 250 µg (vehicle-treated mice) or 25 µg (MC-treated mice) microsomal protein (Pohl and Fouts, 1980). Reactions were initiated by the addition of 1 mM NADPH and terminated by the addition of 2.5 ml ice-cold methanol after either 2 min (MC-treated mice) or 4 min (vehicle-treated mice). Resorufin production was monitored fluorometrically (excitation and emission wavelengths of 550 and 585 nm, respectively) and quantitation was achieved via comparison to a resorufin calibration curve. Product formation was linear with respect to protein concentration and incubation time. 7-Ethoxyresorufin and resorufin were purchased from Sigma Chemical Company (St. Louis, MO).

Testosterone hydroxylation activity. Microsomes (25 µg protein) were incubated with 25 µM [4-$^{14}$C]testosterone (60 to 67 nCi) and NADPH (1 mM) in 50 mM Hepes (pH 7.6)/15 mM MgCl$_2$/0.1 mM EDTA at 37°C for 20 min in a final reaction volume of 0.1 ml. Enzymatic activity was terminated by the addition of 50 µl tetrahydrofuran. Testosterone metabolites were separated on silica gel thin-layer chromatography plates (J.T. Baker, Phillipsburg, NJ) with chloroform:ethyl acetate:ethanol (4:1:0.7) as the mobile phase (Waxman et al., 1983).
Radioactive metabolites were localized by autoradiography, identified by comparison with co-chromatographed authentic standards (testosterone, 16α-OH testosterone, 7α-OH testosterone, 6β-OH testosterone). Relative quantitation was performed by Phosphorimager analysis (Molecular Dynamics, Sunnyvale, CA) using IPLab Gel software (Signal Analytics, Vienna, VA). Selected bands were scraped from each plate and radioactivity was quantitated by liquid scintillation spectrometry, thus permitting the conversion of arbitrary Phosphorimager units into dpm and hence nmol of product formed. Product formation was linear with respect to protein concentration and incubation time. Testosterone was obtained as a controlled substance from Health Canada (Ottawa, Ontario). [4-14C]Testosterone (specific activity 48.0 or 53.6 mCi/mm; radiochemical purity >97%) was obtained from PerkinElmer Life Sciences, Inc. (Boston, MA). 16α-OH testosterone was purchased from Sigma Chemical Company (St. Louis, MO). 7α-OH testosterone and 6β-OH testosterone were purchased from Steraloids Inc. (Wilton, NH).

**Immunoblot analysis.** Microsomal protein (5 µg) from each mouse liver sample was resolved by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose (Hybond-ECL, Amersham Biosciences Inc., Baie d’Urfé, Quebec). For detection of CYP1A1 apoprotein, mouse monoclonal antibody 1-31-2 (Dr. H.V. Gelboin, National Cancer Institute, Bethesda, MD) was used at a 1:5,000 dilution, followed by a sheep anti-mouse Ig-horseradish peroxidase conjugate (Amersham) at a dilution of 1:5,000. For detection of CYP2D9 apoprotein, a rabbit anti-CYP2D9 polyclonal antibody (Dr. M. Negishi, National Institute of Environmental Health Sciences, Research Triangle Park, NC) was used at a 1:6,000 dilution, followed by a donkey anti-rabbit Ig-horseradish peroxidase conjugate (Amersham) at a dilution of 1:5,000. For detection of CYP3A apoproteins, four different primary antibodies (Ab 1 to 4) were used. Primary Ab 1, mouse monoclonal antibody 2-13-1 developed against rat CYP3A1/3A2 (Dr. H.V.
Gelboin, National Cancer Institute, Bethesda, MD), was used at a 1:50,000 dilution, followed by a sheep anti-mouse Ig-horseradish peroxidase conjugate (Amersham) at a dilution of 1:10,000. Primary Ab 2, a rabbit polyclonal antibody developed against rat CYP3A1 (Dr. A. Parkinson, XenoTech, Lenexa, KS), was used at a 1:10,000 dilution, followed by a donkey anti-rabbit Ig-horseradish peroxidase conjugate (Amersham) at a dilution of 1:5,000. Primary Ab 3, a rabbit polyclonal antibody developed against rat CYP3A2 (BD Gentest, Bedford, MA), was used at a 1:50,000 dilution, followed by a donkey anti-rabbit Ig-horseradish peroxidase conjugate (Amersham) at a dilution of 1:5,000. Primary Ab 4, a rabbit polyclonal antibody developed against rat CYP3A2 (Dr. S. Imaoka, Kwansei Gakuin University, Sanda, Japan), was used at a 1:10,000 dilution, followed by a donkey anti-rabbit Ig-horseradish peroxidase conjugate (Amersham) at a dilution of 1:5,000. An enhanced chemiluminescence system (ECL, Amersham) was used for protein detection, and films were scanned on a HP Scanjet 3970 scanner (Hewlett-Packard Company, Palo Alto, CA) and relative quantitation was performed using IPLabGel software. CYP1A1 immunoblots were used as a qualitative positive control response; all other immunoblot quantitative analyses were performed under conditions that yielded a linear relationship between amount of microsomal protein and immunoreactive signal intensity.

**Analysis of mRNA levels by semiquantitative reverse transcriptase-polymerase chain reaction (RT-PCR).** Total RNA was isolated from liver tissue by the acid guanidinium thiocyanate-phenol-chloroform extraction method (Chomczynski and Sacchi, 1987) using Tri- Reagent (Sigma Chemical Company, St. Louis, MO). RNA samples were then treated with 20 U DNase I (Amersham) at 37°C for 20 min to remove genomic DNA contamination. RNA yield and purity were assessed by determining the A_{260}/A_{280} ratio (≥ 1.7 for all samples), and RNA
integrity was assessed by comparing the relative intensities of the 28S and 18S rRNA bands as visualized on ethidium bromide-stained agarose gels. For the reverse transcription step, RNA (1 µg) was incubated with oligo d(T)$_{15}$ (2 µg; Roche Diagnostics, Laval, Quebec) at 60°C for 5 min. Primer-annealed samples were then incubated in a final volume of 40 µl with MMLV-reverse transcriptase (400 U; Invitrogen Corporation, Carlsbad, CA), RNA Guard (60 U; Amersham), 1 mM of each 2’-deoxynucleoside 5’-triphosphate (dNTP; Invitrogen), 10 mM dithiothreitol, and 1X RT buffer containing 50 mM Tris/75 mM KCl/3 mM MgCl$_2$. Reactions were allowed to proceed for 60 min at 37°C, followed by incubation at 70°C for 10 min. PCR primer sequences and cycling parameters are shown in Table 1. All PCR reactions began with a hot start phase, typically 5 min at 95°C, and ended with a final extension phase, typically 7 min at 72°C. Each 50-µl PCR reaction contained input cDNA derived from 25 or 50 ng RNA, Taq polymerase (10 U; Invitrogen), an appropriate concentration of each primer (0.2 to 0.5 µM), 1.6 mM of each dNTP, and 1X PCR buffer containing 20 mM Tris/50 mM KCl/3 mM MgCl$_2$. PCR products were separated on a 6% polyacrylamide gel, stained with Vistra Green (Amersham), and quantitated by Phosphorimager analysis using IPLabGel software. All target mRNA signals were normalized to the internal reference standard, β-actin. CYP1A1 RT-PCR was used as a qualitative positive control response; PCR conditions (input cDNA, cycle number) for all other targets were optimized to yield product within the exponential range of amplification.

PCR primers were synthesized by Integrated DNA Technologies, Inc. (IDT, Coralville, IA). Primer sequences were designed using Primer3 software [frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi] and the possibility of secondary structure formation was checked using IDT BioTools [www.idtdna.com]. The specificity of primers against the mouse genome...
was confirmed by BLAST search [www.ncbi.nlm.nih.gov/BLAST/] and by Primer-UniGene Selectivity (PUNS) analysis (Boutros and Okey, 2004).

**Statistical analysis.** Data are presented as mean ± S.D. of determinations from the specified number of mice. All statistical analyses were performed on the original raw data and not on the percent control data presented in the figures. Data were analyzed initially using a randomized design two-way analysis of variance to identify significant drug and time effects. If a significant drug effect was identified, Student’s t tests were performed to identify the time-points at which the mean value for MC-treated mice differed from the mean for the corresponding vehicle controls. If a significant time effect was identified, randomized design one-way analysis of variance followed by post hoc Newman-Keuls test was performed to identify time-dependent differences within the vehicle control groups and within the MC-treated groups. In all cases, a result was considered to be statistically significant if $p \leq 0.05$. 

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RESULTS

We have examined the effects of a single intraperitoneal dose of MC (80 mg/kg) on the expression of selected constitutive P450 enzymes and GH signaling components in the liver of male C57BL/6 mice. This MC dose was not associated with any observable signs of toxicity. Mouse body weight was decreased slightly by MC treatment at days 1 and 7 (Table 2), and liver weight was increased in MC-treated mice after 4 days to 122% of vehicle control (Table 2). The liver to body weight ratio was increased in MC-treated mice after 2, 4 and 7 days to levels of 108%, 123% and 117% of vehicle controls, respectively (Table 2). These effects are similar to what we have observed previously in MC-treated rats (Jones and Riddick, 1996; Lee and Riddick, 2000) and likely reflect the ability of MC to cause proliferation of hepatic smooth endoplasmic reticulum and/or mild hepatic inflammation.

As measures of general hepatic microsomal function, we monitored total P450 and heme content and NADPH P450 oxidoreductase activity (Table 3). MC increased total hepatic microsomal P450 content at all time-points and this response reached its peak at day 2 (235% of vehicle control). Similarly, MC increased total hepatic microsomal heme content at all time-points and this response reached its peak at day 4 (177% of vehicle control). NADPH P450 oxidoreductase activity was elevated by MC at all time-points except day 1 and this response peaked at day 7 (133% of vehicle control).

CYP1A1 induction was monitored in this study as a well-characterized positive control response to MC treatment. As a measure of CYP1A1 catalytic function, EROD activity was strongly induced by MC treatment at all time-points and this response peaked at day 3 (4022% of...
vehicle control) (Table 3). Dramatic induction of CYP1A1 by MC at all time-points was also confirmed at the mRNA and protein levels (data not shown).

Under \textit{in vivo} experimental conditions in which the AHR is activated and CYP1A1 is induced dramatically by MC, we have also examined alterations in the expression of constitutive hepatic P450s and components of the GH signaling cascade. Testosterone is primarily hydroxylated by hepatic microsomes from male mice at the 16\(\alpha\), 7\(\alpha\), and 6\(\beta\)-positions (Fig. 1). Although the P450 isozyme selectivity of steroid hydroxylation reactions is not as clearly defined for mouse as it is for rat, we have used testosterone hydroxylation at 16\(\alpha\), 7\(\alpha\), and 6\(\beta\)-positions as selective catalytic markers for CYP2D9 (Harada and Negishi, 1984), CYP2A12 (Iwasaki et al., 1993), and CYP3A forms (Bornheim and Correia, 1990), respectively.

Mouse hepatic CYP2D9 is a male-specific steroid 16\(\alpha\)-hydroxylase and the expression of this protein is clearly regulated by pulsatile GH in a STAT5b-dependent manner (Udy et al., 1997; Davey et al., 1999). MC caused a mild decrease in testosterone 16\(\alpha\)-hydroxylation activity and this response was maximal after 4 days (73\% of vehicle control) (Fig. 1). CYP2D9 expression was also studied at the protein level using a well-characterized polyclonal antibody known to recognize three protein bands (a, b, c) in microsomes from male mice; the middle band “b” has been identified as the male-specific CYP2D9 (Fig. 2A) (Udy et al., 1997; Davey et al., 1999). CYP2D9 protein levels were decreased by MC at days 3, 4 and 7 with maximal suppression being achieved at days 4 and 7 (58\% of vehicle control) (Fig. 2). CYP2D9 expression at the mRNA level was assessed by RT-PCR (Fig. 2B), revealing a significant suppression of CYP2D9 mRNA by MC, with maximal suppression apparent at day 7 (72\% of control) (Fig. 2). Similar results were obtained by “processed” northern analysis (Negishi et al.,...
1991), although statistical significance was not achieved using this analytical method (data not shown).

As a measure of CYP3A catalytic activity, we found that testosterone 6ß-hydroxylation was not altered by MC treatment (Fig. 1). Under conditions in which this catalytic activity was not altered, we observed very dramatic loss of CYP3A immunoreactive protein triggered by MC treatment (Fig. 3). The mouse CYP3A subfamily consists of eight members (CYP3A11, 13, 16, 25, 41, 44, 57, 59) (Nelson et al., 2004) and the specificity of antibody probes against these proteins is poorly characterized. We have used four independent anti-rat CYP3A primary antibodies (Ab 1 to 4) to probe mouse hepatic microsomes in this study (Fig. 3). Primary Ab 1, a mouse monoclonal developed against rat CYP3A1/3A2, has uncharacterized specificity toward mouse proteins; this antibody revealed a profound loss of CYP3A immunoreactivity caused by MC at all time-points with a maximal suppression observed at day 2 (14% of vehicle control) (Fig. 3). Primary Ab 2, a rabbit polyclonal developed against rat CYP3A1, has been suggested to recognize mouse CYP3A11 and 3A13 (Warrington et al., 2000). CYP3A11 is the predominant subfamily member in the liver of male mice (Yanagimoto et al., 1997), whereas expression of CYP3A13 in mouse liver is much lower (Sakuma et al., 2000). Focusing on the upper band “a” recognized by this antibody, thought to represent CYP3A11, we found that MC decreased CYP3A11 immunoreactivity at days 2, 3 and 4 with the maximum suppression observed at day 2 (58% of vehicle control) (Fig. 3). Primary Ab 3, a rabbit polyclonal developed against rat CYP3A2, has been suggested to recognize mouse CYP3A11 and 3A13 (Mori et al., 2001). Focusing on the upper band “a” recognized by this antibody, thought to represent CYP3A11, we found that MC decreased CYP3A11 immunoreactivity at days 2, 3 and 4 with the maximum suppression observed at day 2 (40% of vehicle control) (Fig. 3). Primary Ab 4, a
rabbit polyclonal developed against rat CYP3A2, has uncharacterized specificity toward mouse proteins (Ashino et al., 2004); this antibody revealed a marked loss of CYP3A immunoreactivity caused by MC at all time-points with a maximal suppression observed at day 2 (38% of vehicle control) (Fig. 3). We also used RT-PCR to monitor the expression of four CYP3A subfamily members at the mRNA level (Fig. 4); in addition to CYP3A11 and 3A13 described above, we also assessed mRNA for CYP3A25 (Dai et al., 2001) and the female-specific CYP3A41 (Sakuma et al., 2002). Modest suppression of CYP3A11 mRNA levels by MC was observed at days 1 and 2 (66 to 70% of vehicle control) (Fig. 4). MC increased CYP3A13 mRNA levels at days 2, 3 and 4, with the maximum increase observed at day 3 (153% of vehicle control) (Fig. 4). Modest suppression of CYP3A25 mRNA levels by MC was observed at days 3 and 4 (71 to 82% of vehicle control) (Fig. 4). CYP3A41 mRNA was detected only in female mice and this sex specificity was not affected by MC treatment (Fig. 4). Changes in mRNA levels for various members of the CYP3A subfamily could not account for the profound loss of CYP3A immunoreactivity caused by MC.

As a measure of CYP2A12 catalytic activity, we found that testosterone 7α-hydroxylation was only modestly decreased by MC at day 3 to 83% of vehicle control levels (Fig. 1). Similarly, MC had no effect on CYP2A12 mRNA levels (data not shown).

We next examined whether the suppression of CYP2D9 by MC could be related to alterations in the levels of expression of key components of the hepatic GH signaling cascade. Since the male-specific hepatic expression of CYP2D9 is primarily regulated by pulsatile GH via the GHR-JAK2-STAT5b cascade, we focused on this signal transduction pathway (Fig. 5). Using two independent sets of PCR primers for RT-PCR analysis, we found that GHR mRNA levels are decreased by MC with maximal suppression observed from day 3 through day 7 (51 to
58% of vehicle control) (Fig. 5). Expression of JAK2 and STAT5 at the mRNA level was not altered by MC treatment (Fig. 5).

To determine if other genes that are under GH control via the GHR-JAK2-STAT5 signaling pathway are suppressed by MC, we examined the hepatic expression of two additional STAT5 target genes: Cis and Mup2 (Fig. 6). CIS, a specific member of the suppressor of cytokine signaling (SOCS) family, is induced by GH via a STAT5-dependent transcriptional mechanism and plays a role in the negative feed-back loop that inhibits GHR signaling (Landsman and Waxman, 2005). MUP2 is a member of the family of α2-microglobulin-related liver secretory proteins that form a significant component of protein in mouse urine; the male-predominance of MUP2 is also due to pulsatile GH signaling via a STAT5-dependent mechanism (Udy et al., 1997). Hepatic expression of CIS mRNA was not altered by MC treatment; however, MUP2 mRNA levels were decreased by MC at days 2 and 7 with a maximal suppression observed at day 7 (45% of vehicle control).
DISCUSSION

PAHs and HAHS cause AHR-dependent P450 induction; however, the mechanisms by which these compounds down-regulate constitutive P450s and other genes are not understood (Riddick et al., 2003). In this study, we analyzed the effects of MC on the expression of the major mouse hepatic P450s involved in testosterone hydroxylation, components of the GHR-JAK2-STAT5 signaling pathway, and selected STAT5 target genes.

The first major finding is that the expression of mouse hepatic Cyp2d9 is suppressed by MC via a pre-translational mechanism. This is important because it parallels our work on hepatic CYP2C11 regulation conducted in a rat model. MC down-regulates rat CYP2C11 at the transcriptional level in vivo (Jones and Riddick, 1996; Lee and Riddick, 2000) and in primary rat hepatocytes (Safa et al., 1997; Bhathena et al., 2002). There are similarities between the gene products encoded by rat CYP2C11 and mouse Cyp2d9. Both proteins are major steroid 16α-hydroxylation enzymes and are expressed nearly exclusively in the liver of male animals; this sex specificity is determined by the pulsatile GH secretion pattern characteristic of males. The GHR-JAK2-STAT5b signaling pathway is key in determining the male-specific pattern of expression of both CYP2C11 (Park and Waxman, 2001) and Cyp2d9 (Udy et al., 1997; Davey et al., 1999). The Cyp2d9 suppression response in mouse liver is of lesser magnitude than the CYP2C11 suppression response previously characterized in rat. We found that CYP2D9 mRNA, protein and catalytic activity was decreased by 27 to 42% by treatment of male mice with MC at 80 mg/kg; in contrast, we previously showed that CYP2C11 mRNA, protein and catalytic activity was decreased by 59 to 66% by treatment of male rats with MC at 50 mg/kg (Jones and Riddick, 1996). Despite this magnitude difference, our finding that both rat CYP2C11 and
mouse Cyp2d9 are suppressed by MC suggests that the GHR-JAK2-STAT5b cascade, a key shared physiological regulatory pathway for both genes, may be targeted for disruption by PAHs.

This possibility is supported by our second major finding; an additional hepatic STAT5 target gene, Mup2, is also suppressed by MC in male mice. Like Cyp2d9, the male-predominance of Mup2 expression is also due to pulsatile GH signaling via STAT5 (Udy et al., 1997). Our finding that MUP2 mRNA levels are decreased by MC agrees with a similar recent observation (Nukaya et al., 2004). We employed a single intraperitoneal injection of MC at 80 mg/kg and then euthanized mice at 1, 2, 3, 4 and 7 days following injection. Nukaya et al. (2004) treated mice with MC at a dose of 80 mg/kg intraperitoneally once daily for two days and mice were euthanized 24 h after the final dose. Perhaps related to this difference in MC dosing, our results differ from those of Nukaya et al. (2004) in an important way. We found that the expression of an additional STAT5 target gene, Cis, was not altered by MC; in contrast, Nukaya et al. (2004) reported that hepatic CIS mRNA levels were decreased by MC in wild-type mice but not in Ahr-null mice. The STAT5 target gene product, whey acidic protein (WAP), was also reported to be down-regulated at the mRNA level by MC in mouse liver in an AHR-dependent manner (Nukaya et al., 2004). Using two primer sets for RT-PCR analysis, we could not detect WAP mRNA in mouse liver, consistent with the selective expression of WAP mRNA in mammary tissue (Vorderstrasse et al., 2004). We suggest that some mouse hepatic genes that are regulated by the GHR-JAK2-STAT5b pathway (e.g. Cyp2d9 and Mup2), but not others (e.g. Cis), are targeted for suppression by PAHs. Thus PAHs may modulate the expression of a subset of STAT5 target genes via mechanisms that depend on specific regulatory elements found in particular genes rather than via mechanisms that involve disruption of more upstream common elements of the GHR-JAK2-STAT5b cascade.
In light of this question, we examined the effects of MC on the expression of mouse hepatic GHR, JAK2 and STAT5a/b mRNA. Our third major finding is that mouse hepatic GHR mRNA levels are decreased by MC. Effects of PAHs and HAHs on the hepatic GHR-JAK2-STAT5b signaling pathway remain controversial. We found that MC interferes with the ability of GH to stimulate hepatic \textit{CYP2C11} expression in the liver of hypophysectomized male rats (Timsit and Riddick, 2000). However, MC did not alter the ability of GH to stimulate rat STAT5b phosphorylation, nuclear localization and binding to DNA (Timsit and Riddick, 2002). The present study revealed that MC decreases mouse hepatic GHR expression at the mRNA level, suggesting that the observed decreased expression of specific STAT5 target genes may be at least partially caused by disrupted expression of this key upstream component in the GH signaling pathway. Nukaya et al. (2004) found that MC caused a dramatic loss of mouse GHR and JAK2 mRNA and this was accompanied by a decrease in binding of STAT5 to DNA. Our new findings suggest that the mouse \textit{Ghr} gene may be an important target for disruption by PAHs and HAHs (Nukaya et al., 2004); the responsiveness of this gene to aromatic hydrocarbons and the role that this process plays in the impaired growth and wasting syndrome associated with these compounds deserve further study. To solidify the mechanistic link between altered GHR mRNA, STAT5b function, and \textit{Cyp2d9} expression, it will be necessary to measure the protein levels and activation status of the components of this signaling cascade.

The focus has been on changes triggered by MC at the mRNA level, suggesting pre-translational and likely transcriptional mechanisms. Most changes in gene expression caused by PAHs and HAHs result from binding of the activated AHR complex to DRE sequences located in the 5'-flanking region of target genes (Riddick et al., 2003), and we have conducted the present study under conditions in which the hepatic AHR is strongly activated by MC as
evidenced by the dramatic induction of CYP1A1. Definitive evidence for the role of the AHR in the down-regulation of GH-controlled genes is limited. Structure-activity relationship data support a role for the AHR in CYP2C11 suppression (Safa et al., 1997). Activated AHR binds to a DRE-like sequence in the CYP2C11 5′-flank; however, the functional implications are not known (Bhathena et al., 2002). Work with Ahr-null mice showed that the effects of MC on GH signaling components and on STAT5 targets are dependent on functional AHR (Nukaya et al., 2004). The mouse Ghr gene may be a primary target for suppression by PAHs and this is supported by the presence of putative DREs in the Ghr promoter (Nukaya et al., 2004). Future mechanistic studies will focus on the functional consequences of AHR binding to DRE-like sequences in the mouse Ghr and Cyp2d9 genes. It will also be interesting to compare the pre-translational suppression of Cyp2d9 in wild-type versus Ahr-null mice in order to probe the role of the AHR in this new biological response.

The fourth major finding is that mouse hepatic CYP3A immunoreactive protein levels are dramatically decreased by MC. This represents one of the most pronounced protein down-regulation effects ever observed in response to PAH treatment, with CYP3A levels reaching as low as 14% of vehicle controls. In rats, MC causes initial suppression of CYP3A protein followed by a later increase above control levels, consistent with differential regulation of CYP3A subfamily members in an isozyme- and time-dependent manner (Jones and Riddick, 1996). CYP3A11 is the predominant CYP3A subfamily form expressed in the liver of male mice (Yanagimoto et al., 1997) and there is strong evidence that CYP3A11 is the main immunoreactive protein recognized by the antibodies used here. Although MC may also affect other mouse CYP3A proteins, it seems clear that CYP3A11 immunoreactive protein levels are decreased strongly by MC. This dramatic drop in CYP3A11 protein occurs without a
corresponding change in testosterone 6ß-hydroxylation activity and only relatively small decreases (CYP3A11, 3A25) or increases (CYP3A13) in CYP3A mRNA levels are observed. Although CYP3A11 mRNA suppression may be important, we suspect that a large component of the CYP3A11 protein response may be due to direct effects on MC and/or its metabolites on hepatic CYP3A proteins. Future studies will address whether MC is metabolically activated to reactive products that target mouse CYP3A proteins for destruction. In addition to examining this in \( Ahr \)-null mice, it is desirable to test if mice deficient in hepatic NADPH P450 oxidoreductase and therefore lacking P450-dependent metabolism are resistant to this CYP3A protein destruction response. It remains puzzling how a profound loss of CYP3A11 immunoreactive protein can occur with no corresponding change in a marker catalytic activity.

In conclusion, our results show that under \textit{in vivo} conditions resulting in strong AHR activation, MC suppresses mouse \( Cyp2d9 \), a pulsatile GH- and STAT5b-dependent male-specific gene, via a pre-translational mechanism that appears to involve disrupted GH signaling. Mouse CYP3A protein levels are dramatically decreased by MC, perhaps via a post-translational mechanism. Genetically modified mouse models will facilitate future mechanistic studies of the role of the AHR and other key proteins in these two novel biological responses to PAH exposure.
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REFERENCES


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FIGURE LEGENDS

FIG. 1. Time-course of the effects of MC administration to male mice on hepatic testosterone hydroxylation activity. A, thin-layer chromatogram showing the regioselective hydroxylation of $[^{14}C]$testosterone by mouse hepatic microsomes. “Blank” indicates an incubation carried out in the absence of microsomal protein. The labels on the right side indicate the distance of migration of nonradioactive authentic standards for testosterone (T) and $6\beta$-, $7\alpha$-, and $16\alpha$-OH testosterone. Male mice were treated with vehicle (V) or MC (M) and euthanized at the indicated time-points following injection; untreated female mice are included for comparison. Representative results are shown for one mouse sample from each treatment group. B, quantitative Phosphorimager analysis of testosterone hydroxylation activity. Results for MC-treated mice are expressed as a percentage of the mean for the vehicle-treated controls at each time-point. Data are expressed as mean ± S.D. of determinations from four microsomal samples prepared from male mice or the mean of determinations from two microsomal samples prepared from female mice. Each microsomal sample was prepared from pooled livers from two or three individual mice. Similar results were obtained in a total of three independent chromatographic analyses. Mean testosterone hydroxylation activity (nmol/min/mg protein) for the vehicle control group at day 1 were as follows: $16\alpha$, $0.82 ± 0.08$; $7\alpha$, $0.50 ± 0.05$; $6\beta$, $0.44 ± 0.08$. Data for vehicle- and MC-treated male mice were analyzed initially using a randomized design two-way analysis of variance to identify significant drug and time effects; for clarity of presentation, only significant drug effects are shown. *, significantly different ($p ≤ 0.05$) from vehicle control at a given time-point, based on Student’s t test.
FIG. 2. Time-course of the effects of MC administration to male mice on hepatic CYP2D9 protein and mRNA levels.  A, immunoblot analysis of microsomal protein (5 µg) using polyclonal antibody directed against mouse CYP2D9.  The labels on the left side indicate that this antibody recognizes three protein bands (a, b, c) in mouse hepatic microsomes and the middle band “b” represents the male-specific CYP2D9.  B, RT-PCR analysis of CYP2D9 and β-actin mRNA levels as visualized on Vistra Green-stained polyacrylamide gels. Male mice were treated with vehicle (V) or MC (M) and euthanized at the indicated time-points following injection; untreated female mice are included for comparison. Representative results are shown for one mouse sample from each treatment group.  C, semiquantitative image analysis of CYP2D9 protein and mRNA expression. Results for MC-treated mice are expressed as a percentage of the mean for the vehicle-treated controls at each time-point. Immunoblot data are expressed as mean ± S.D. of determinations from four microsomal samples prepared from male mice or the mean of determinations from two microsomal samples prepared from female mice. Each microsomal sample was prepared from pooled livers from two or three individual mice. RT-PCR data are expressed as mean ± S.D. of determinations from four individual male mice or the mean of determinations from two individual female mice, normalized to β-actin. Similar results were obtained in a total of three independent immunoblot analyses and two independent PCR analyses. Data for vehicle- and MC-treated male mice were analyzed initially using a randomized design two-way analysis of variance to identify significant drug and time effects; for clarity of presentation, only significant drug effects are shown. *, significantly different (p ≤ 0.05) from vehicle control at a given time-point, based on Student’s t test.
FIG. 3. Time-course of the effects of MC administration to male mice on hepatic CYP3A protein levels. A to D, immunoblot analysis of microsomal protein (5 µg) using four independent primary antibodies directed against rat CYP3A forms. A, primary Ab 1, a mouse monoclonal developed against rat CYP3A1/3A2, has uncharacterized specificity toward mouse proteins. B, primary Ab 2, a rabbit polyclonal developed against rat CYP3A1, has been suggested to recognize mouse CYP3A11 (band “a”) and 3A13 (band “b”) as shown by the labels on the left side. C, primary Ab 3, a rabbit polyclonal developed against rat CYP3A2, has been suggested to recognize mouse CYP3A11 (band “a”) and 3A13 (band “b”) as shown by the labels on the left side. D, primary Ab 4, a rabbit polyclonal developed against rat CYP3A2, has uncharacterized specificity toward mouse proteins. Male mice were treated with vehicle (V) or MC (M) and euthanized at the indicated time-points following injection; untreated female mice are included for comparison. Representative results are shown for one mouse sample from each treatment group. E, semiquantitative image analysis of CYP3A protein expression. Results for MC-treated mice are expressed as a percentage of the mean for the vehicle-treated controls at each time-point. Data are expressed as mean ± S.D. of determinations from four microsomal samples prepared from male mice or the mean of determinations from two microsomal samples prepared from female mice. Each microsomal sample was prepared from pooled livers from two or three individual mice. Similar results were obtained in a total of two or three independent immunoblot analyses. Data for vehicle- and MC-treated male mice were analyzed initially using a randomized design two-way analysis of variance to identify significant drug and time effects; for clarity of presentation, only significant drug effects are shown. *, significantly different ($p \leq 0.05$) from vehicle control at a given time-point, based on Student’s $t$ test.
FIG. 4. Time-course of the effects of MC administration to male mice on hepatic CYP3A mRNA levels. A to D, RT-PCR analysis of CYP3A11 (A), CYP3A13 (B), CYP3A25 (C), and CYP3A41 (D) and β-actin mRNA levels as visualized on Vistra Green-stained polyacrylamide gels. Male mice were treated with vehicle (V) or MC (M) and euthanized at the indicated time-points following injection; untreated female mice are included for comparison. Representative results are shown for one mouse sample from each treatment group. E, semiquantitative Phosphorimager analysis of CYP3A mRNA expression. Results for MC-treated mice are expressed as a percentage of the mean for the vehicle-treated controls at each time-point. Data are expressed as mean ± S.D. of determinations from four individual male mice or the mean of determinations from two to four individual female mice, normalized to β-actin. Similar results were obtained in a total of two independent PCR analyses. Data for vehicle- and MC-treated male mice were analyzed initially using a randomized design two-way analysis of variance to identify significant drug and time effects; for clarity of presentation, only significant drug effects are shown. *, significantly different (p ≤ 0.05) from vehicle control at a given time-point, based on Student’s t test.

FIG. 5. Time-course of the effects of MC administration to male mice on hepatic GHR, JAK2 and STAT5a/b mRNA levels. A to E, RT-PCR analysis of GHR using two primer sets (A, B), JAK2 using two primer sets (C, D), and STAT5a/b (E) and β-actin mRNA levels as visualized on Vistra Green-stained polyacrylamide gels. Male mice were treated with vehicle (V) or MC (M) and euthanized at the indicated time-points following injection; untreated female mice are included for comparison. Representative results are shown for one mouse sample from each treatment group. F, semiquantitative Phosphorimager analysis of GHR, JAK2 and STAT5a/b
mRNA expression. Results for MC-treated mice are expressed as a percentage of the mean for the vehicle-treated controls at each time-point. Data are expressed as mean ± S.D. of determinations from four individual male mice or the mean of determinations from two individual female mice, normalized to β-actin. Similar results were obtained in a total of two to three independent PCR analyses. Data for vehicle- and MC-treated male mice were analyzed initially using a randomized design two-way analysis of variance to identify significant drug and time effects; for clarity of presentation, only significant drug effects are shown. *, significantly different (\( p \leq 0.05 \)) from vehicle control at a given time-point, based on Student’s \( t \) test.

FIG. 6. Time-course of the effects of MC administration to male mice on hepatic CIS and MUP2 mRNA levels. A to B, RT-PCR analysis of CIS (A) and MUP2 (B) and β-actin mRNA levels as visualized on Vistra Green-stained polyacrylamide gels. Male mice were treated with vehicle (V) or MC (M) and euthanized at the indicated time-points following injection; untreated female mice are included for comparison. Representative results are shown for one mouse sample from each treatment group. C, semiquantitative Phosphorimager analysis of CIS and MUP2 mRNA expression. Results for MC-treated mice are expressed as a percentage of the mean for the vehicle-treated controls at each time-point. Data are expressed as mean ± S.D. of determinations from four individual male mice or the mean of determinations from two individual female mice, normalized to β-actin. Similar results were obtained in a total of two independent PCR analyses. Data for vehicle- and MC-treated male mice were analyzed initially using a randomized design two-way analysis of variance to identify significant drug and time effects; for clarity of presentation, only significant drug effects are shown. **, significantly different (\( p \leq 0.01 \)) from vehicle control at a given time-point, based on Student’s \( t \) test.
### TABLE 1. Primer sequences and thermal cycling conditions used for measurement of steady-state mRNA levels in mouse liver by semi-quantitative RT-PCR.

<table>
<thead>
<tr>
<th>Target</th>
<th>Forward and reverse PCR primer sequences</th>
<th>PCR product size (bp)</th>
<th>Thermal cycling conditions (s/°C) (denature; anneal; extend) x cycles</th>
<th>Reference</th>
</tr>
</thead>
</table>
| CYP1A1     | 5′-CCCACAGCACCACACAGAGATA-3′  
5′-AAGTAGGAGGGGAGGCAATGTC-3′ | 500                   | (30 s/94°C; 30 s/55°C; 45 s/72°C) x 21                                   | (Giannone et al., 1998) |
| CYP2A12    | 5′-TGGTGCTTTATGGATACGATGC-3′  
5′-CCCAGAATCAGATGGGCACG-3′ | 1254                  | (45 s/94°C; 45 s/58°C; 45 s/72°C) x 17                                   | (Su et al., 1996)       |
| CYP2D9     | 5′-CTTTGGGGAACATTGGTCTCCAG-3′  
5′-AAGAATACCATAGACTCCAG-3′ | 347                   | (30 s/94°C; 30 s/55°C; 45 s/72°C) x 21                                   | (Sueyoshi et al., 1999) |
| CYP3A11    | 5′-CGCCTTCTCCTGCTGTCACA-3′  
5′-CTTGGCTTTCTGCTCAATG-3′ | 260                   | (30 s/94°C; 40 s/55°C; 30 s/72°C) x 21                                   | (Yamada et al., 2002)   |
| CYP3A13    | 5′-CCCTGCTGTCTCCAACCTT-3′  
5′-TGCCATCCCTTTCATGTT-3′ | 390                   | (30 s/94°C; 30 s/55°C; 45 s/72°C) x 21                                   | (Choudhary et al., 2003)|
| CYP3A25    | 5′-CCGTTACTTGCCACCATTT-3′  
5′-GTCTTTCTGCTAGTGTCG-3′ | 390                   | (30 s/94°C; 30 s/55°C; 45 s/72°C) x 21                                   | (Choudhary et al., 2003)|
| CYP3A41    | 5′-TGCCACTCTTGGTCTACAG-3′  
5′-AGTCAACTCTTTAAATCCCTT-3′ | 408                   | (30 s/94°C; 30 s/55°C; 45 s/72°C) x 19                                   | (Yamada et al., 2002)   |
| GHR        | 5′-AATGCAGATGTCCTGAGGGA-3′  
5′-ATATCTGCTGCTCAAGACATCT-3′ | 1223                  | (60 s/94°C; 30 s/58°C; 90 s/72°C) x 21                                   | (Nukaya et al., 2004)   |
| GHR        | 5′-CAGTCACCAGCAGCACAATT-3′  
5′-TCCAGCAGAGACAATAATCCGG-3′ | 311                   | (30 s/94°C; 30 s/55°C; 45 s/72°C) x 21                                   | new design               |
For analysis of GHR and JAK2, two independent sets of PCR primers were used; one set was derived from the published literature and a second set was based on our own design.

The relative level of expression for each hepatic target mRNA was normalized for the level of expression of the internal reference standard, β-actin. With the exception of CYP3A25 and MUP2, all RT-PCR assays were run as duplex reactions with both β-actin and the target cDNA being amplified according to the thermal cycling conditions listed for the specific target. For analysis of CYP3A25 and MUP2, β-actin cDNA was amplified in a separate reaction according to the thermal cycling conditions listed for β-actin.
TABLE 2. *Time-course of the effects of MC administration to male mice on body weight, liver weight, and liver to body weight ratio.*

Data are expressed as mean ± S.D. of determinations from eight to ten individual male mice or four individual female mice.

<table>
<thead>
<tr>
<th>Time (days)</th>
<th>Treatment</th>
<th>Final body weight (g)</th>
<th>Liver weight (g)</th>
<th>Liver to body weight ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Vehicle</td>
<td>24.8 ± 0.8</td>
<td>1.25 ± 0.09</td>
<td>0.051 ± 0.003</td>
</tr>
<tr>
<td></td>
<td>MC</td>
<td>23.9 ± 0.9 *</td>
<td>1.25 ± 0.09</td>
<td>0.052 ± 0.003</td>
</tr>
<tr>
<td>2</td>
<td>Vehicle</td>
<td>24.9 ± 1.3</td>
<td>1.33 ± 0.10</td>
<td>0.053 ± 0.002</td>
</tr>
<tr>
<td></td>
<td>MC</td>
<td>23.9 ± 1.3</td>
<td>1.36 ± 0.09</td>
<td>0.057 ± 0.002 **</td>
</tr>
<tr>
<td>3</td>
<td>Vehicle</td>
<td>25.3 ± 1.8</td>
<td>1.30 ± 0.15</td>
<td>0.051 ± 0.002</td>
</tr>
<tr>
<td></td>
<td>MC</td>
<td>24.7 ± 1.6</td>
<td>1.36 ± 0.27</td>
<td>0.055 ± 0.009</td>
</tr>
<tr>
<td>4</td>
<td>Vehicle</td>
<td>25.5 ± 1.6</td>
<td>1.34 ± 0.17</td>
<td>0.053 ± 0.004</td>
</tr>
<tr>
<td></td>
<td>MC</td>
<td>25.0 ± 0.7</td>
<td>1.63 ± 0.09 **</td>
<td>0.065 ± 0.003 ** ††</td>
</tr>
<tr>
<td>7</td>
<td>Vehicle</td>
<td>27.1 ± 0.9 †††</td>
<td>1.46 ± 0.08 †</td>
<td>0.054 ± 0.002</td>
</tr>
<tr>
<td></td>
<td>MC</td>
<td>24.7 ± 1.3 ***</td>
<td>1.55 ± 0.22 †</td>
<td>0.063 ± 0.007 ** ††</td>
</tr>
</tbody>
</table>

Female Untreated

<table>
<thead>
<tr>
<th></th>
<th>Final body weight (g)</th>
<th>Liver weight (g)</th>
<th>Liver to body weight ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>19.6 ± 1.0</td>
<td>0.99 ± 0.03</td>
<td>0.050 ± 0.002</td>
</tr>
</tbody>
</table>

Data for vehicle- and MC-treated male mice were analyzed initially using a randomized design two-way analysis of variance to identify significant drug and time effects.

*, Significantly different (p ≤ 0.05) from vehicle control at a given time-point; **, significantly different (p ≤ 0.01) from vehicle control at a given time-point; †††, significantly different (p ≤ 0.05) from vehicle control at a given time-point, based on Student’s t test.

†, Significantly different (p ≤ 0.05) from corresponding treatment group at day 1; ††, significantly different (p ≤ 0.05) from corresponding treatment group at days 1, 2 and 3; ††††, significantly different (p ≤ 0.05) from corresponding treatment group at days 1, 2, 3 and 4, based on randomized design one-way analysis of variance and post hoc Newman-Keuls test.
TABLE 3. Time-course of the effects of MC administration to male mice on hepatic microsomal P450 and heme levels, NADPH P450 oxidoreductase activity, and EROD activity.

Data are expressed as mean ± S.D. of determinations from four microsomal samples prepared from male mice or the mean of determinations from two microsomal samples prepared from female mice. Each microsomal sample was prepared from pooled livers from two or three individual mice. NADPH P450 oxidoreductase activity is based on triplicate determinations per sample, P450 content and EROD activity are based on duplicate determinations per sample, and heme content is based on a single determination per sample.

<table>
<thead>
<tr>
<th>Time (days)</th>
<th>Treatment</th>
<th>P450 (nmol/mg protein)</th>
<th>Heme (nmol/mg protein)</th>
<th>NADPH P450 oxidoreductase (nmol/min/mg protein)</th>
<th>EROD (nmol/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Vehicle</td>
<td>0.90 ± 0.16</td>
<td>1.84 ± 0.07</td>
<td>282 ± 10</td>
<td>0.39 ± 0.08</td>
</tr>
<tr>
<td></td>
<td>MC</td>
<td>1.56 ± 0.12 ***</td>
<td>2.56 ± 0.11 **</td>
<td>305 ± 19</td>
<td>11.49 ± 1.46 ***</td>
</tr>
<tr>
<td>2</td>
<td>Vehicle</td>
<td>0.95 ± 0.05</td>
<td>1.95 ± 0.15</td>
<td>314 ± 7 †</td>
<td>0.58 ± 0.10</td>
</tr>
<tr>
<td></td>
<td>MC</td>
<td>2.22 ± 0.15 *** †</td>
<td>3.40 ± 0.16 ** †</td>
<td>340 ± 18 * †</td>
<td>18.91 ± 2.38 *** †††††</td>
</tr>
<tr>
<td>3</td>
<td>Vehicle</td>
<td>1.11 ± 0.05 †</td>
<td>2.10 ± 0.14 †</td>
<td>308 ± 6 †</td>
<td>0.52 ± 0.18</td>
</tr>
<tr>
<td></td>
<td>MC</td>
<td>2.44 ± 0.20 *** †</td>
<td>3.65 ± 0.09 ** †</td>
<td>358 ± 17 * †</td>
<td>21.00 ± 1.44 *** †††††</td>
</tr>
<tr>
<td>4</td>
<td>Vehicle</td>
<td>1.03 ± 0.08</td>
<td>2.13 ± 0.13 †</td>
<td>297 ± 19</td>
<td>0.60 ± 0.12</td>
</tr>
<tr>
<td></td>
<td>MC</td>
<td>2.39 ± 0.07 *** †</td>
<td>3.76 ± 0.38 ** †</td>
<td>378 ± 14 * †††</td>
<td>21.63 ± 0.65 *** †††††</td>
</tr>
<tr>
<td>7</td>
<td>Vehicle</td>
<td>1.04 ± 0.03</td>
<td>2.05 ± 0.09</td>
<td>273 ± 5 †††††</td>
<td>0.59 ± 0.12</td>
</tr>
<tr>
<td></td>
<td>MC</td>
<td>2.06 ± 0.31 *** ††††</td>
<td>3.28 ± 0.47 ** †</td>
<td>363 ± 25 * ††††</td>
<td>13.39 ± 3.19 ***</td>
</tr>
</tbody>
</table>

Data for vehicle- and MC-treated male mice were analyzed initially using a randomized design two-way analysis of variance to identify significant drug and time effects.

*, significantly different (p ≤ 0.05) from vehicle control at a given time-point; **, significantly different (p ≤ 0.01) from vehicle control at a given time-point; †††, significantly different (p ≤ 0.001) from vehicle control at a given time-point, based on Student’s t test.

†, Significantly different (p ≤ 0.05) from corresponding treatment group at day 1; ††, significantly different (p ≤ 0.05) from corresponding treatment group at day 2; ††††, significantly different (p ≤ 0.05) from corresponding treatment group at days 1 and 2; †††††, significantly different (p ≤ 0.05) from corresponding treatment group at days 1 and 3; ††††††, significantly different (p ≤ 0.01) from corresponding treatment group at days 1 and 7; †††††††, significantly different (p ≤ 0.05) from corresponding treatment group at days 2, 3 and 4, based on randomized design one-way analysis of variance and post hoc Newman-Keuls test.
Figure 1

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Figure 2

A  (~49.5 kDa) CYP2D9 = b

B  β-actin (450 bp) 
CYP2D9 (347 bp)

Days after MC exposure

C

Relative expression level (% of vehicle control)

Days after MC exposure

Female

CYP2D9 protein

CYP2D9 mRNA
**Figure 3**

A. (~56 to 58 kDa) CYP3A

B. (~58 kDa) CYP3A11? = a
   (~57.5 kDa) CYP3A13? = b

C. (~58 kDa) CYP3A11? = a
   (~57.5 kDa) CYP3A13? = b

D. (~56 to 58 kDa) CYP3A

E. Relative expression level (% of vehicle control)

Days after MC exposure

- Ab 1
- Ab 2, band a
- Ab 3, band a
- Ab 4
**Figure 4**

The figure shows a gel electrophoresis image with bands stained for different genes. The bands are labeled with the following information:

- **A**: β-actin (450 bp), CYP3A11 (260 bp)
- **B**: β-actin (450 bp), CYP3A13 (390 bp)
- **C**: CYP3A25 (390 bp)
- **D**: CYP3A41 (408 bp)
- **E**: Relative expression level (% of vehicle control)

The x-axis represents the days after MC exposure (1 day, 2 days, 3 days, 4 days, 7 days) and the y-axis represents the relative expression level. The bars are annotated with asterisks to indicate statistical significance, and the bars are grouped by gender (V for vehicle control, M for treated group). The y-axis values range from 0 to 225.

Legend:
- **Black**: CYP3A11
- **White**: CYP3A13
- **Gray**: CYP3A25

The figure illustrates the expression levels of CYP3A11, CYP3A13, and CYP3A25 in female mice after MC exposure over different time points.
Figure 5

A

B

C

D

E

F

Relative expression level (% of vehicle control)

Days after MC exposure
Figure 6

A

V M V M V M V M V M Female
1 day 2 days 3 days 4 days 7 days

B

β-actin (450 bp)
CIS (353 bp)

β-actin (450 bp)
MUP2 (241 bp)

C

Relative expression level (% of vehicle control)

CIS
MUP2

Days after MC exposure

1 2 3 4 7 Female

0 100 200 300 400