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Cytochrome P450 2C11 5'-flanking region and promoter mediate in vivo suppression by 3-methylcholanthrene

Rana M. Sawaya and David S. Riddick

Department of Pharmacology & Toxicology, Medical Sciences Building,

University of Toronto, Toronto, Ontario, Canada M5S 1A8

Running Title: In vivo suppression of *CYP2C11* by 3-methylcholanthrene

Corresponding author: David S. Riddick, Department of Pharmacology & Toxicology, Medical Sciences Building, University of Toronto, Toronto, Ontario, Canada M5S1A8

Tel. (416) 978-0813; Fax. (416) 978-6395;

E-mail: david.riddick@utoronto.ca

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Abbreviations: **AHR**, aryl hydrocarbon receptor; **ARNT**, aryl hydrocarbon receptor nuclear translocator; **bp**, base pair; **DRE**, dioxin-responsive element; **GH**, growth hormone; **HNF**, hepatocyte nuclear factor; **HYPX**, hypophysectomized; **kb**, kilobase; **JAK2**, Janus kinase 2; **MC**, 3-methylcholanthrene; **NF**, nuclear factor; **P450**, cytochrome P450; **PAH**, polycyclic aromatic hydrocarbon; **PCR**, polymerase chain reaction; **RT**, reverse transcription; **STAT5**, signal transducer and activator of transcription 5; **TCDD**, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; **TF**, transcription factor

ABSTRACT

Aromatic hydrocarbons such as 3-methylcholanthrene (MC) elicit toxic and adaptive responses through the aryl hydrocarbon receptor (AHR). Aromatic hydrocarbons act via an unknown mechanism to suppress the transcription of *CYP2C11*, a growth hormone-regulated gene encoding the male-specific rat hepatic cytochrome P450 2C11. We hypothesize that suppression of *CYP2C11* by aromatic hydrocarbons is mediated by the gene's promoter and 5'-flank. Using hydrodynamics-based injections to deliver plasmid DNA to the liver of live rats, we studied the MC-responsiveness of luciferase constructs containing 10.1-kb, 5.6-kb and 2.4-kb of the *CYP2C11* 5'-flank. MC suppressed *CYP2C11*-luciferase activity of the 10.1-kb and 5.6-kb constructs to below 50% of vehicle levels by 24 h and 72 h. Luciferase activity of the 2.4-kb *CYP2C11* construct was decreased to 63% of vehicle levels 24 h after MC treatment, but no suppression was detected by 72h. Negative regulatory element(s) responsible for *CYP2C11* reporter suppression by MC exist in the proximal 2.4-kb of the 5'-flank; however, additional cis-acting elements located between -5.6-kb and -2.4-kb mediate persistent reporter suppression. As a positive control for AHR activation, MC dramatically induced the luciferase activity of a *Cyp1a1*-driven luciferase plasmid under AHR control. Modulation of reporter gene activity by MC was accompanied by induction of endogenous CYP1A1 and suppression of endogenous CYP2C11 mRNA/protein. This is the first demonstration of aromatic hydrocarbon-mediated suppression of a *CYP2C11*-luciferase construct and this finding suggests that the 5'-flanking region and promoter mediate down-regulation of this gene in the intact rat.

Aromatic hydrocarbons are environmental contaminants that are present in almost every aspect of the global ecosystem. Non-halogenated polycyclic aromatic hydrocarbons (PAHs), represented by the laboratory chemical 3-methylcholanthrene (MC), are formed during incomplete combustion of organic materials and are found in automobile exhaust, furnace gas, cigarette smoke and grilled meat. PAHs are bioactivated and the resulting metabolites covalently bind DNA to initiate carcinogenesis. PAH exposure can pose a serious threat to human health and has been linked to the development of human lung and stomach cancers (Lee and Shim, 2007). Scientific interest in aromatic hydrocarbons stems from the toxicity and endocrine-disrupting potential of this important class of chemicals. It is therefore important to understand the mechanisms of aromatic hydrocarbon-mediated toxicity, particularly pathways implicated in the modulation of genes encoding cytochromes P450 (P450) that are involved in the metabolism of xenobiotics and endogenous substrates.

MC and related aromatic hydrocarbons are ligands for the aryl hydrocarbon receptor (AHR), which is a ligand-activated transcription factor (TF) belonging to the basic-helix-loop-helix/Per-ARNT-Sim superfamily. The AHR resides in the cytoplasm in complex with two molecules of the 90-kDa heat shock protein, p23, and the AHR-interacting protein. Upon ligand binding, the AHR migrates into the nucleus and forms a heterodimer with the AHR nuclear translocator (ARNT). The resulting complex can recognize and bind dioxin-responsive elements (DREs) located in the 5'-flanking region of various target genes; modulation of the expression of these genes can elicit toxic and adaptive responses in mammals (Okey et al., 1994).

Aromatic hydrocarbons can up-regulate and down-regulate P450 gene expression. Mechanisms of AHR-mediated induction of P450s are partly understood as they have been

studied extensively; however, our understanding of P450 suppression is less clear (Riddick et al., 2004). Data from our laboratory (Jones and Riddick, 1996), in agreement with earlier studies [reviewed in (Riddick et al., 2003)] as well as recent studies (Shaban et al., 2005; Caron et al., 2006), report that exposure of male rats to aromatic hydrocarbons results in the suppression of hepatic CYP2C11 catalytic activity, protein and mRNA levels via an unknown pre-translational mechanism.

CYP2C11 is the major male-specific constitutive P450 present in the rat liver. The male-characteristic pulsatile pattern of growth hormone (GH) secretion is the main physiological signal regulating the expression of this gene. This regulation occurs at the transcriptional level by a combination of direct and indirect actions involving the Janus kinase 2 (JAK2)-signal transducer and activator of transcription 5b (STAT5b) pathway as well as hepatic nuclear factors (HNFs) (Holloway et al., 2006; Waxman and O'Connor, 2006).

In vivo suppression of *CYP2C11* by MC is at least partially regulated at the level of gene transcription as shown by nuclear run-on analysis (Lee and Riddick, 2000). We showed previously that MC and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) do not down-regulate expression of *CYP2C11*-luciferase reporter constructs containing up to 10.1-kb of the *CYP2C11* 5'-flank in transient transfection assays in cell culture (Bhathena et al., 2002; Sawaya and Riddick, 2008). Lack of suppression of *CYP2C11*-luciferase constructs by aromatic hydrocarbons in these previous cell culture studies suggests this gene should be studied in vivo where the full complement of TFs, co-activators, co-repressors and endocrine factors necessary for physiological *CYP2C11* regulation are present. To overcome the limitation of cell culture transfection studies, we implemented the hydrodynamics-based approach to study reporter plasmids in the living rat (Liu et al., 1999). In rats this technique results in high levels of

plasmid DNA expression in hepatocytes upon rapid injection of a large volume (equivalent to 8-12% of the rat's body weight) of DNA-containing solution into the tail vein (Maruyama et al., 2002).

We hypothesize that the in vivo suppression of *CYP2C11* by aromatic hydrocarbons is mediated by the gene's promoter and 5'-flank. To identify regulatory sequences involved in mediating transcriptional down-regulation of this gene, we have studied the MC-responsiveness of luciferase constructs under the control of various lengths of the *CYP2C11* 5'-flank in living rats using the hydrodynamics-based approach.

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MATERIALS AND METHODS

Reporter gene constructs. The following plasmids from Promega Corporation (Madison, WI) were used as controls: the promoterless pGL3-Basic and pRL-TK (*Renilla* luciferase). pGudluc1.1 contains the luciferase gene under regulation of a 480-bp fragment from the mouse *Cyp1a1* 5'-flank containing four DREs driven by the mouse mammary tumor virus (MMTV) promoter (Garrison et al., 1996).

Luciferase plasmids (-10.1-2C11) and (-5.6-2C11) containing extended regions of the *CYP2C11* 5'-flank amplified by the polymerase chain reaction (PCR) and cloned into the promoterless pGL3-Basic vector were generated and characterized as described previously (Sawaya and Riddick, 2008). Generation of the (-2.4-2C11) plasmid, which was originally named (-2390-2C11)-pGL3, was described previously (Bhathena et al., 2002). The following stretches of *CYP2C11* 5'-flank sequence are contained in these reporter plasmids: (-10.1-2C11), -10,061 to +20; (-5.6-2C11), -5,616 to +17; (-2.4-2C11), -2,390 to +21. All numbering of nucleotide positions is according to version 3.4 of the November 2004 rat genomic assembly found on the UCSC Genome Browser website [http://genome.ucsc.edu/cgi-bin/hgGateway] relative to the start site of transcription.

Large-scale Plasmid Preparation. The Endo-Free plasmid purification Maxi and Giga kits (Qiagen, Valencia, CA) were used for large-scale plasmid preparation of pGL3-Basic, (-5.6-2C11), (-2.4-2C11) and pRL-TK. The pGudluc1.1 and (-10.1-2C11) endotoxin-free plasmids were prepared and purified at large-scale by Aldevron (Fargo, ND). All plasmids were resuspended in buffer TE (pH 8) and stored at -20°C until use.

Animals and Treatment. Male Fischer 344 rats (7-9 weeks of age; 150-200 g) were purchased from Charles River Canada (St.-Constant, Quebec, Canada). Rats were fed Harlan Teklad rodent laboratory chow and water ad libitum. All animal experimentation was approved by the University of Toronto Animal Care Committee and rats were cared for in accordance with guidelines of the Canadian Council on Animal Care. Rats were housed two animals per cage, and exposed to a 12-h light cycle followed by a 12-h dark cycle. Animals were acclimatized to living conditions in the Division of Comparative Medicine at the University of Toronto for 7 days before experimental procedures were begun.

Prior to hydrodynamics-based injections, rats were anesthetized by isoflurane inhalation (5% isoflurane in oxygen for induction; 2% isoflurane in oxygen for maintenance). Rats received a single intraperitoneal (i.p.) injection of either MC (80 mg/kg; Aldrich Chemical Company, Milwaukee, WI; 98% purity) or an equivalent volume of vehicle (sterile Mazola corn oil). Rats were then injected via the tail vein with a firefly luciferase plasmid and the pRL-TK *Renilla* luciferase plasmid dissolved in sterile lactated Ringer's solution (130 mM Na, 4 mM K, 1.4 mM Ca, 109 mM Cl, 28 mM lactate; Baxter Co., Mississauga, Ontario, Canada) at a volume equivalent to 8% of the rat's body weight. Injection time never exceeded 10 s. The *Renilla* construct was used to normalize for DNA transfection efficiency into the liver. Tail vein injections were performed using a 22-guage, 1-inch long catheter connected to a 25 ml-capacity syringe. Each rat received 20 μg of firefly luciferase plasmid and 1.75 μg of *Renilla* plasmid DNA per ml of Ringer's solution injected, with the following exceptions. Rats euthanized at 24 and 72 h after injection with the (-5.6-2C11) plasmid and rats euthanized at 6 h after injection with the (-10.1-2C11) plasmid received 5 μg of firefly luciferase plasmid and 0.5 μg of *Renilla* plasmid DNA per ml of Ringer's solution. These plasmid amounts fall within optimal ranges

required for high-level reporter gene expression in the liver (Liu et al., 1999; Maruyama et al., 2002).

Rats were euthanized by decapitation at 24 h or 72 h following high-volume injections. Rats receiving the (-10.1-2C11) plasmid were also studied at an earlier time-point of 6 h post-injection. The liver of each rat was perfused in situ with ice-cold 1.15% KCl. Livers were excised and weighed. Small pieces of individual livers (~ 0.1 g) were frozen in liquid nitrogen and stored at -70°C until RNA isolation was performed. Fresh liver (1.5 g) isolated from the left-lobe of each rat was homogenized in 1 ml of 1X passive lysis buffer (Promega). Homogenates were centrifuged at 4°C for 25 min at 15 000 x *g*. The supernatant was stored at -70°C until dual luciferase assays were performed. Thawed supernatant (20 μl) was mixed with 100 μl luciferase assay reagent II (Promega). Firefly luciferase activity was measured using a TD-20/20 luminometer (Turner Designs, Inc., Sunnyvale, CA). Stop and glow buffer (100 μl) was then added and *Renilla* luciferase activity was measured. The firefly luciferase activity was normalized to *Renilla* luciferase activity.

The remaining liver (~ 8 g) was homogenized in 4 volumes of cold phosphate-buffered KCl (1.15% KCl, 10 mM potassium phosphate, pH 7.4), and microsomes were isolated by differential centrifugation (McCluskey et al., 1986). Microsomal pellets 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. Microsomal protein concentrations were determined by the method of Bradford (1976).

Analysis of CYP1A1 and β-actin mRNA Levels by RT-PCR. Total RNA was isolated from liver tissue by the acid guanidinium thiocyanate-phenol-chloroform extraction method using TRI Reagent (Sigma Chemical Company, St. Louis, MO). To remove genomic DNA contamination,

all RNA samples were treated with 20 U of DNase I (GE Healthcare Bio-Sciences, Baie d'Urfé, Quebec, Canada) for 25 min at 37°C. The yield and purity of RNA was assessed by determining the A_{260}/A_{280} ratio, and the RNA integrity was determined by comparing the 28S and 18S rRNA bands as visualized on ethidium bromide-stained agarose gels. Reverse-transcription (RT) was performed by incubating 1 µg RNA with oligo$(dT)_{15}$ (2 µg; Roche Diagnostics, Laval, Quebec, Canada) at 60°C for 5 min. Samples were then incubated in a final volume of 40 µl with Moloney murine leukemia virus-reverse transcriptase (400 U; Invitrogen Corporation, Carlsbad, CA), RNA Guard (60 U; GE Healthcare Bio-Sciences), a 1 mM concentration of each 2'-deoxynucleoside 5'-triphosphate (Invitrogen), 10 mM dithiothreitol, and 1X RT buffer containing 50 mM Tris, 75 mM KCl, 3 mM $MgCl_2$. Reactions proceeded for 60 min at 37°C, followed by incubation at 70°C for 10 min.

All PCR primers were obtained from Integrated DNA Technologies Inc. (Coralville, IA). PCR primer sequences and cycling conditions are shown in Table 1. Each 50-µl PCR reaction contained input cDNA derived from 50 ng of RNA, *Taq* DNA polymerase (2.5 U; Invitrogen), 0.2 µM of each primer, a 0.2 mM concentration of each 2'-deoxynucleoside 5'-triphosphate (Invitrogen), and 1X PCR buffer containing 20 mM Tris/50 mM KCl/3 mM $MgCl_2$. PCR products were separated on a 6% non-denaturing polyacrylamide gel, stained with Vistra Green (GE Healthcare Bio-Sciences) and imaged using the STORM Phosphorimager (Molecular Dynamics, Sunnyvale, CA). The presence of CYP1A1 mRNA was used as a qualitative positive control for MC treatment and not subjected to quantitative analysis.

Analysis of CYP2C11 mRNA Levels by Real-Time Quantitative RT-PCR. Primers and probes used for real-time analyses were synthesized by Integrated DNA Technologies, Inc. Table 2 contains all primer and probe sequences. Molecular probes were labeled at the 5'-end

with reporter fluorescent dye 6-carboxyfluorescein and the quencher fluorescent dye Iowa Black at the 3'-end. RNA was reverse-transcribed as described above. The MX4000 real-time PCR system (Stratagene, La Jolla, CA) was used for real-time PCR reactions. Each 25-µl reaction contained a final concentration of 0.3 µM (CYP2C11) or 0.4 µM (β-actin) primers, 0.2 µM (CYP2C11) or 0.3 µM (β-actin) probe, input cDNA (derived from 50 ng of RNA) and the 2X Brilliant QPCR Master Mix (Stratagene). A standard curve consisting of a 10-fold dilution series derived from pooling cDNA from several rats was run with each real-time PCR reaction to determine the reaction efficiency. Each dilution used in the standard curve was performed in triplicate. Cycling conditions consisted of an initial 10 min denaturation at 95°C, followed by 40 cycles of: 95°C, 30 s; 55°C, 1 min; 72°C, 30 s. Normalized expression (NE) was determined for each sample using the calculation $NE = E^{Ct\,(\beta\text{-actin})} / E^{Ct\,(CYP2C11)}$, where E is the efficiency of the PCR amplification for each gene product and C_t is the threshold cycle at which fluorescence was detected (Simon, 2003).

Immunoblot Analysis. Microsomal protein (5 µg for CYP1A1 and 3.5 µg for CYP2C11 analysis) from each liver was resolved by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (Hybond-ECL; GE Healthcare Bio-Sciences). For CYP1A1 detection, mouse monoclonal antibody 1-31-2 (Dr. H. V. Gelboin, National Cancer Institute, Bethesda, MD) was used at a dilution of 1:5000 in TNT (20 mM Tris, pH 7.4, 150 mM NaCl, and 0.1% Tween-20) containing 5% skim milk powder. Blots were then incubated with sheep anti-mouse Ig-horseradish peroxidase conjugate (GE Healthcare Bio-Sciences) at a dilution of 1:5000. For CYP2C11 protein detection, a rabbit anti-CYP2C11 polyclonal antibody [Dr. E. T. Morgan, Emory University, Atlanta, GA; (Morgan et al., 1985)] was used at a dilution of 1:5000 in TNT containing 5% skim milk powder, followed by a donkey anti-rabbit Ig-

horseradish peroxidase conjugate (GE Healthcare Bio-Sciences) at a 1:5000 dilution. An enhanced chemiluminescence system (ECL; GE Healthcare Bio-Sciences) was used for protein detection, and films were scanned on an HP Scanjet 3970 scanner (Hewlett-Packard Company, Palo Alto, CA). Relative quantitation was performed using IPLabGel software (Signal Analytics, Vienna, VA). CYP1A1 immunoblots were used as a qualitative positive control for MC treatment and not subjected to quantitative analysis. CYP2C11 quantitative analyses were performed under conditions that yielded a linear relationship between amount of microsomal protein and immunoreactive signal intensity.

Statistical Analysis. Data are presented as a percentage of the mean for the vehicle-treated controls and each bar represents the mean ± S.D. of determinations from three or four rats. All statistical analyses were performed on the raw data rather than the percent control data presented in the figures. Student's *t* tests were performed to determine whether the mean value for MC-treated rats differed from the mean value for the corresponding vehicle control rats at each time point. Statistical significance of linear correlation data was determined by the Pearson correlation test. In all cases, a result was considered to be statistically significant if $p \leq 0.05$.

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RESULTS

We have studied how MC affects luciferase plasmids driven by various lengths of the *CYP2C11* 5'-flank in living rats. Based on previous bioinformatic analyses (Sawaya and Riddick, 2008), we have focused on the proximal 10.1-kb region of the 5'-flank. This region is particularly enriched for DRE-like motifs (also known as AHRE-I elements) and also contains one perfect match to the AHRE-II consensus sequence, a novel site first identified in the *CYP1A2* gene that binds an unknown factor that recruits the AHR•ARNT complex as a co-activator (Boutros et al., 2004; Sogawa et al., 2004). This region also contains putative sites for TFs involved in GH regulation of this gene (STAT5, HNF-3) (Park and Waxman, 2001; Timsit and Riddick, 2002) and NF-κB response elements which can mediate suppression of this gene by inflammatory cytokines (Iber et al., 2000).

Using the hydrodynamics-based technique, rats received a tail vein injection consisting of one of five firefly luciferase plasmids [pGudluc1.1, pGL3-Basic, (-10.1-2C11), (-5.6-2C11) or (-2.4-2C11)] and the *Renilla* luciferase plasmid (pRL-TK) to normalize for *in vivo* transfection efficiency. MC or vehicle corn oil was administered to rats via i.p. injection immediately prior to the high-volume tail vein injection and rats were euthanized at 24 or 72 h post-treatment; however, rats receiving the (-10.1-2C11) plasmid were also euthanized at an earlier time-point of 6 h following treatment.

Hepatic luciferase activity of our positive control plasmid pGudluc1.1 was increased approximately 2000-fold by 24 h and 3000–fold by 72 h following MC administration (Fig. 1A). This confirmed that the administered dose of MC (80 mg/kg) increased transcription of a reporter

gene delivered to the liver by the hydrodynamics-based approach. No observable signs of toxicity were associated with this dose of MC.

Under in vivo conditions in which MC activates the AHR to induce pGudluc1.1 reporter gene transcriptional activity, we examined the MC-responsiveness of the four other firefly luciferase reporter plasmids: pGL3-Basic and three *CYP2C11* constructs containing 10.1-kb, 5.6-kb and 2.4-kb of 5'-flanking region (Fig. 1B). pGL3-Basic is a luciferase reporter plasmid devoid of an eukaryotic promoter, thus serving as a negative control for aromatic hydrocarbon responsiveness. MC induced pGL3-Basic luciferase activity slightly at both time-points studied, but this response was not statistically significant. At 24 h post-treatment, all *CYP2C11* constructs were down-regulated by MC compared to vehicle-treated rats. The most pronounced down-regulation was observed with (-5.6-2C11), which was suppressed to 36% of vehicle levels. (-2.4-2C11) showed the smallest magnitude of suppression 24 h post-treatment, being decreased to 63% of vehicle levels. At 72 h, luciferase activity of (-10.1-2C11) and (-5.6-2C11) plasmids was strikingly down-regulated in response to MC treatment by 69% and 76%, respectively. Suppression of (-5.6-2C11) did not achieve statistical significance at 72 h since vehicle-treated rats displayed large variation in basal luciferase activity, whereas the variation at 24 h was minimal (Table 3). MC up-regulated (-2.4-2C11) luciferase activity by 53% at 72 h; however, this was not statistically significant. Since all plasmids displayed a decline in luciferase activity by MC at 24 h, we examined an earlier time-point of 6 h post-injection in rats receiving the (-10.1-2C11) plasmid, to gain a clearer understanding of the time course involved in *CYP2C11* reporter suppression. Regardless of chemical treatment, large variation in normalized luciferase activity was observed at this early time-point (Table 3). There was a 43% decline in luciferase activity 6 h following MC administration, an effect that was not statistically significant (Fig. 1B).

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For *CYP2C11* constructs that were suppressed by MC at both 24 h and 72 h, basal luciferase activity in vehicle-treated rats was always higher at 72 h after injection (Table 3). The opposite trend was observed for pGudluc1.1 and pGL3-Basic luciferase activity, which displayed high levels of activity at 24 h, tapering off by 72 h post-treatment. All *Renilla* luciferase levels were relatively similar within a single plasmid group at the same time-point, indicating consistent transfection efficiencies between rats. *Renilla* luciferase values were higher when examined at 24 h post-treatment and were considerably lower by 72 h for all rats.

The effectiveness of the MC treatment was further confirmed by monitoring induction of endogenous CYP1A1 protein in rats receiving high-volume tail vein injections as a qualitative positive control response. MC markedly induced CYP1A1 protein (Fig. 2A) at all time-points regardless of plasmid administered, although the extent of induction varied between individual rats. The effects of MC administration on endogenous hepatic CYP2C11 expression in rats receiving hydrodynamics-based injections were examined at the protein level (Fig. 2B,C). Suppression of CYP2C11 protein levels by MC was detected at 24 h (62-77% of vehicle levels); however, statistical significance was achieved only in rats receiving pGudluc1.1, (-10.1-2C11) and (-2.4-2C11). Dramatic down-regulation of CYP2C11 protein was observed at 72 h in MC-treated rats receiving pGL3-Basic, pGudluc1.1 and (-5.6- 2C11) with levels falling to 24-54% of vehicle levels. Rats receiving (-2.4-2C11) showed no overall changes in CYP2C11 protein levels between vehicle- and MC-treatment at 72 h. These rats displayed high levels of variation in CYP2C11 protein with standard deviations ranging from 45-54% of the mean. No differences in CYP2C11 protein levels were detected between MC- and vehicle-treated rats at 6 h.

As an additional qualitative positive control, MC markedly induced CYP1A1 mRNA (Fig. 3A,B) at all time-points regardless of plasmid administered, although the extent of

induction varied between individual rats. We used real-time quantitative RT-PCR to determine whether endogenous hepatic CYP2C11 mRNA expression was down-regulated by MC in rats receiving hydrodynamics-based injections (Fig. 3C). At 24 h, modest trends for CYP2C11 mRNA suppression were observed in MC-treated rats receiving high-volume injections (67-76% of vehicle levels), but statistical significance was only achieved in rats receiving (-5.6-2C11). Suppression of endogenous CYP2C11 mRNA levels was detected at 72 h in all rats (52-69% of vehicle control) except rats receiving pGL3-Basic. Statistical significance at 72 h was achieved in rats receiving pGudluc1.1 and (-10.1-2C11), since large variation in CYP2C11 mRNA expression was observed in vehicle-treated rats receiving (-5.6-2C11) and (-2.4-2C11) (data not shown). At 6 h post-treatment, no suppression of CYP2C11 mRNA levels following MC treatment was observed.

In general, CYP2C11 protein levels paralleled CYP2C11 mRNA levels (Fig. 4). A significant positive correlation was observed between endogenous CYP2C11 mRNA and protein levels in rats receiving all plasmids by high-volume tail vein injection except for rats in the (-2.4-2C11) plasmid group. For *CYP2C11* constructs strongly suppressed by MC [(-10.1-2C11) and (-5.6-2C11)], luciferase activity was positively correlated with endogenous CYP2C11 mRNA levels (Fig. 5A,B). Luciferase activity of (-2.4-2C11) was down-regulated at 24 h following MC treatment but showed a trend for an increase at 72 h, resulting in significant negative correlation between luciferase activity and CYP2C11 mRNA expression (Fig. 5C). pGudluc1.1 luciferase activity displayed a slight trend for negative correlation with endogenous CYP2C11 mRNA levels; however, this was not statistically significant (Fig. 5D). There was no significant relationship between pGL3-Basic luciferase activity and endogenous CYP2C11 mRNA (data not shown).

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DISCUSSION

CYP2C11-luciferase constructs containing extended stretches of the 5'-flank were hydrodynamically introduced into rat hepatocytes in vivo. Our findings indicate that negative regulatory element(s) responsible for *CYP2C11* reporter suppression by MC exist in the proximal 2.4-kb of the 5'-flank and that additional cis-acting elements located between -5.6-kb and -2.4-kb mediate persistent reporter suppression.

Endogenous CYP2C11 protein and mRNA levels are quite variable in both vehicle- and MC-treated rats receiving high-volume injections, a finding that coincides with variability in our qualitative assessment of CYP1A1 induction (Fig. 2A, 3A). This variability, together with the small numbers of animals used in this study, results in some data trends that do not achieve statistical significance. Overall there was a positive correlation between CYP2C11 mRNA and protein levels in individual rats that reached significance in all but one of the five plasmid treatment groups. This further supports a pre-translational mechanism for the down-regulation of CYP2C11 protein by MC. The half-life of CYP2C11 mRNA in primary rat hepatocytes has been reported to be 9.8 h (Iber et al., 2000) and 16 h (Bhathena et al., 2002). Since MC is able to cause partial suppression of the rate of gene transcription within 6 h (Lee and Riddick, 2000) and blockage of transcription is not complete due to ongoing MC metabolism, we included time-points that would give us a realistic window for detecting suppression of endogenous *CYP2C11* and reporter plasmids: 24 h, 72 h, as well as 6 h in rats receiving (-10.1-2C11). A prior study in our laboratory found that maximal suppression of endogenous CYP2C11 protein and mRNA occurs between 72 h to 120 h following in vivo MC treatment (Jones and Riddick, 1996). In the present study, there was an overall trend for down-regulation of endogenous CYP2C11

expression at 24 h and 72 h, but not at 6 h (Fig. 2C, 3C).

Endogenous CYP2C11 levels in rats receiving high-volume injections were not uniform in their response to MC between the five plasmid groups. This may result if the high-volume injection itself elicits an inflammatory response in rats, stimulating the release of pro-inflammatory cytokines that are implicated in decreasing *CYP2C11* transcription (Iber et al., 2000). Although we used procedures to generate endotoxin-free plasmids, injection of plasmids containing variable endotoxin concentrations may down-regulate endogenous CYP2C11 levels (Cheng et al., 2003) in both vehicle- and MC-treated rats. Mechanistic insight into variations in endogenous CYP2C11 levels following hydrodynamics-based injections could be gained by measuring pro-inflammatory cytokine and endotoxin levels in future studies. Transient hepatotoxicity is also a consequence of high-volume tail vein injection that may impact expression of endogenous CYP2C11. Serum liver enzyme levels increase transiently following high-volume tail vein injections, returning to baseline within 2-3 days (Liu et al., 1999; Budker et al., 2006); this coincides with normal liver morphology at 24 h post-injection (Budker et al., 2006). Other biochemical markers of hepatic toxicity are unchanged following hydrodynamics-based injections (Liu et al., 1999). Although we did not measure serum liver enzymes in this study, veterinary staff observed no clinical signs of hepatotoxicity and histology performed on selected animals showed good hepatocyte morphology with some small isolated foci of hepatic necrosis at 2-3 days post-injection.

Interestingly, *CYP2C11*-luciferase plasmids strongly suppressed by MC [(-5.6-2C11) and (-10.1-2C11)] displayed a positive correlation between luciferase activity and endogenous CYP2C11 mRNA levels indicating that luciferase activity paralleled the response of the endogenous gene. Luciferase activity of (-2.4-2C11), which was weakly suppressed by MC at

24 h, was negatively correlated with CYP2C11 mRNA expression. This correlation may be skewed by the unexpected trend for induction of this plasmid at 72 h while endogenous CYP2C11 mRNA showed trends for suppression.

All of our *CYP2C11*-luciferase constructs were suppressed at 24 h post-treatment suggesting that TF binding sites located in the proximal 2.4-kb of the 5'-flank are required for transient suppression by MC. By 72 h post-treatment, luciferase activity from the (-10.1-2C11) and (-5.6-2C11) plasmids remains down-regulated. This suggests that negative regulatory elements located between -5.6-kb and -2.4-kb mediate persistent suppression by MC. Although the precise mechanism of *CYP2C11* suppression remains to be elucidated, possible mechanisms can be postulated based on the presence of putative DRE-like, AHRE-II, NF-κB, STAT5 and HNF-3 sequences located in the proximal 5.6-kb of the 5'-flank (Sawaya and Riddick, 2008). We envision three possible mechanisms by which MC suppresses *CYP2C11* expression: direct AHR-mediated suppression via DREs and/or AHRE-II; interference with GH signaling needed to maintain *CYP2C11* transcription; and/or suppression involving inflammatory cytokines activated by MC.

Structure activity relationship studies first introduced the notion that *CYP2C11* suppression coincided with the stimulation of AHR-mediated responses [reviewed in (Riddick et al., 2003)]. Our laboratory demonstrated that the transformed AHR binds with high affinity to a DRE-like sequence at positions -1546 to -1533 of the *CYP2C11* 5'-flank (Bhathena et al., 2002). A functional role for this DRE in the paradoxical induction of *CYP2C11*-luciferase activity by TCDD and MC in HepG2 cells was determined by site-directed mutagenesis (Sawaya and Riddick, 2008). Multiple DRE-like elements and/or the AHRE-II motif located in the proximal 5.6-kb of the *CYP2C11* 5'-flank may function as inhibitory DREs, which confer AHR-mediated

suppression of other genes following TCDD treatment [reviewed in (Riddick et al., 2003)]. To verify the involvement of the AHR in suppression of our *CYP2C11*-luciferase constructs we attempted, but did not succeed, in antagonizing the receptor in vivo using the AHR antagonist/partial agonist α-naphthoflavone (data not shown).

The second mechanistic possibility involves interference by MC with hormonal signaling pathways involved in *CYP2C11* expression. The mechanisms behind suppression of numerous P450s following aromatic hydrocarbon treatment are unknown (Kurose et al., 1998; Lee et al., 2006; Ovando et al., 2006; Ning et al., 2008) but several components of the GH receptor/JAK2/STAT5b pathway have been shown to be targets for disruption by MC in mice (Nukaya et al., 2004; Lee et al., 2006). In hypophysectomized (hypx) male rats, MC interferes with the ability of exogenous GH to stimulate hepatic CYP2C11 expression (Timsit and Riddick, 2000). Ethylbenzene, a simple aromatic hydrocarbon, can decrease CYP2C11 protein only in intact but not in hypx rats (Serron et al., 2001). Such findings suggest that *CYP2C11* down-regulation by aromatic hydrocarbons may occur by a mechanism that disrupts the ability of GH to maintain constitutive *CYP2C11* expression.

A third possible mechanism of MC-mediated *CYP2C11* suppression involves inflammatory cytokines. Increased NF-κB binding activity and protein levels have been reported following exposure to the PAH benzo[*a*]pyrene (Weng et al., 2004). Inflammatory cytokines activated by MC could act to suppress our constructs by directly binding to NF-κB responsive elements on the *CYP2C11* 5'-flank (Iber et al., 2000) or by interfering with GH signaling (Ahmed et al., 2007).

Previous studies of our *CYP2C11*-luciferase constructs in HepG2 cells revealed paradoxical induction of the reporter gene that is AHR-dependent and DRE-mediated (Sawaya

and Riddick, 2008). Conversely, we found that these same reporter plasmids were suppressed by MC treatment when studied in the living rat, a response that is analogous to the behavior of the endogenous *CYP2C11* gene. Similarly, Sasaki et al. (1999) found that studying GH regulation of rat *CYP2C12* reporter constructs in living rats was more mechanistically accurate compared to when these constructs were studied in HepG2 cells. Reporter gene results obtained in cell lines may not reliably predict in vivo responses of the endogenous gene to xenobiotic treatment.

Studying mechanisms of *CYP2C11* suppression has useful implications from both a clinical and molecular viewpoint. P450 suppression is of clinical significance as shown by altered drug toxicity in patients during inflammatory conditions (Morgan et al., 2008). Although the sex difference of hepatic P450 expression is not as obvious in humans as in rats, it does exist and appears to be under hormonal control (Dhir et al., 2006). Recently, human *CYP2C8* was shown to be down-regulated in primary human hepatocytes treated with MC (Ning et al., 2008). Consequently, studies of molecular mechanisms involved in control of rat P450s have the potential to reveal fundamental mechanistic pathways that could be conserved in other mammals. We plan to use *CYP2C11* as a model system to understand how PAHs decrease the transcription of other hormonally-regulated genes.

This is the first demonstration of aromatic hydrocarbon-mediated suppression of a *CYP2C11*-luciferase construct in any biological system. Sequences responsible for *CYP2C11* reporter suppression by MC exist in the proximal 2.4-kb of the 5'-flank and additional cis-acting elements located between -5.6-kb and -2.4-kb mediate persistent reporter suppression. Additional deletion constructs and site-directed mutants introduced to living rats by the hydrodynamics approach will more precisely define the sequences mediating suppression, while in vivo chromatin immunoprecitation assays will allow us to study protein-DNA interactions in

the *CYP2C11* gene in its native chromosomal context. To determine whether pituitary factors are required to mediate suppression by MC, *CYP2C11*-luciferase constructs should be studied in hypx rats with or without GH replacement. The involvement of the AHR in reporter suppression may be verified with the use of an effective in vivo AHR antagonist in the rat.

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FOOTNOTES

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Address correspondence to: David S. Riddick, Department of Pharmacology & Toxicology, Medical Sciences Building, University of Toronto, Toronto, Ontario, Canada, M5S 1A8. E-mail: david.riddick@utoronto.ca

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FIGURE LEGENDS

FIG. 1. Effect of MC on hepatic reporter gene activity in rats receiving hydrodynamics-based injections. Rats received one of five firefly luciferase plasmids: (A) pGudluc1.1 or (B) pGL3-Basic, (-10.1-2C11), (-5.6-2C11), (-2.4-2C11), as well as the pRL-TK *Renilla* construct, and were given i.p. injections of vehicle corn oil (CO) or 80 mg/kg MC. Rats were euthanized 6 h, 24 h or 72 h later as indicated. Hepatic lysates were prepared from each rat and firefly luciferase activity was measured and normalized to *Renilla* luciferase activity. Results for MC-treated rats are expressed as a percentage of the mean for the vehicle-treated controls at each time point. Each bar represents the mean ± S.D. of determinations from three or four rats. Asterisks indicate significant differences from vehicle-treated rats at a given time point (* $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$); based on a two-tailed Student's *t* test for pGL3-Basic luciferase data, and a one-tailed Student's *t* test for all *CYP2C11* constructs and pGudluc1.1 luciferase data.

FIG. 2. Immunoblot analysis of endogenous hepatic CYP1A1 and CYP2C11 protein levels following vehicle or MC treatment in rats receiving hydrodynamics-based injections. Rats received one of five firefly luciferase plasmids: pGL3-Basic, pGudluc1.1, (-10.1-2C11), (-5.6-2C11) or (-2.4-2C11) as well as the pRL-TK *Renilla* construct, and were given i.p. injections of vehicle (V) or MC (M). Rats were euthanized 6 h, 24 h or 72 h later as indicated. A, immunoblot analysis of hepatic microsomal protein (5 µg) using a CYP1A1 monoclonal antibody. B, immunoblot analysis of hepatic microsomal protein (3.5 µg) using a CYP2C11 polyclonal antibody. Representative immunoblots are shown for one rat sample selected from the three or four rats within each treatment group. C, relative quantitation of CYP2C11

apoprotein levels. Results for MC-treated rats are presented as a percentage of the mean for the vehicle-treated control rats at each time point. Data are expressed as mean ± S.D. of determinations from microsomal samples isolated from three or four rats. Similar results were obtained in two or three independent immunoblot analyses. Asterisks indicate significant differences from vehicle-treated rats at a given time point (* $p \leq 0.05$; ** $p \leq 0.01$); based on a one-tailed Student's *t* test.

FIG. 3. Effect of MC administration on endogenous hepatic CYP1A1 and CYP2C11 mRNA levels in rats receiving hydrodynamics-based injections. Rats received one of five firefly luciferase plasmids: pGL3-Basic, pGudluc1.1, (-10.1-2C11), (-5.6-2C11) or (-2.4-2C11) as well as the pRL-TK *Renilla* construct, and were given i.p. injections of vehicle (V) or MC (M). Rats were euthanized 6 h, 24 h or 72 h later as indicated. RT-PCR analysis of CYP1A1 (A) and β-actin (B) mRNA as visualized on Vistra Green-stained polyacrylamide gels. Representative results are shown for one rat sample selected from the three or four rats within each treatment group. C, relative quantitation of CYP2C11 mRNA levels as determined by real-time RT-PCR using molecular beacons. CYP2C11 signal was normalized to β-actin. Results for MC-treated rats are presented as a percentage of the mean for the vehicle-treated control rats at each time point. Data are expressed as mean ± S.D. of determinations from hepatic RNA samples isolated from three or four rats. Asterisks indicate significant differences from vehicle-treated rats at a given time point (* $p \leq 0.05$; ** $p \leq 0.01$); based on a one-tailed Student's *t* test.

FIG. 4. Correlation between endogenous hepatic CYP2C11 protein levels and CYP2C11 mRNA levels in rats receiving the following luciferase plasmids by hydrodynamics-based injection:

(A) (-10.1-2C11), (B) (-5.6-2C11), (C) (-2.4-2C11), (D) pGL3-Basic or (E) pGudluc1. The equations of the lines of best fit were generated by least-squares linear regression analysis. Statistical significance was determined using the two-tailed Pearson correlation test with a significance level set at $p \leq 0.05$.

FIG. 5. Correlation between hepatic luciferase activity and endogenous CYP2C11 mRNA levels in rats receiving the following luciferase plasmids by hydrodynamics-based injection: (A) (-10.1-2C11), (B) (-5.6-2C11), (C) (-2.4-2C11), or (D) pGudluc1. The equations of the lines of best fit were generated by least-squares linear regression analysis. Statistical significance was determined using the two-tailed Pearson correlation test with a significance level set at $p \leq 0.05$.

TABLE 1. *Primer sequences and thermal cycling conditions used for analysis of steady-state mRNA levels in rat liver by RT-PCR.*

Target	Forward and Reverse PCR Primer Sequences	PCR product size	Thermal cycling parameters (denature; anneal; extend) x cycles	Reference
CYP1A1	5'- 627-ACGTTATGACCACGATGACC-646-3' (FP) 5'- 1299-AGGCCGGAACTCGTTTG-1283-3' (RP)	672 bp	(20s/94°; 20s/52°; 40s/72°) x 17	Franc et al., 2001
β-actin	5'-344-ACCGTGAAAAGATGACCCAG-363-3' (FP) 5'-1031-GAGCCACCAATCCACACAG-1011-3' (RP)	688 bp	(20s/94°; 20s/52°; 40s/72°) x 17	Franc et al., 2001

FP, forward primer; RP, reverse primer

TABLE 2. *Primer and probe sequences used for measurements of steady-state mRNA levels in rat liver by real-time quantitative RT-PCR.*

	CYP2C11	β-actin
Forward Primer	5'-1219-TTTGACCCTGGTCACTTTCT-1238-3'	5'-357-GACCCAGATCATGTTTGAGACCTTC-381-3'
Reverse Primer	5'-1318-GGGCTTCTCCTGCACATATC-1299-3'	5'-465-GGAGTCCATCACAATGCCAGTG-444-3'
Probe	5'-1272-CTCTTTCCTGCTGAGAATGGCATAAAG-1298-3'	5'-441-ACGACCAGAGGCATACAGGGACAACACAG-413-3'
Product Size	100 bp	109 bp
Reference	New design	Tijet et al., 2006

TABLE 3. *Hepatic luciferase activity in rats receiving hydrodynamics-based injections.*

	6 h		24 h		72 h	
Plasmid delivered	Vehicle	MC	Vehicle	MC	Vehicle	MC
pGL3-Basic	N.D.	N.D.	0.60 ± 0.07	0.71 ± 0.08	0.21 ± 0.01	0.28 ± 0.10
pGudluc1.1	N.D.	N.D.	0.15 ± 0.02	305.95 ± 62.22	0.10 ± 0.03	335.05 ± 256.84
(-10.1-2C11)	5.29 ± 3.62	3.00 ± 1.79	153.72 ± 19.73	74.84 ± 50.13	4225.88 ± 2386.38	1330.27 ± 485.98
(-5.6-2C11)	N.D.	N.D.	1.95 ± 0.25	0.70 ± 0.36	49.65 ± 38.53	11.79 ± 1.74
(-2.4-2C11)	N.D.	N.D.	3.50 ± 0.42	2.22 ± 0.51	1.48 ± 0.83	2.28 ± 1.32

All data are expressed in arbitrary light units (normalized for *Renilla* activity) as mean ± S.D. of determinations from hepatic lysates prepared from three or four rats. Statistically significant effects of MC are summarized in Fig. 1.

N.D., not determined

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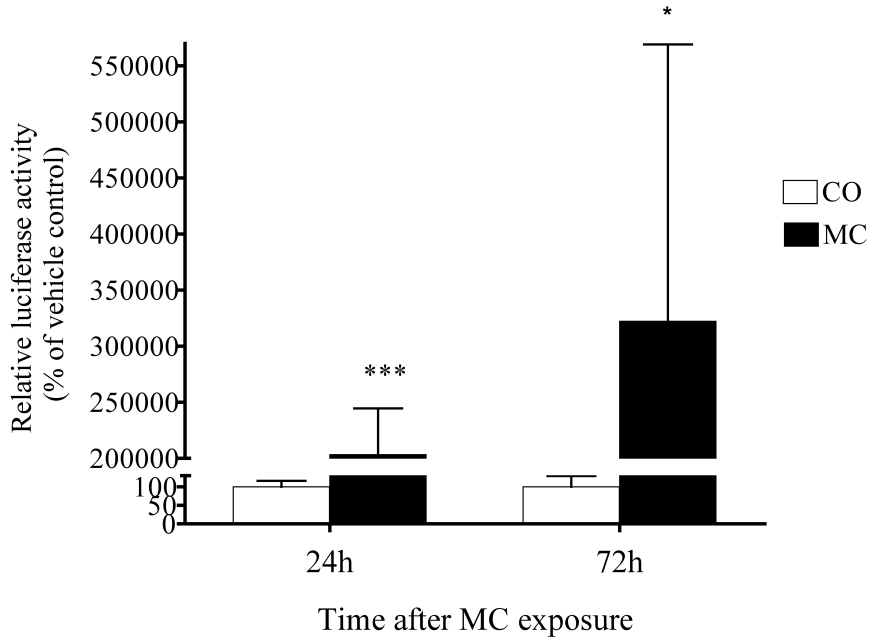
Figure 1

A



B

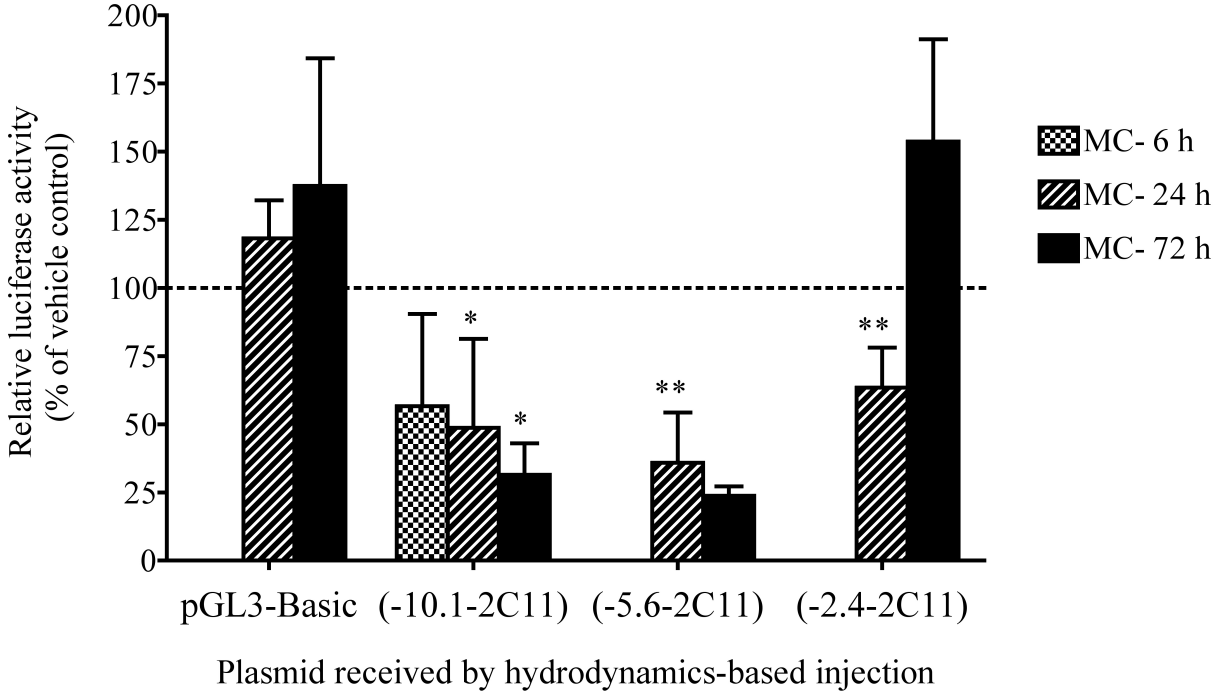


Figure 2

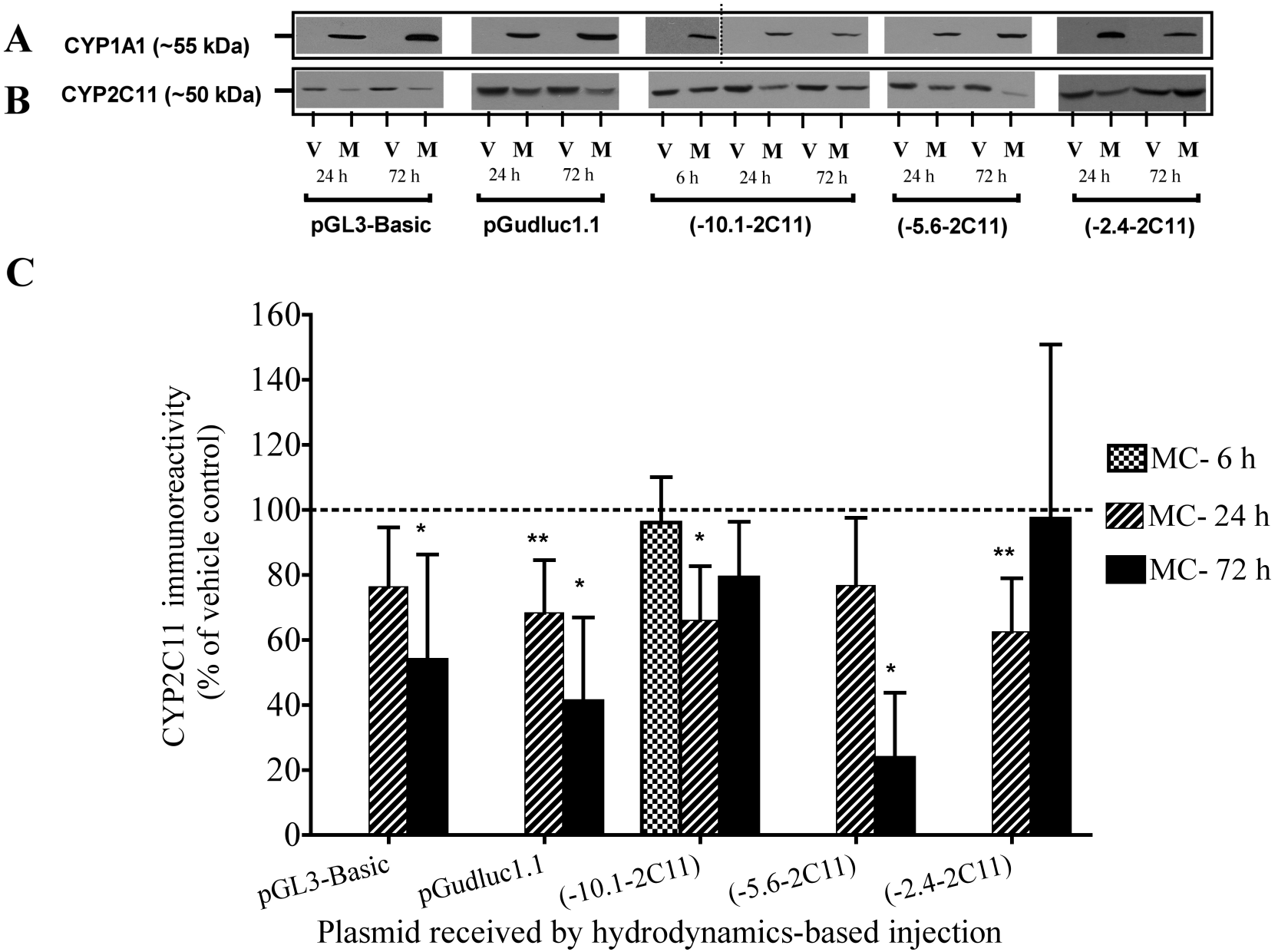


Figure 3

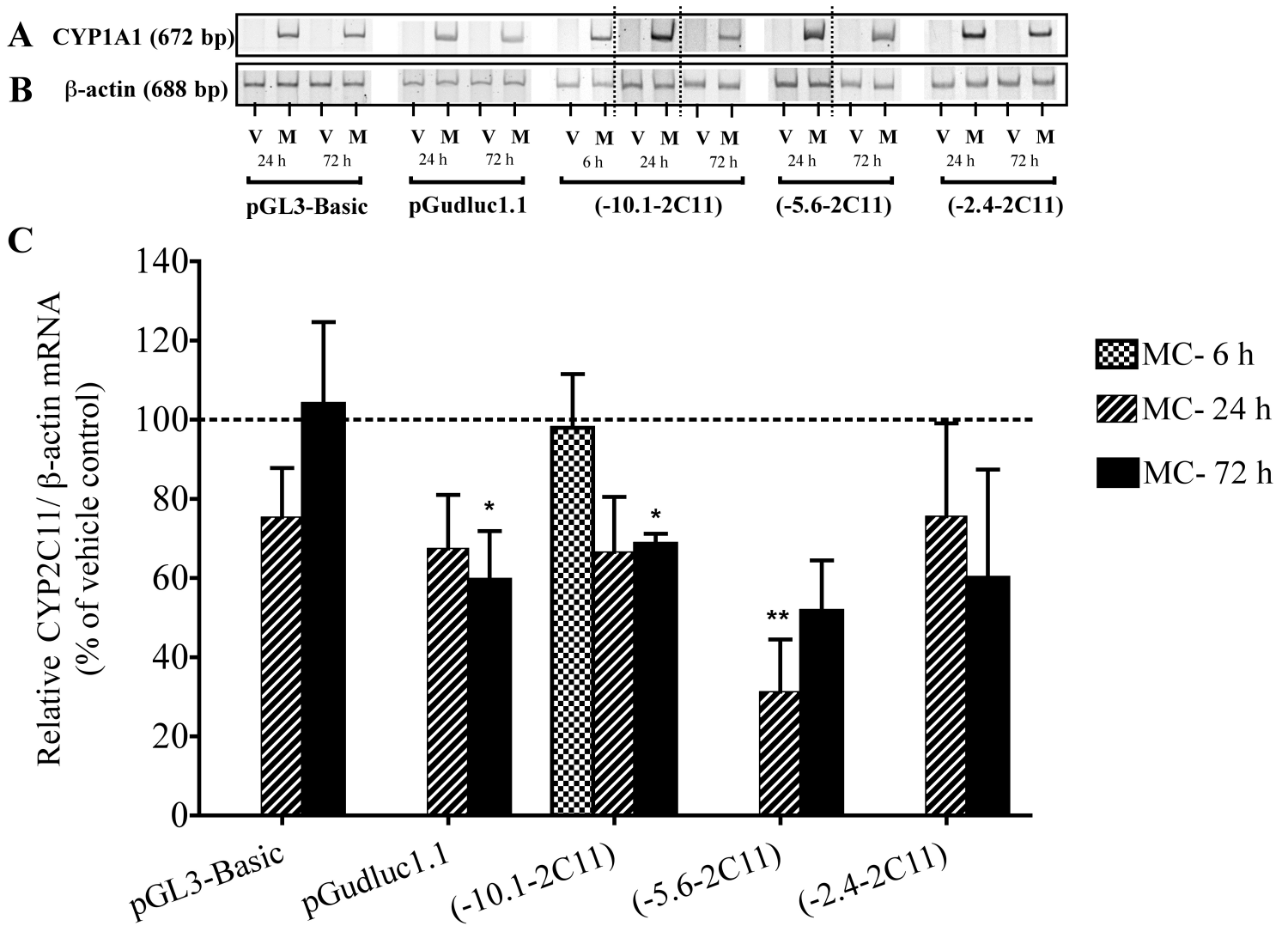


Figure 4

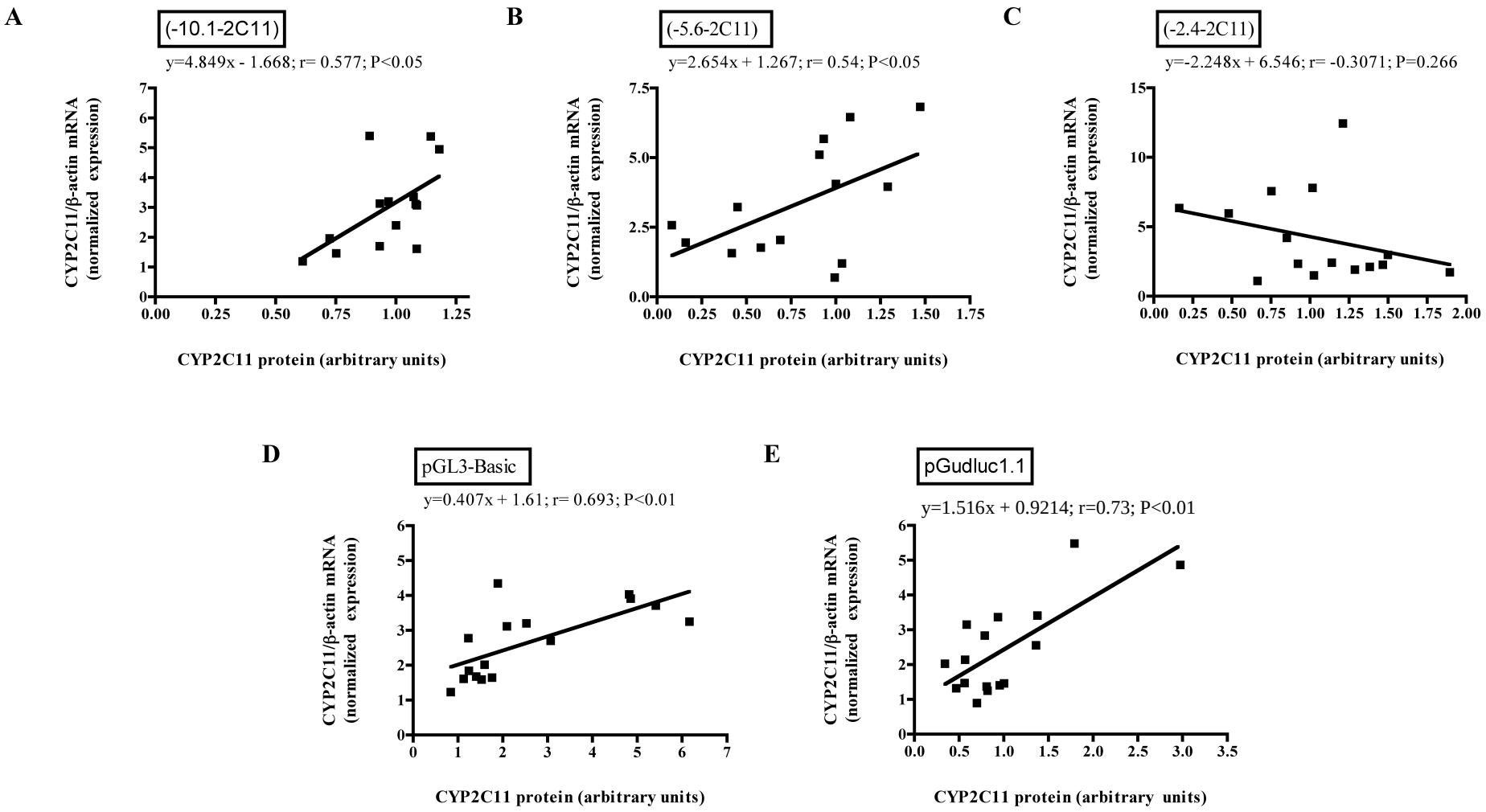


Figure 5