The aryl hydrocarbon receptor pathway and the response to 3-methylcholanthrene are altered in the liver of adrenalectomized rats

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ABBREVIATIONS: ADX, adrenalectomy or adrenalectomized; AHH, aryl hydrocarbon hydroxylase; AHR, aryl hydrocarbon receptor; AHRE, aromatic hydrocarbon response element; ARNT, aryl hydrocarbon receptor nuclear translocator; β-NF, β-naphthoflavone; B[a]P, benzo[a]pyrene; DME, drug-metabolizing enzyme; DEX, dexamethasone; ECL, enhanced chemiluminescence; EROD, 7-ethoxyresorufin O-deethylase; GR, glucocorticoid receptor; MC, 3-methylcholanthrene; P450, cytochrome P450; POR, NADPH-cytochrome P450 oxidoreductase; TAT, tyrosine aminotransferase; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; VEH, vehicle.
Abstract

The aryl hydrocarbon receptor (AHR) is activated by 3-methylcholanthrene (MC), a polycyclic aromatic hydrocarbon, and environmental contaminants, such as 2,3,7,8-tetrachlorodibenzo-\textit{p}-dioxin. Adrenalectomized (ADX) rats have decreased hepatic AHR protein and lower levels of MC-induced CYP1B1 mRNA. To further characterize the effects of decreased AHR protein and the response to MC in ADX rats, we measured AHR-mediated responses in the liver of SHAM and ADX rats, 6 and 54 h after MC treatment. CYP1A2 mRNA was suppressed by 46-60% four days after ADX in vehicle-treated animals. AHR mRNA was induced 4-fold, 6 h after MC in SHAM rats, but no induction was observed in ADX rats. The MC-induced 7-ethoxyresorufin O-deethylase (EROD) activity in ADX rats was 35% of the activity in the MC-treated SHAM group at 6 h. At 54 h post-treatment, the induction of EROD activity by MC was more pronounced in ADX rats compared to 6 h. To assess the overall capacity for hepatic P450-mediated metabolism, we measured NADPH-cytochrome P450 oxidoreductase (POR) activity. POR activity was decreased by 50% following ADX. We have shown that the response to MC in ADX rats is suppressed for some, but not all, AHR-mediated responses and that reduced POR activity following ADX could contribute to a decreased capacity for P450-dependent metabolism. The current study contributes to our understanding of how adrenal-dependent factors modulate the AHR pathway and the response to MC in vivo.
Introduction

The aryl hydrocarbon receptor (AHR) regulates xenobiotic biotransformation via transcriptional control of various drug-metabolizing enzymes (DMEs), including phase I cytochromes P450 (P450) and phase II conjugating enzymes (Mimura and Fujii-Kuriyama, 2003). Many chemicals bind to the AHR, including the environmental contaminant 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), and the model polycyclic aromatic hydrocarbon, 3-methylcholanthrene (MC). These exogenous ligands and their adaptive up-regulation of the CYP1 family of enzymes via the AHR have been well characterized (Denison and Whitlock, 1995). Functions of the AHR in the absence of exogenous ligand have also been reported (Nguyen and Bradfield, 2008). CYP1A1 and CYP1B1 are typically extra-hepatic enzymes with negligible basal hepatic expression, which is inducible to varying degrees following activation of the AHR. On the other hand, hepatic CYP1A2 is expressed constitutively but is also inducible following AHR ligand exposure. Both constitutive and inducible CYP1A2 expression is regulated by the AHR, as shown by reduced basal and inducible CYP1A2 in the liver of Ahr-null mice (Tijet et al., 2006). The activity of P450 enzymes depends on the transfer of electrons from NADPH via NADPH-cytochrome P450 oxidoreductase (POR). In turn, the level of POR can influence the level of P450 activity, as shown in hypophysectomized rats that display decreased microsomal P450 activity that can be restored with the addition of exogenous POR (Waxman et al., 1989).

The AHR is a cytosolic transcription factor that translocates to the nucleus and heterodimerizes with its nuclear partner, the aryl hydrocarbon receptor nuclear translocator (ARNT), upon ligand activation. The regulation of the receptor itself and the functional impact
of changes in the level of the receptor in vivo are not well documented. However, its physiological, adaptive and toxicological importance has been highlighted with evidence from Ahr-null mice showing aberrant hepatic vascular development (Lahvis et al., 2005) and a muted adaptive and toxic response to TCDD exposure (Fernandez-Salguero et al., 1996; Tijet et al., 2006). With respect to regulation of the receptor, the level of the AHR is altered following exposure to aromatic hydrocarbons, with pronounced depletion of AHR protein in cell culture and in rodent liver (Pollenz, 2002), whereas induction of AHR mRNA by AHR ligands in vivo has been noted (Franc et al., 2001). Putative aromatic hydrocarbon response elements (AHREs) have been reported in the 5’-flanking region of the rat AHR gene (Harper et al., 2006).

Few endogenous regulators of AHR expression have been characterized definitively. Glucocorticoids are putative endogenous regulators of AHR expression and function in rat liver. We reported that adrenalectomized (ADX) rats have decreased hepatic AHR protein four days after surgery and lower levels of MC-induced CYP1B1 mRNA relative to SHAM 6 h after treatment (Mullen Grey and Riddick, 2009). There are conflicting reports concerning the effect of ADX on the induction of hepatic DMEs by MC. MC-induced aryl hydrocarbon hydroxylase (AHH) activity (Nebert and Gelboin, 1969) and benzo[a]pyrene (B[a]P) metabolism (Bogdanffy et al., 1982) are decreased in the liver of ADX rats compared to SHAM rats. However, MC-induced 7-ethoxyresorufin O-deethylolation (EROD) activity is nearly identical in the liver of ADX and SHAM rats (Sherratt et al., 1989). Overall, adrenal hormones contribute to the maintenance of hepatic P450 activity in rats. For example, CYP2C11 is a major constitutive P450 found in the liver of male rats and is suppressed by ADX (Murray, 2000). Where MC-induced hepatic enzyme activity is compromised in ADX rats, CYP2C11 is not likely the enzyme responsible because it is not MC-inducible, but is actually suppressed by MC (Jones and
Therefore, the mechanistic basis for differences in MC-induced enzyme activity between SHAM and ADX rats has not been elucidated. In addition to influencing hepatic drug metabolism, adrenal glands express constitutive CYP1B1 and inducible CYP1A1 (Bhattacharyya et al., 1995), and participate in P450-mediated steroidogenesis (Nebert and Russell, 2002).

We set out to study AHR-dependent up-regulation of hepatic DMEs by MC, or the adaptive response (Schmidt and Bradfield, 1996), in ADX and SHAM rats, building on our recent demonstration of decreased hepatic AHR protein in ADX rats (Mullen Grey and Riddick, 2009). The purpose of this in vivo study was to characterize the functional impact of ADX on hepatic expression of POR and its role in P450-mediated metabolism, as well as AHR-dependent constitutive expression of CYP1A2 and the adaptive response to MC. We hypothesized that the lower level of hepatic AHR protein in ADX rats will result in lowered basal CYP1A2 expression and a compromised adaptive response to MC as assessed with multiple AHR target genes.
Materials and Methods

Materials and reagents. Oligonucleotide primers for real-time RT-PCR analysis of AHR, CYP1A1, CYP1A2, CYP1B1 and POR mRNA were commercially synthesized by Integrated DNA Technologies (Coralville, IA). Cartridge-purified oligonucleotide primers for β-actin real-time RT-PCR were commercially synthesized by ACGT Corp. (Toronto, ON, Canada). DNase I and enhanced chemiluminescence (ECL) detection reagent were obtained from GE Healthcare Bio-Sciences Inc. (Baie d’Urfé, QC, Canada). Oligo d(T)15 was purchased from Roche Diagnostics (Laval, QC, Canada). Power SYBR Green Master Mix for real-time RT-PCR was purchased from Applied Biosystems (Foster City, CA). MC (98% purity) was purchased from Aldrich Chemical Co. (Milwaukee, WI). Tri-Reagent, cytochrome c and NADPH were obtained from the Sigma Chemical Co. (St. Louis, MO). RiboLock RNase inhibitor was purchased from MBI Fermentas (Burlington, ON, Canada). A rabbit polyclonal antibody directed against the N-terminal fragment of the AHR encoded by the mouse b-1 allele (amino acid 1-402) was purchased from Enzo Life Sciences International Inc. (Plymouth Meeting, PA). A mouse monoclonal antibody directed against the full-length rat CYP1A2 protein was from Affinity Bioreagents (Golden, CO). A rabbit polyclonal antibody raised against amino acids 1-300 of human POR was obtained from Santa Cruz Biotechnologies (Santa Cruz, CA). A mouse monoclonal antibody directed against rat CYP1A1 (1-31-2) was obtained from Dr. Harry Gelboin (National Cancer Institute, Bethesda, MD).

Animals and treatment. Male Fischer 344 rats were purchased from Charles River Laboratories Canada (St. Constant, QC, Canada). Rats were allowed to acclimatize to housing conditions (two rats per cage, 12-h light/12-h dark cycle with lights on at 7 am, ad libitum access
to Purina Rodent Laboratory Chow No. 5001 and water/saline) and handling for 7 days after arrival in the Division of Comparative Medicine, University of Toronto. Bilateral ADX and SHAM operations were performed at 8 weeks of age by University of Toronto animal surgical technicians. Rats recovered for 4 days following surgery before commencement of the treatment regimen. Following surgery, rats were housed individually in opaque cages to reduce environmental stress. ADX rats received 0.9% sodium chloride in drinking water for the remainder of the study. Body weights were monitored during this time to establish the effectiveness of ADX surgery and to monitor general health status. All animals were cared for in accordance with the principles of the Canadian Council on Animal Care and all animal experimentation was approved by the University of Toronto Animal Care Committee.

To assess the response to aromatic hydrocarbon treatment, groups of four to seven SHAM and ADX rats were treated by gavage with MC (20 mg/kg) or corn oil vehicle at four days after surgery. Liver tissue was collected 6 or 54 h later to quantify expression of several target genes at the mRNA, protein and catalytic activity levels. The liver was processed for RNA isolation and subcellular fractionation as described previously (Timsit et al., 2002). Briefly, each liver was perfused in situ with 50 ml of ice-cold HEGD buffer (25 mM HEPES, 1.5 mM EDTA, 10% glycerol, 1 mM dithiothreitol, pH 7.4), excised and wet weight was recorded.

Approximately 0.1 g portions of tissue from individual livers were frozen in liquid nitrogen and stored at -70°C for subsequent RNA isolation. The remaining liver was homogenized in 4 volumes of HEGD buffer, and cytosol was prepared by differential centrifugation. Several aliquots of liver cytosol and homogenate were frozen and stored in liquid nitrogen until use. Microsomes were isolated by differential centrifugation using 100-150 mg of snap frozen liver tissue homogenized in 2 ml of phosphate-buffered KCl (1.15% KCl, 10 mM potassium
phosphate, pH 7.4). Microsomal pellets were resuspended in 500 µl of storage buffer (10 mM Tris, 20% glycerol, 1 mM EDTA, pH 7.4) and stored at -70°C until use. Cytosolic, microsomal and homogenate protein concentrations were determined by the method of Bradford (1976).

**Immunoblot analysis.** The methods for SDS-polyacrylamide gel electrophoresis, transfer to nitrocellulose membrane, blocking and ECL detection of cytosolic AHR protein are described elsewhere (Mullen Grey and Riddick, 2009). For CYP1A1 detection, 2.5 µg of liver homogenate protein was loaded and the anti-CYP1A1 antibody was used at a 1:3,000 dilution in TNT (20 mM Tris, pH 7.4, 150 mM NaCl, 0.1% Tween-20) containing 5% skim milk powder with thimerasol, followed by incubation with a sheep anti-mouse Ig-horseradish peroxidase conjugate at a 1:3,000 dilution in TNT containing 5% skim milk powder with thimerasol. For CYP1A2 detection, 2.5 µg of liver homogenate protein was loaded and the anti-CYP1A2 antibody was used at a 1:40,000 dilution in TNT containing 5% skim milk powder with thimerasol, followed by incubation with a sheep anti-mouse Ig-horseradish peroxidase conjugate at a 1:10,000 dilution in TNT containing 5% skim milk powder with thimerasol. For POR detection, 2 µg of liver homogenate protein was loaded and the anti-POR antibody was used at a 1:1,000 dilution in TNT containing 5% skim milk powder with thimerasol, followed by incubation with a donkey anti-rabbit Ig-horseradish peroxidase conjugate at a 1:5,000 dilution in TNT containing 5% skim milk powder with thimerasol. Following ECL detection of target proteins, relative quantitation was performed by scanning processed films on a HP Scanjet 3970 scanner (Hewlett-Packard Company, Palo Alto, CA) and using IPLabGel software (Signal Analytics, Vienna, VA). Analysis was performed under optimized conditions for each lot of antibody used; a linear relationship between amount of protein loaded and immunoreactive signal intensity was confirmed.
Analysis of AHR, CYP1A1, CYP1A2, CYP1B1, POR and β-actin mRNA levels by real-time quantitative RT-PCR. The method for RNA isolation and reverse transcription were carried out as described previously (Mullen Grey and Riddick, 2009) unless otherwise indicated. The RNase inhibitor used for the RT step was RiboLock (80 U). Primers for PCR that were previously or newly designed were verified according to up-to-date published sequences for rat AHR mRNA (Genbank accession number NM_013149), rat CYP1A1 mRNA (Genbank accession number NM_012540), rat CYP1A2 mRNA (Genbank accession number NM_012541), rat CYP1B1 mRNA (Genbank accession number NM_12940), rat POR mRNA (Genbank accession number NM_031576), and rat β-actin mRNA (Genbank accession number NM_031144). Samples were analyzed in triplicate using the ABI Prism 7500 Sequence Detection System. Each PCR reaction was prepared to a final volume of 10 μl, including an optimized amount of input cDNA (derived from 1 or 10 ng RNA), optimized final primer concentration (100-300 nM) and 5 μl of 2X Power SYBR Green Master Mix. The primer sequences are listed in Table 1. Primers were designed as described previously (Mullen Grey and Riddick, 2009).

For AHR and β-actin, the comparative threshold cycle (ΔΔCt) relative quantitation method was employed to calculate the level of mRNA by real-time RT-PCR. The efficiencies of the AHR and β-actin real-time PCR reactions were validated as equivalent (data not shown), as required for accuracy by the ΔΔCt method used. Cycling conditions were: initial cycle of 2 min at 50°C and 10 min at 95°C, followed by 40 cycles at 95°C for 15 s, 58°C for 15 s and 72°C for 45 s. Ct values for each sample were normalized to β-actin mRNA (ΔCt), and the calibrator sample (ΔΔCt). Relative fold change (RQ) was calculated as $2^{-\Delta\Delta Ct}$.
For CYP1A1, CYP1A2, CYP1B1, POR and β-actin, the relative standard curve method was used for calculation of the level of mRNA by real-time RT-PCR. Cycling conditions were: initial cycle of 2 min at 50°C and 10 min at 95°C, followed by 40 cycles at 95°C for 15 s, 64°C for 1 min. A standard curve was prepared for each target mRNA and β-actin from MC-treated rat liver cDNA for relative quantitation. Using the relative standard curve method according to Applied Biosystems, the quantity of target and β-actin mRNA was interpolated using the determined Ct value for each sample and the standard curve plotting Ct vs. log RNA input. The target mRNA level for each sample was then normalized to β-actin mRNA, and the fold difference for each sample was expressed relative to the normalized calibrator sample.

For all PCR reactions, regardless of the method of calculation used, a vehicle-treated SHAM sample was used as the calibrator sample on each plate (except for CYP1B1, in which case a MC-treated SHAM sample was used due to low mRNA abundance in vehicle-treated animals). The mean RQ (ΔΔCt) or mean fold difference (relative standard curve) for each treatment group was expressed as a percentage of the 6 h SHAM vehicle-treated group (except for CYP1B1, in which case data were expressed as a percentage of the 54 h SHAM vehicle-treated group). All mRNAs were measured at 6 and 54 h following MC or vehicle treatment in SHAM and ADX rats, corresponding to four or six days after surgery to assess the magnitude of the response to aromatic hydrocarbon exposure following ADX.

**EROD Activity.** EROD activity was assayed at 37°C in 1.25-ml (final volume) incubation mixtures containing 1.5 μM 7-ethoxyresorufin, 1 mM NADPH, hepatic microsomal protein (200 μg for vehicle-treated rats and 20 μg for MC-treated rats) and reaction buffer (0.1 M HEPES, 60 μM EDTA, 5 mM MgSO₄, pH 7.8) (Pohl and Fouts, 1980). Reactions were terminated by the addition of 2.5 ml ice-cold methanol after either 2 min (MC-treated rats) or
4 min (vehicle-treated rats). Resorufin production was monitored fluorometrically (excitation and emission wavelengths of 550 nm and 585 nm, respectively) and quantitation was achieved via comparison to a resorufin calibration curve. The resorufin formation in a blank reaction in which microsomes were omitted was subtracted from each sample. Product formation was linear with respect to protein concentration and incubation time.

**POR enzyme activity.** Microsomes (15 µg protein) were assayed at room temperature in 0.5-ml (final volume) incubation mixtures containing 300 mM potassium phosphate (pH 7.7) and 70 µM cytochrome c. 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 an extinction coefficient of 21 mM⁻¹cm⁻¹ (Strobel and Dignam, 1978).

**Statistical Analysis.** All data are expressed as means ± S.D. 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 three-way ANOVA to identify significant influences of the three independent variables in a given experiment (variable 1 = surgery; variable 2 = MC treatment; variable 3 = time). Post-hoc analysis for the planned comparisons (surgery effect, treatment effect or time effect) was performed to assess whether there were significant differences between particular groups. Post-hoc tests were Bonferroni corrected for multiple comparisons and were performed using the online tool provided by Graphpad Software (www.graphpad.com). In all cases, a result was considered statistically significant if \( p < 0.05 \).
Results

The decrease in cytosolic AHR protein in the liver of ADX rats is sustained 6 days after surgery while the MC-elicited depletion of AHR protein observed 6 h after treatment is reversed at 54 h. We previously reported that ADX decreased the level of AHR protein 4 days after surgery and that the induction of \textit{CYP1B1}, an AHR target gene, by MC was diminished in conjunction with the lowered receptor levels (Mullen Grey and Riddick, 2009). To further characterize the impact of ADX on the AHR and the response to MC, we have measured several AHR target genes and indices of P450-dependent metabolism in ADX rats. We measured cytosolic AHR protein in the liver of SHAM and ADX rats 6 and 54 h following vehicle or MC treatment, corresponding to 4 and 6 days after the surgical intervention. Using immunoblot analysis, the single band observed migrating at approximately 106 kDa represented immunoreactive AHR protein (Fig. 1a). We confirmed our previous finding that the hepatic level of cytosolic AHR protein was decreased 4 days after surgery where vehicle-treated ADX rats had 25% of the level of AHR protein observed in vehicle-treated SHAM rats (Fig. 1b). The decreased level of AHR protein in vehicle-treated ADX rats persisted at 6 days after surgery, although some recovery was noted with levels at 65% of time-matched vehicle-treated SHAM rats. The well-documented depletion of hepatic cytosolic AHR protein following ligand exposure (Pollenz, 2002) was observed in both SHAM and ADX rats 6 h after MC treatment but the levels of AHR protein returned to at least vehicle-treated levels 54 h after MC administration. Measurements of AHR protein in whole liver homogenate yielded similar results to liver cytosol indicating that the depletion by MC was not due only to an alteration of subcellular localization of the AHR (data not shown).
The level of hepatic AHR mRNA is induced in SHAM but not ADX rats 6 h after administration of the AHR agonist MC. Up-regulation of the AHR gene by TCDD in vivo has been reported previously (Franc et al., 2001). To determine whether MC altered the level of AHR mRNA in rat liver and whether the decrease in AHR protein after ADX had an impact on the response to MC, the level of AHR mRNA was measured by real time RT-PCR (Fig. 2). AHR mRNA was induced 4-fold by MC in the liver of SHAM rats at the 6 h time point. No induction by MC was observed in ADX rats at the early time point. At the later time point, the induction by MC was no longer apparent in SHAM rats and no differences were observed between SHAM and ADX rats following vehicle or MC treatment at this time point.

Hepatic CYP1A1 mRNA and protein induction by MC occurs in both SHAM and ADX rats but the statistical significance of the mRNA induction is not sustained at 54 h. CYP1A1 is an AHR target gene commonly used to confirm activation of the AHR following ligand exposure. We used real-time RT-PCR to measure CYP1A1 mRNA following MC treatment (Fig. 3). The level of CYP1A1 mRNA was induced by MC in both SHAM and ADX rats 6 h after treatment by approximately 1200- and 600-fold, respectively. At 54 h, the level of CYP1A1 mRNA in MC-treated SHAM rats returned to vehicle-treated levels. At this later time point, the level of CYP1A1 mRNA in MC-treated ADX rats could be described as intermediate, in that it was not significantly different from the induction observed at 6 h in MC-treated ADX rats or the level observed at 54 h in the vehicle-treated ADX group. CYP1A1 protein was measured in whole liver homogenate using immunoblot analysis with an antibody directed against rat CYP1A1. Representative gel images show a single band migrating at approximately 55 kDa (Fig. 4a). Consistent with its low basal expression in the liver, CYP1A1 protein was not
detectable in homogenate from vehicle-treated rat liver. There were no statistically significant differences between the different MC-treated groups (Fig. 4b). Detectable CYP1A1 protein was observed in all MC-treated SHAM rats at both time points and in all MC-treated ADX rats at 54 h. However, only five of the seven ADX rats had detectable CYP1A1 protein at the 6 h time point after MC treatment.

**Moderate and transient induction of hepatic CYP1A2 mRNA by MC occurs in ADX rats while the basal level of CYP1A2 mRNA is decreased following ADX.** *CYP1A2* is a constitutively expressed gene that is a target for regulation by the AHR under basal conditions and following exposure to exogenous AHR ligands. We measured the level of hepatic CYP1A2 mRNA by real-time RT-PCR (Fig. 5). CYP1A2 mRNA was induced 7-fold in MC-treated ADX rats 6 h after treatment. The level of CYP1A2 mRNA in MC-treated SHAM rats was not significantly different from that observed in MC-treated ADX rats at the early time point. The effect of MC on hepatic CYP1A2 mRNA did not persist at the later time point. We also assessed whether ADX and the accompanying decrease in AHR protein had an impact on the basal level of CYP1A2 mRNA in vehicle-treated rats. Although the data in Fig. 5 showed no significant difference in this regard, we pursued this further by measuring CYP1A2 mRNA levels in an independent set of rats from the “acute study” reported in our previous paper (Mullen Grey and Riddick, 2009) in order to increase our statistical power. The experimental and surgical timing in this previous set of rats was identical to the protocol used in the study presented here but CYP1A2 mRNA levels were not previously measured or reported. The level of hepatic CYP1A2 mRNA in vehicle-treated ADX rats in the “acute study” was 40% of vehicle-treated SHAM rats and this difference was statistically significant (*p* = 0.0399; data not shown).
Similar induction of hepatic CYP1A2 protein occurs in MC-treated SHAM and ADX rats but the basal level of CYP1A2 protein shows a decreasing trend following ADX. CYP1A2 immunoreactive protein was measured at 6 and 54 h post-MC treatment in whole liver homogenate from SHAM and ADX rats and representative gel images showed a single band migrating at approximately 53 kDa (Fig. 6a). Whereas immunoreactive CYP1A1 protein was not detected in vehicle-treated liver homogenate from SHAM or ADX rats, CYP1A2 protein was detectable in the liver homogenate from all vehicle-treated SHAM rats. Vehicle-treated ADX rats had 6% of the level of CYP1A2 protein compared to time-matched vehicle-treated SHAM animals four days after surgery (6 h time point) and 44% at six days after surgery (54 h time point) (Fig. 6b). Three of the six ADX rats at the 6 h time point and one of the four ADX rats at the 54 h time point had undetectable basal CYP1A2 protein levels. These differences between vehicle-treated SHAM and ADX rats for hepatic CYP1A2 protein did not reach statistical significance at either time point. The induction of CYP1A2 protein was observed in all MC-treated SHAM and ADX rats. There were no differences with respect to the level of MC-inducible CYP1A2 protein between SHAM and ADX rats.

**Induction of CYP1B1 mRNA by MC is not sustained 54 h after treatment.** We previously reported that the induction of CYP1B1 mRNA by MC was suppressed by 50% in ADX rats compared to SHAM 6 h after MC treatment (Mullen Grey and Riddick, 2009). Here we report the findings for hepatic CYP1B1 mRNA measured in ADX and SHAM rats 54 h after MC treatment, corresponding to 6 days after surgery (Fig. 7). The induction of CYP1B1 mRNA by MC in SHAM rats was no longer apparent at 54 h. The level of CYP1B1 mRNA in MC-treated ADX rats at 54 h was 5-fold higher than vehicle-treated animals but this difference was not
statistically significant. We attempted to measure CYP1B1 immunoreactive protein in liver homogenate and microsomes by immunoblot analysis. Limited by the relatively low abundance of hepatic CYP1B1 protein, the low dose of MC used (20 mg/kg) and the sensitivity of the two commercially-available antibodies tested, we were not able to reliably detect immunoreactive CYP1B1 protein in either liver homogenate or microsomes from vehicle- or MC-treated rats.

**Induction of hepatic EROD activity by MC is more pronounced at the later time point in ADX rats.** The effect of ADX on the induction of CYP1A1 enzyme activity by MC was assessed in rat liver microsomes using an EROD assay (Fig. 8). At 6 h, EROD activity was induced 10-fold in liver microsomes from MC-treated SHAM rats compared to vehicle-treated rats. The induction by MC in SHAM rats at 54 h was greater, representing a 21-fold induction over the time-matched vehicle-treated SHAM group and a 1.3-fold increase relative to the MC-treated SHAM group at 6 h. The MC-induced EROD activity in ADX rats was 35% of the activity in the MC-treated SHAM group at 6 h. At 54 h post-treatment, the 26-fold induction of EROD activity by MC in ADX rats was more pronounced compared to 6 h. This level of EROD activity represented a 2.5-fold increase over that observed in MC-treated ADX rats at 6 h and was 67% of the level observed in time-matched MC-treated SHAM rats.

**Hepatic POR protein and activity are decreased in ADX rats.** The level of hepatic POR mRNA was measured by real-time RT-PCR in SHAM and ADX rats following vehicle or MC treatment (Fig. 9). POR mRNA levels were not altered by surgery or MC treatment. Hepatic POR immunoreactive protein was measured at 6 and 54 h post-MC treatment in whole liver homogenate from SHAM and ADX rats and representative gel images show a single band
migrating at approximately 78 kDa (Fig. 10a). Treatment with MC did not affect hepatic POR protein in either SHAM or ADX rats at either time point. At the 6 h time point, 4 days after surgery, ADX rats showed a 70% decrease in hepatic POR protein compared to SHAM rats (Fig. 10b), whether treated with vehicle or MC. While this effect appeared to persist at the later time point to a varying degree, the difference in POR protein levels between treatment-matched SHAM and ADX rats was only statistically significant at the early time point. Measurement of microsomal cytochrome c reduction was used to assess the hepatic POR activity of SHAM and ADX rats following vehicle or MC treatment (Fig. 11). Similar to our hepatic POR protein data, POR catalytic activity was decreased by 50% in ADX rats compared to treatment- and time-matched SHAM rats. This difference was statistically significant at both time points and in both vehicle- and MC-treated groups.
We demonstrated that ADX rats differ from their SHAM counterparts in basal hepatic 
POR protein and activity, constitutive hepatic CYP1A2, and some AHR-mediated responses to 
MC treatment. This is an important follow-up to our report showing decreased hepatic AHR 
protein and decreased MC-induced CYP1B1 mRNA at 4 days following ADX (Mullen Grey and 
Riddick, 2009). We report here that the response to MC was altered for AHR mRNA and 
CYP1A1 enzyme activity, where the induction in SHAM rats was absent or decreased in ADX 
rats at the early time point. A delayed response to MC was apparent in ADX rats, whereby 
CYP1A1 enzyme activity was induced to a greater extent at the later time point. A tendency for a 
similar delayed, or more pronounced, response at the later time point in ADX rats was also 
observed for CYP1A1 mRNA and protein, and CYP1B1 mRNA, but these differences were not 
statistically significant. Hepatic POR protein and enzyme activity were also lower in ADX rats, 
suggesting an overall diminished capacity for P450-mediated biotransformation. CYP1A2 
mRNA, basally regulated by the AHR, was also decreased in vehicle-treated ADX rats when 
compared to SHAM.

The auto-regulation of AHR mRNA has been shown in rat liver (Kondraganti et al., 
2005) whereby one dose of MC induced AHR mRNA. Putative AHREs were identified in the 5’-
flanking region of the rat AHR gene (Harper et al., 2006), suggesting that the gene might be a 
transcriptional target of the AHR. We showed that 6 h after a single dose of MC, AHR mRNA 
was induced 4-fold in SHAM rats compared to vehicle; for MC-treated ADX rats, no induction 
was observed. This lack of AHR mRNA induction coincided with a 75% decrease in hepatic 
AHR protein observed in ADX rats four days after surgery. In vehicle-treated ADX rats, the
The constitutive level of hepatic CYP1A2 mRNA was decreased four days after surgery to approximately 40% of vehicle-treated SHAM levels. We hypothesize that the lowered level of hepatic AHR protein contributes to the decreased basal expression of CYP1A2 and diminished induction of AHR mRNA by MC in ADX rats.

A direct contribution of glucocorticoids to the basal regulation of CYP1A2 mRNA cannot be entirely ruled out, as ADX decreased constitutive CYP1A2 expression. However, glucocorticoid regulation of hepatic CYP1A2 mRNA was not observed in ADX rats treated with methylprednisolone (Almon et al., 2005). Dexamethasone (DEX) potentiates CYP1A2 mRNA induction by the AHR ligand β-naphthoflavone (β-NF) in primary rat hepatocytes (Sidhu and Omiecinski, 1995), but not CYP1A2 induction by MC in rat liver in vivo (Sherratt et al., 1989). The contribution of glucocorticoids to CYP1A2 mRNA regulation appears to occur via alteration of AHR levels caused by ADX and perhaps by potentiation of induction in the presence of exogenous AHR ligands, but evidence for a direct effect of glucocorticoids on CYP1A2 mRNA levels is not apparent.

Compared to the AHR mRNA auto-induction response, we did not observe the same trend of diminished induction by MC in ADX rats for CYP1A1 and CYP1A2 mRNA, both transcriptional targets of the AHR. For CYP1A1, this was surprising given that DEX treatment is known to potentiate CYP1A1 mRNA induction by β-NF in rat hepatocytes (Sidhu and Omiecinski, 1995) and CYP1A1 induction by MC in rat liver in vivo (Sherratt et al., 1989). Glucocorticoid response elements identified in intron 1 of the rat CYP1A1 gene are responsible for the glucocorticoid potentiation effect on AHR-mediated induction, but glucocorticoids alone do not influence the regulation of CYP1A1 (Linder et al., 1999). The effects of ADX on glucocorticoid-dependent transcriptional responses also depend on timing of steroid
administration and surgery, as we noted for the glucocorticoid-regulated gene, tyrosine aminotransferase (TAT) (Mullen Grey and Riddick, 2009). Although controversial, a post-transcriptional component to CYP1A2 regulation by MC (Silver and Krauter, 1988) could help explain why the decreased AHR protein levels following ADX did not result in a diminished CYP1A2 mRNA induction response to MC. Our findings demonstrate that endogenous levels of adrenal-dependent factors (e.g. glucocorticoids) can influence AHR target gene regulation. However, the impact that lowered AHR protein levels caused by ADX has on MC responsiveness is target-specific, likely owing to differences in mRNA stabilities and/or the specific regulatory milieu for each gene.

Four days after surgery and 6 h after MC, the differences between SHAM and ADX rats suggest that ADX has important functional impacts on hepatic drug metabolism. The level of hepatic POR activity was decreased in ADX rats relative to treatment-matched SHAM rats and the induction of hepatic CYP1A1 enzyme activity by MC was decreased in ADX rats. For hepatic CYP1A1 protein, the fold-induction by MC relative to vehicle was not reported because immunoreactive protein in vehicle-treated samples was undetectable. The level of hepatic CYP1A1 immunoreactive protein in MC-treated ADX rats was 50% of levels in MC-treated SHAM rats. This difference was not statistically significant but the functional impact was pronounced for the difference in MC-induced CYP1A1 enzyme activity between ADX and SHAM rats. It is likely that the compromised hepatic CYP1A1 enzyme activity in ADX rats at 6 h is partly due to decreased POR activity and to the trend for decreased CYP1A1 protein in MC-treated ADX rats. The level of POR activity influences the capacity for P450-dependent catalysis; for example, hepatic POR activity is decreased in hypophysectomized rats and consequently, P450-dependent catalytic activity is compromised (Waxman et al., 1989).
lowered level of AHR protein in ADX rats could also contribute to the decreased induction of CYP1A1 enzyme activity at 6 h relative to SHAM. Mice with hypomorphic Ahr alleles have decreased AHR protein and function, and TCDD-induced EROD activity is diminished (Walisser et al., 2004). Our results provide further mechanistic insight into previous findings of decreased MC-induced hepatic AHH activity (Nebert and Gelboin, 1969) and decreased MC-induced hepatic B[a]P metabolism (Bogdanffy et al., 1982) in ADX rats.

As mentioned above, certain early responses (6 h) to MC in ADX rats were impaired in comparison to SHAM rats. However, assessments at 54 h indicated that the response to MC was either delayed or more pronounced in ADX rats. While decreased induction of CYP1A1 enzyme activity was observed 6 h after MC in ADX rats, the induction of CYP1A1 enzyme activity by MC at 54 h was more pronounced, despite the decrease in POR activity sustained in ADX rats at this time. Hepatic levels of MC drop to less than 1% of administered dose 14 h after a single treatment (Bresnick et al., 1967). This rapid hepatic clearance of MC is due in large part to biotransformation by P450 enzymes; CYP1A1 and CYP1B1 are primarily responsible for metabolism of MC-like polycyclic aromatic hydrocarbons, following induction (Shimada, 2006). We hypothesize that at the early time point, down-regulation of constitutive P450s (e.g. CYP2C11) (Murray, 2000), lower MC-induced CYP1A1 activity, and lower POR activity, all act to slow the hepatic clearance of MC in ADX rats relative to SHAM rats. Consequently, higher hepatic levels of parent MC may persist in ADX rats leading to activation of AHR protein, present at recovering levels at the 54 h time point. Although not yet confirmed in our experiments, this slower clearance could account for the delayed MC induction profile in ADX rats, as shown for CYP1A1 enzyme activity, with a similar trend observed for CYP1A1 mRNA, CYP1A1 protein and CYP1B1 mRNA.
There is some published evidence that ADX decreases hepatic POR activity (Castro et al., 1970; Sherratt et al., 1989). Cortisone acetate (5 mg/kg/day x 8 days) restores hepatic POR activity in ADX rats (Castro et al., 1970). DEX (10 mg/kg/day x 2 days) increases hepatic POR activity in SHAM and ADX rats (Sherratt et al., 1989) and DEX (10 mg/kg single dose) increases hepatic POR activity in neonatal and adolescent rats (Linder and Prough, 1993). Higher doses of DEX (80 mg/kg) induced hepatic POR mRNA, apparently via mRNA stabilization (Simmons et al., 1987). Such doses of DEX, however, might activate the pregnane X receptor, which is activated by DEX at concentrations higher than those required for glucocorticoid receptor (GR) activation (Zhang et al., 1999). POR mRNA levels were not decreased by ADX under the conditions of this experiment, but since the mRNA for TAT, a prototypical GR-regulated gene, was not decreased by ADX (Mullen Grey and Riddick, 2009), the glucocorticoid regulation of the rat POR gene cannot be excluded. Our preliminary data suggest that low doses of DEX induce rat hepatic POR mRNA (data not shown). We are currently studying the role of the GR in this response.

In conclusion, we have demonstrated that: (1) in ADX rats showing decreased hepatic AHR protein levels, basal hepatic CYP1A2 mRNA, MC-induced AHR mRNA and EROD activity are suppressed; (2) the induction of hepatic CYP1A1 enzyme activity by MC is more pronounced at the later time point in ADX rats; and (3) ADX rats have decreased hepatic POR protein and activity. This study contributes to our understanding of how adrenal-dependent factors modulate the hepatic AHR pathway and the response to MC and related environmental contaminants in vivo.
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Authorship Contributions

Participated in research design: Mullen Grey and Riddick.

Conducted experiments: Mullen Grey and Riddick.

Contributed new reagents or analytic tools: not applicable.

Performed data analysis: Mullen Grey and Riddick.

Wrote or contributed to the writing of the manuscript: Mullen Grey and Riddick.

Other: Riddick acquired funding for the research.
References


Footnotes

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Figure Legends

Fig. 1. Hepatic cytosolic AHR protein in SHAM and ADX rats following treatment with MC or corn oil vehicle (VEH). Liver cytosol was collected 6 h or 54 h after a single gavage treatment administered four days after surgery. Cytosolic protein was then subjected to immunoblot analysis. a, Representative immunoblot analysis of cytosolic protein (30 µg) from each rat using polyclonal AHR antibody, showing two samples from each treatment group. Lines between lanes indicate non-adjacent wells run on the gel. b, Semiquantitative image analysis of AHR protein levels. Results are presented as a percentage of the mean for the SHAM vehicle-treated group at 6 h, and each bar represents the mean ± S.D. of determinations from 4-7 rats. *Significantly different (p < 0.05) from surgery- and time-matched vehicle-control (“treatment effect”); †significantly different (p < 0.05) from treatment- and time-matched SHAM-control (“surgery effect”); ‡significantly different (p < 0.05) from treatment- and surgery-matched 6 h group (“time effect”), based on three-way ANOVA followed by a post-hoc test Bonferroni corrected for multiple comparisons. nd: not detectable.

Fig. 2. Hepatic AHR mRNA levels in SHAM and ADX rats following treatment with MC or corn oil vehicle (VEH), as quantified by real-time RT-PCR. Results are presented as a percentage of the mean for the SHAM vehicle-treated group at 6 h, and each bar represents the mean ± S.D. of determinations from 4-7 rats. *Significantly different (p < 0.05) from SHAM-VEH 6 h group (“treatment effect”); †significantly different (p < 0.05) from SHAM-MC 6 h group (“surgery effect”), based on three-way ANOVA followed by a post-hoc test Bonferroni corrected for multiple comparisons.
FIG. 3. Hepatic CYP1A1 mRNA levels in SHAM and ADX rats following treatment with MC or corn oil vehicle (VEH), as quantified by real-time RT-PCR. Results are presented as a percentage of the mean for the SHAM vehicle-treated group at 6 h, and each bar represents the mean ± S.D. of determinations from 4-7 rats. *Significantly different (p < 0.05) from surgery-matched vehicle-treated 6 h group (“treatment effect”); ‡significantly different (p < 0.05) from SHAM-MC 6 h group (“time effect”), based on three-way ANOVA followed by a post-hoc test Bonferroni corrected for multiple comparisons.

FIG. 4. Hepatic homogenate CYP1A1 protein in SHAM and ADX rats following treatment with MC or corn oil vehicle (VEH). Liver homogenate was collected 6 h or 54 h after a single gavage treatment administered four days after surgery. Homogenate protein was then subjected to immunoblot analysis. a, Representative immunoblot analysis of homogenate protein (2.5 µg) from each rat using monoclonal CYP1A1 antibody, showing two samples from each treatment group. Lines between lanes indicate non-adjacent wells run on the gel. b, Semiquantitative image analysis of CYP1A1 protein levels. Results are presented as a percentage of the mean for the SHAM MC-treated group at 6 h, and each bar represents the mean ± S.D. of determinations from 4-7 rats. No statistically significant differences were detected (p > 0.05) based on three-way ANOVA followed by a post-hoc test Bonferroni corrected for multiple comparisons. nd: not detectable.

FIG. 5. Hepatic CYP1A2 mRNA levels in SHAM and ADX rats following treatment with MC or corn oil vehicle (VEH), as quantified by real-time RT-PCR. Results are presented as a percentage of the mean for the SHAM vehicle-treated group at 6 h, and each bar represents the...
mean ± S.D. of determinations from 4-7 rats. *Significantly different ($p < 0.05$) from ADX-VEH 6 h group (“treatment effect”); ‡significantly different ($p < 0.05$) from ADX-MC 6 h group (“time effect”), based on three-way ANOVA followed by a post-hoc test Bonferroni corrected for multiple comparisons.

FIG. 6. Hepatic homogenate CYP1A2 protein in SHAM and ADX rats following treatment with MC or corn oil vehicle (VEH). Liver homogenate was collected 6 h or 54 h after a single gavage treatment administered four days after surgery. Homogenate protein was then subjected to immunoblot analysis. a, Representative immunoblot analysis of homogenate protein (2.5 µg) from each rat using monoclonal CYP1A2 antibody, showing two samples from each treatment group. Lines between lanes indicate non-adjacent wells run on the gel. b, Semiquantitative image analysis of CYP1A2 protein levels. Results are presented as a percentage of the mean for the SHAM vehicle-treated group at 6 h, and each bar represents the mean ± S.D. of determinations from 4-7 rats. *Significantly different ($p < 0.05$) from surgery- and time-matched vehicle-control (“treatment effect”); ‡significantly different ($p < 0.05$) from ADX-MC 6 h group (“time effect”), based on three-way ANOVA followed by a post-hoc test Bonferroni corrected for multiple comparisons.

FIG. 7. Hepatic CYP1B1 mRNA levels in SHAM and ADX rats at 54 h following treatment with MC or corn oil vehicle (VEH), as quantified by real-time RT-PCR. Results are presented as a percentage of the mean for the SHAM vehicle-treated group at 54 h, and each bar represents the mean ± S.D. of determinations from 4 rats. No statistically significant differences were detected
(p > 0.05) based on three-way ANOVA followed by a post-hoc test Bonferroni corrected for multiple comparisons.

**Fig. 8.** Hepatic CYP1A1 catalytic activity in SHAM and ADX rats following treatment with MC or corn oil vehicle (VEH), as assessed by microsomal EROD activity. Each bar represents the mean ± S.D. of determinations from 4-7 rats. *Significantly different (p < 0.05) from surgery-and time-matched vehicle-control (“treatment effect”); †significantly different (p < 0.05) from time-matched SHAM-MC group (“surgery effect”); ‡significantly different (p < 0.05) from surgery-and treatment-matched 6 h group (“time effect”), based on three-way ANOVA followed by a post-hoc test Bonferroni corrected for multiple comparisons.

**Fig. 9.** Hepatic POR mRNA levels in SHAM and ADX rats following treatment with MC or corn oil vehicle (VEH), as quantified by real-time RT-PCR. Results are presented as a percentage of the mean for the SHAM vehicle-treated group at 6 h, and each bar represents the mean ± S.D. of determinations from 4-7 rats. No statistically significant differences were detected (p > 0.05) based on three-way ANOVA followed by a post-hoc test Bonferroni corrected for multiple comparisons.

**Fig. 10.** Hepatic homogenate POR protein in SHAM and ADX rats following treatment with MC or corn oil vehicle (VEH). Liver homogenate was collected 6 h or 54 h after a single gavage treatment administered four days after surgery. Homogenate protein was then subjected to immunoblot analysis. a, Representative immunoblot analysis of homogenate protein (2 µg) from each rat using polyclonal POR antibody, showing two samples from each treatment group. Lines
between lanes indicate non-adjacent wells run on the gel. b, Semiquantitative image analysis of POR protein levels. Results are presented as a percentage of the mean for the SHAM vehicle-treated group at 6 h, and each bar represents the mean ± S.D. of determinations from 4-7 rats. †Significantly different (p < 0.05) from treatment-matched SHAM 6 h group (“surgery effect”), based on three-way ANOVA followed by a post-hoc test Bonferroni corrected for multiple comparisons.

**FIG. 11.** Hepatic POR catalytic activity in SHAM and ADX rats following treatment with MC or corn oil vehicle (VEH), as assessed by microsomal cytochrome c reduction. Each bar represents the mean ± S.D. of determinations from 4-7 rats. †Significantly different (p < 0.05) from treatment- and time-matched SHAM-control (“surgery effect”), based on three-way ANOVA followed by a post-hoc test Bonferroni corrected for multiple comparisons.
### TABLE 1

**Primer sequences for target genes measured at the mRNA level by real-time quantitative RT-PCR**

<table>
<thead>
<tr>
<th>Target</th>
<th>Forward and Reverse PCR Primer Sequences</th>
<th>PCR product size (bp)</th>
<th>Reference</th>
</tr>
</thead>
</table>
| AHR    | 5’-GCTGTGATGCCAAGGGCAGCT-3’  
5’-TGAAGCATGTCAGGGCCTG-3’ | 100                   | New Design |
| CYP1A1 | 5’-GAATGCCAATGTCCAGCTCTCA-3’  
5’-TACCAGGTACATGAGGCTCAAA-3’ | 115                   | Tijet et al., 2006 |
| CYP1A2 | 5’-CGAGGGACACCTCACTGAAT-3’  
5’-TCCACTGCTCTCATACTGTCTTT-3’ | 93                    | New design |
| CYP1B1 | 5’-CTCATCCTCTCTTACCAGATACTCCG-3’  
5’-GCCAGGTATGTCAGGGTGGGTTC-3’ | 117                   | Vondracek et al., 2006 |
| POR   | 5’-GCCTGCCTGAGATCGACAAG-3’  
5’-GGTGGGCCCTCTCCGTATG-3’ | 64                    | Muguruma et al., 2006 |
| β-actin | 5’-GACCCAGATCATGTGTTGGAGACCT-3’  
5’-GGACTCCATCATGATGGAAGG-3’ | 109                   | Tijet et al., 2006 |

*The POR primer sequences were originally designed based on the mouse cDNA sequence. The underlined and bolded base was changed to match the rat cDNA sequence.*
Figure 1

(a) Relative AHR Immunoreactivity (% 6h SHAM-VEH control)

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AHR ~106kDa

(b) Relative AHR Immunoreactivity (% 6h SHAM-VEH control)

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<tr>
<td></td>
<td>VEH</td>
<td>MC</td>
<td>VEH</td>
<td>MC</td>
</tr>
</tbody>
</table>

Figure 1
Figure 2

AHR mRNA / β-actin
(% 6h SHAM-VEH control)
Figure 3

CYP1A1 mRNA / β-actin
(% 6h SHAM-VEH control)
Figure 4

Relative CYP1A1 Immunoreactivity (% 6h SHAM-MC group)

SHAM 6h VEH MC
ADX 6h VEH MC
SHAM 54h VEH MC
ADX 54h VEH MC

CYP1A1 ~55 kDa
Figure 5

CYP1A2 mRNA / β-actin (% 6h SHAM-VEH control)

VEH
MC

SHAM  ADX  SHAM  ADX
6h  54h
Figure 6

Relative CYP1A2 Immunoreactivity (% 6h SHAM-VEH control)

- **CYP1A2 ~53 kDa**

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<th>54h SHAM</th>
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* * *

Figure 6
CYP1B1 mRNA / β-actin
(% SHAM-VEH control)
**Figure 8**

EROD activity (nmol / min / mg protein)

- **SHAM**
  - 6h
  - 54h

- **ADX**
  - 6h
  - 54h

- **MC**
  - Open bars

- **VEH**
  - Solid bars

* denotes significant difference from SHAM.
† denotes significant difference from ADX.
‡ denotes significant difference between 6h and 54h within the same group.

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Figure 9

POR mRNA / β-actin
(% 6h SHAM-VEH control)
Figure 10

(a) Relative POR Immunoreactivity (% 6h SHAM-VEH control)

(b) POR Immunoreactivity (6h SHAM-VEH control)

VEH vs MC

Figure 10
Figure 11

POR activity (nmol / min / mg protein)

- SHAM 6h
- SHAM 54h
- ADX
- ADX

MC VEH

† † ††