Glucocorticoid and adrenalectomy effects on the rat aryl hydrocarbon receptor pathway depend on the dosing regimen and post-surgical time

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Abstract

The aryl hydrocarbon receptor (AHR) is a ligand-activated transcription factor that mediates the effects of aromatic hydrocarbons, such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin and 3-methylcholanthrene (MC); the prototypical response is induction of drug-metabolizing enzymes. Factors that regulate AHR levels *in vivo* are poorly understood and it is also not clear how AHR levels affect aromatic hydrocarbon responsiveness. Our interest in pituitary-dependent regulation of AHR levels was prompted by two findings from our laboratory: (1) hypophysectomized rats have reduced hepatic levels of AHR protein; and (2) glucocorticoids increase AHR expression and aromatic hydrocarbon responsiveness in rodent hepatoma cells. To study whether adrenalectomy and glucocorticoids contribute to hormone-dependent regulation of the hepatic AHR pathway, male adrenalectomized (ADX) or SHAM-ADX rats were treated with dexamethasone (DEX) or vehicle. AHR protein was depleted by 50-60% at four days after ADX, but was not altered by DEX treatment. To assess whether the observed AHR depletion affected aromatic hydrocarbon responsiveness, the induction of hepatic cytochrome P450 1B1 (CYP1B1) mRNA by MC was measured as an AHR-mediated adaptive response. MC-induced hepatic CYP1B1 mRNA was reduced by 50% in ADX rats relative to SHAM-ADX. Exogenous glucocorticoid treatment (DEX – 1.5 mg/kg) induced hepatic AHR nuclear translocator (ARNT) mRNA by up to 9-fold at 3 and 6 h after dosing, with no corresponding change in ARNT protein levels. These data demonstrate that: (1) adrenal-dependent factors contribute to the physiological maintenance of hepatic AHR protein levels; (2) the depletion of hepatic AHR protein in ADX rats coincided with a diminished adaptive response to MC; and (3) exogenous glucocorticoid treatment increases hepatic ARNT mRNA levels regardless of adrenal status. This model is useful for studying the mechanisms of AHR and ARNT regulation and for further characterization of the impact of AHR protein depletion on the response to aromatic hydrocarbons *in vivo*.

Keywords: aryl hydrocarbon receptor, adrenalectomy, aryl hydrocarbon receptor nuclear translocator, glucocorticoids, 3-methylcholanthrene, cytochrome P450 1B1

1. Introduction

Exogenous aromatic hydrocarbons bind to and activate the aryl hydrocarbon receptor (AHR). These ligands include 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), a halogenated aromatic hydrocarbon, and 3-methylcholanthrene (MC), a non-halogenated polycyclic aromatic hydrocarbon (PAH) [1]. The AHR is the chemical sensor and mediator of the majority of the toxic effects of aromatic hydrocarbons. The importance of the AHR in the effects of aromatic hydrocarbons was exemplified by observations that *Ahr*-null mice do not show the characteristic toxicities associated with TCDD exposure and the vast majority of TCDD-induced hepatic transcriptional changes are lost in *Ahr*-null mice [2].

The AHR is a cytosolic, ligand-activated transcription factor. Ligand binding elicits activation of the AHR complex and translocation to the nucleus, followed by heterodimerization of the liganded AHR with its nuclear partner, the AHR nuclear translocator (ARNT). The AHR•ARNT heterodimer binds to specific regulatory elements on DNA, termed aromatic hydrocarbon response elements (AHREs) contained within the regulatory regions of aromatic hydrocarbon-responsive genes. The best-documented aromatic hydrocarbon-responsive genes encode drug-metabolizing enzymes (DMEs). Particularly well-studied are the members of the *CYP1* family, *CYP1A1*, *1A2* and *1B1* [3], and several phase II conjugating enzymes [4].

The AHR is ubiquitously expressed, but the levels of AHR mRNA and protein vary between tissues. Highest mRNA expression is observed in lung, liver, kidney, placenta and thymus, while lower expression is observed in the heart [5]. Thymus and liver are highly sensitive to effects of TCDD [6], which might be influenced by the relatively high level of the AHR in these tissues. Since the level of AHR varies in a tissue-selective manner, AHR levels are likely regulated by endogenous factors.

Hormones are potential endogenous regulators of AHR expression levels and AHR function. AHR regulation by pituitary hormones was observed by Burke et al. [7]. They observed that hepatic CYP1A-catalyzed enzymatic reactions induced by MC were attenuated in hypophysectomized (HYPX) rats. We previously observed that HYPX significantly reduced hepatic AHR protein and ligand-binding in male rats [8]. It is not apparent which pituitary-dependent factors that were depleted with HYPX are responsible for the reduced hepatic AHR protein and ligand-binding.

We set out to identify plausible pituitary-dependent factors involved in regulation of hepatic AHR levels. Adrenal glucocorticoids are pituitary-dependent hormones that have been shown to modulate responses to aromatic hydrocarbons. For example, physiological stress in rats involves an elevation of plasma corticosterone (CORT), the predominant rodent glucocorticoid. In rats under stress, there was enhanced hepatic ethoxyresorufin-O-deethylation (EROD) activity following PAH administration, a measure of CYP1A1 activity [9]. In cultured rodent cells, glucocorticoids potentiate CYP1A1 inducibility by aromatic hydrocarbons [10-12]. Glucocorticoid potentiation of PAH-induced CYP1A1 was also observed in rats [13, 14], whereas glucocorticoids alone had no effect on CYP1A1 protein level or EROD activity. There is a functional glucocorticoid response element (GRE) in intron I of the *CYP1A1* gene [15], which contributes in part to the glucocorticoid-potentiated PAH-induced CYP1A1 activity. The GRE in intron I is conserved between mouse, rat and human *CYP1A1* genes. However, this glucocorticoid-mediated potentiation was observed only in rodent hepatoma cells/hepatocytes and in rat liver, while induction of CYP1A1 protein [16] and EROD activity [17] by MC was *inhibited* by glucocorticoids in human hepatocytes. The disparity between the human and rodent responses indicates that the presence of the GRE in intron I does not alone confer the potentiation effect of glucocorticoids on CYP1A1 induction by PAHs. Most recently, a bi-directional cross-talk between the AHR and the glucocorticoid receptor (GR) has been observed in HO23 human ovarian granulosa cells, in which PAHs enhance glucocorticoid responsiveness and glucocorticoids inhibit PAH responsiveness [18]. This recent paper concludes with the suggestion that *in vivo* animal studies will be needed to investigate the physiological significance of this *in vitro* crosstalk, an issue addressed in the present study.

In addition to the evidence above that implicates glucocorticoids in the response to aromatic hydrocarbons, glucocorticoids are also putative regulators of the level of hepatic AHR expression. Glucocorticoids increased AHR levels and enhanced expression of AHR target genes in H4IIE rat hepatoma cells [12, 19]. Glucocorticoids repressed AHR mRNA levels in human hepatocytes [17] and in rat mammary fibroblasts [20], indicating a possible cell-specific response. In HepG2 human hepatoma cells, dexamethasone (DEX) reduced AHR mRNA levels [18, 21] and TCDD-induced EROD activity, with no effect on AHR protein [21]. Once again, these studies suggest that the positive effect of glucocorticoids on the AHR system occurs in rodent, but not human, liver. Recently we found that DEX increased the level of AHR mRNA,

protein and ligand-binding in Hepa-1 mouse hepatoma cells. A functional impact of the altered level of AHR was observed as DEX pre-treatment enhanced induction of an AHR-activated luciferase reporter by TCDD and MC in Hepa-1 cells [22]. With respect to *in vivo* evidence regarding the glucocorticoid effect on AHR expression, Abbott et al. [23] showed that hydrocortisone increased AHR mRNA and protein in the embryonic mouse palate. A previous study in adrenalectomized (ADX) rats one week after surgery showed no alteration of AHR ligand-binding as measured by isoelectric focusing [24]. The evidence demonstrating a role for glucocorticoids in the positive modulation of the AHR system in cell culture has not been experimentally verified in the adult rodent liver.

Our objective was to study whether removal of the endogenous source of glucocorticoids by ADX, combined with exogenous glucocorticoid treatment, affects the level of AHR in rat liver. Our hypothesis is that ADX rats have lower AHR protein and ligand-binding, as observed in liver of HYPX rats. Our second hypothesis is that glucocorticoid treatment in ADX rats restores the level of AHR protein and ligand-binding to normal levels. In addition to the study of the effects of ADX and glucocorticoids on the level of the AHR, we investigated the functional impact of ADX on the AHR-dependent adaptive response to MC. We hypothesize that a lower level of AHR will result in a reduced adaptive response to MC.

This study documents the *in vivo* contribution of adrenal-dependent endogenous factors to the regulation of the level of AHR mRNA, protein and ligand-binding in rodent liver. We also provide a novel example of how altered AHR levels affect the response to MC *in vivo*. This finding provides mechanistic insight into the *in vivo* functional consequences of altered hepatic AHR protein levels. Glucocorticoid levels fluctuate under different physiological and pathophysiological conditions, and are altered via pharmacological intervention. Knowing the conditions under which AHR levels are altered and whether this alteration results in functional consequences will provide a better understanding of the factors that contribute to the sensitivity to aromatic hydrocarbon exposure and the associated toxicities.

2. Materials and Methods

2.1. Source of Chemicals. [^3H]TCDD (24.8 Ci/mmol; radiochemical purity > 97%) was purchased from Chemsyn Science Laboratories (Lenexa, KS) and 2,3,7,8-tetrachlorodibenzo-furan (TCDF) was obtained from Wellington Laboratories Inc. (Guelph, ON). Oligonucleotide primers for semiquantitative RT-PCR analysis of AHR, ARNT, tyrosine aminotransferase (TAT) and β -actin mRNA and CYP1B1 primers for real-time RT-PCR were commercially synthesized by Integrated DNA Technologies (Coralville, IA). Cartridge-purified oligonucleotide primers for ARNT, TAT and β -actin real-time RT-PCR were commercially synthesized by ACGT Corp. (Toronto, ON). Vistra Green nucleic acid stain, DNase I, RNAGuard and enhanced chemiluminescence (ECL) detection reagent were obtained from GE Healthcare Bio-Sciences Inc. (Baie d'Urfé, QC). Oligo d(T)₁₅ was purchased from Roche Diagnostics (Laval, QC). *Taq* DNA polymerase, MMLV reverse transcriptase and dNTPs for semiquantitative RT-PCR were purchased from Invitrogen Corporation (Carlsbad, CA). 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). DEX and Tri-Reagent were obtained from the Sigma Chemical Co. (St. Louis, MO). Biogel HTP hydroxyapatite (HAP) was purchased from Bio-Rad Laboratories (Hercules, CA). 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 Biomol Research Laboratories Inc. (Plymouth Meeting, PA) [25]. A rabbit polyclonal antibody directed against a C-terminal fragment of human ARNT (amino acid 496-789) was from Novus Biologicals Inc. (Littleton, CO).

2.2. Animals and treatment. Male Fischer 344 rats were purchased from Charles River Laboratories Canada (St. Constant, QC). Rats were allowed to acclimatize to housing conditions (2 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 (acute) or 13 (subacute) 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.

The subacute protocol involved groups of six ADX and SHAM male rats treated with DEX (1 mg/kg) or corn oil vehicle by intraperitoneal injection daily at 3 pm for 7 days. On day 8 of the treatment regimen, rats were euthanized by decapitation at 10 am. For the acute protocol, groups of four or five ADX and SHAM male rats were treated with DEX (1.5 mg/kg) or corn oil vehicle by intraperitoneal injection once, 6 h before euthanasia. Visual examination to confirm removal of adrenal glands was done by two observers for both studies. For the time course study, conditions of the acute study were replicated, but groups of four or five DEX- or vehicle-treated intact rats were euthanized 3, 6, 12 or 27 h after dosing to assess the time-dependent effects of DEX on ARNT and TAT mRNA and ARNT protein. To assess the response to aromatic hydrocarbon treatment, groups of four to seven SHAM and ADX rats were treated by gavage with 20 mg/kg MC or corn oil vehicle at four days after surgery. Liver tissue was collected 6 h later to quantify CYP1B1 mRNA as an index of the AHR-dependent adaptive response.

For all studies, the liver was processed for RNA and cytosol preparation as described previously [8]. 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. Approximately 10 g of each 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. Cytosolic and homogenate protein concentrations were determined by the method of Bradford [26].

2.3. AHR radioligand binding by HAP analysis. HAP analysis to measure the number of TCDD binding sites in rat liver cytosol was performed as described previously [27] with minor modifications described below. Briefly, rat liver cytosol (0.3 ml, ~2 mg protein/ml) was incubated with 2 nM [³H]TCDD with or without the addition of 100-fold molar excess of non-

radioactive TCDF for 2 h at room temperature in a shaking water bath. TCDD and TCDF were added to cytosol in dimethylsulfoxide (DMSO) in 3- μ l volumes. After incubation, 0.2 ml of incubated sample was added to 0.25 ml HAP in HEGD buffer (0.5 g/ml) and incubated for 30 min on ice. The centrifuged samples were then washed three times in HEG buffer with 0.5% (v/v) Tween 80. The remaining radioactivity of each centrifuged sample was then measured by liquid scintillation spectrometry. The number of TCDD binding sites (specific binding) was determined by subtracting the radioactivity in [3 H]TCDD + TCDF (non-specific binding) tubes from that in the [3 H]TCDD + DMSO (total binding) tubes.

2.4. Immunoblot analysis. Cytosolic protein (25-40 μ g) from each rat liver was resolved by SDS-polyacrylamide gel electrophoresis [28] and transferred to nitrocellulose membrane (Hybond-ECL, GE Healthcare Bio-Sciences) for AHR or ARNT detection. Antibody conditions for AHR immunoblotting were optimized previously [22] and confirmed for rat liver cytosol. The anti-AHR antibody was used at a 1:20,000 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 donkey anti-rabbit Ig-horseradish peroxidase conjugate at a 1:5,000 dilution in TNT containing 5% skim milk powder with thimerasol. The anti-ARNT antibody was used at a 1:2,000 (acute and subacute) or 1:3,000 (time course) dilution in TNT containing 5% skim milk powder with thimerasol, followed by incubation with a donkey anti-rabbit Ig-horseradish peroxidase conjugate at a 1:1,000 (acute and subacute) or 1:2,000 (time course) dilution in TNT containing 5% skim milk powder with thimerasol. ECL detection was then performed and the blots were exposed to Bioflex imaging film. Films were processed using a SRX-101A medical film processor (Konica Minolta, Wayne, NJ). Relative quantitation was performed by scanning processed film on an 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.

2.5. Analysis of AHR, ARNT, TAT and β -actin mRNA levels by semiquantitative RT-PCR. Total RNA was isolated from liver tissue by the acid guanidinium thiocyanate-phenol-chloroform extraction method [29] using Tri-Reagent. RNA samples were then treated with 20 U DNase I at

37°C for 25 min to remove genomic DNA contamination. RNA yield and purity were assessed by determining the A_{260}/A_{280} ratio (> 1.7), and RNA integrity was assessed by comparing the relative intensities of the 28S and 18S rRNA bands as visualized on an ethidium bromide-stained agarose gel. RT-PCR was performed according to optimized conditions for annealing temperature, cycle number and cDNA input. The RT step was carried out as described previously [30]. Briefly, total RNA (1 μ g) was incubated with oligo d(T)₁₅ (2 μ g) at 60°C for 5 min. Primer-annealed samples were then incubated in a final volume of 40 μ l with MMLV-reverse transcriptase (400 U), RNA Guard (60 U), 1 mM of each dNTP, 10 mM dithiothreitol, and RT buffer containing 50 mM Tris, 75 mM KCl, and 3 mM MgCl₂. Reactions were allowed to proceed for 60 min at 37°C, followed by incubation at 70°C for 10 min. Primers for PCR that were previously designed were verified according to up-to-date published sequences for rat AHR mRNA (Genbank accession number NM_013149), rat ARNT mRNA (Genbank accession number NM_012780), rat TAT mRNA (Genbank accession number NM_012668), and rat β -actin mRNA (Genbank accession number NM_031144). Corresponding primer sequences and cycling conditions are listed in Table 1. Each 50 μ l quadruplex PCR reaction contained input cDNA derived from 50 ng RNA, *Taq* polymerase (2.5 U; Invitrogen), an appropriate concentration of each primer (0.08 or 0.4 μ M), a 1.6 mM concentration of each dNTP, and 1X PCR buffer containing 20 mM Tris, 50 mM KCl and 3 mM MgCl₂. PCR products were separated on 6% non-denaturing polyacrylamide gels. Gels were stained with Vistra Green and fluorescence was detected using a STORM 860 blue fluorescence scanner (Molecular Dynamics, Sunnyvale, CA). Relative quantitation was performed using IPLabGel software. AHR, ARNT and TAT signals were normalized to the internal reference standard, β -actin. PCR cycling conditions (input cDNA, cycle number) were optimized to yield product within the exponential range of amplification for multiplex PCR.

2.6. Analysis of ARNT, TAT, CYP1B1 and β -actin mRNA levels by real-time quantitative RT-PCR. For all genes measured by real-time quantitative RT-PCR, RNA isolation and reverse transcription were as described above. Samples were analyzed in triplicate using the ABI Prism 7500 Sequence Detection System. To confirm the time course of ARNT and TAT mRNA induction by DEX, real-time quantitative RT-PCR was performed on cDNA from intact rats euthanized 3, 6, 12 or 27 h after a single DEX dose (n=4-5 per group). The efficiencies of the

ARNT, TAT and β -actin real-time PCR reactions were validated as equivalent (data not shown), as required for accuracy by the comparative threshold cycle (Ct) relative quantitation method used. Each PCR reaction was prepared to a final volume of 25 μ l, including input cDNA derived from 25 ng RNA, 100-200 nM final primer concentration and 12.5 μ l of 2X Power SYBR Green Master Mix. Primer sequences and final optimized concentrations are listed in Table 2. 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. A 3 h vehicle-treated sample was used as the calibrator sample and β -actin mRNA was used as the endogenous control for each sample measured. Ct values for each sample were normalized to β -actin mRNA (Δ Ct), and the calibrator sample ($\Delta\Delta$ Ct). Relative fold change (RQ) was calculated as $2^{-\Delta\Delta Ct}$ and the mean RQ for each treatment group was expressed as a percentage of the 3 h vehicle-treated group.

CYP1B1 mRNA was measured at 6 h following MC or vehicle treatment in SHAM and ADX rats four days after surgery to assess the magnitude of the response to aromatic hydrocarbon exposure following ADX. Due to the low abundance of CYP1B1 mRNA in vehicle-treated rat liver, a standard curve was prepared for CYP1B1 and β -actin from MC-treated rat liver cDNA for relative quantitation. Each PCR reaction was prepared to a final volume of 10 μ l, including input cDNA derived from 1 ng RNA, 200 nM final primer concentration and 5 μ l of 2X Power SYBR Green Master Mix. The primer sequences and final optimized concentrations are listed in Table 2. Cycling conditions were: initial cycle of 10 min at 95°C, followed by 40 cycles at 95°C for 15 s, 64°C for 1 min. A vehicle-treated SHAM sample was used as the calibrator sample and β -actin mRNA was used as the endogenous control for each sample measured. 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 CYP1B1 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. The mean fold difference for each treatment group was expressed as a percentage of the vehicle-treated SHAM group.

Forward primers were manually designed so that they spanned an intron-exon boundary and reverse primers were designed using amplicon size specifications and forward primer sequence using Primer3 software [31]. The possibility of secondary structure formation was

checked using Integrated DNA Technologies, Inc. Biotoools (www.idtdna.com). The specificity of primers against the rat genome was confirmed by BLAST search (www.ncbi.nlm.nih.gov/BLAST/) and by Primer-Unigene Selectivity (PUNS) analysis [32].

2.7. Statistical Analysis. All data are expressed as means $\pm$ 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 two-way ANOVA to identify significant influences of the two independent variables in a given experiment (variable 1 = surgery or time; variable 2 = drug treatment). If a significant effect of surgery was identified, a parametric Student's *t*-test or a non-parametric Mann-Whitney test (as appropriate) was performed to compare ADX vs. SHAM groups. If a significant effect of time was identified, a parametric randomized design one-way ANOVA followed by post-hoc Newman-Keuls test or a non-parametric Kruskal-Wallis test followed by post-hoc Dunn's multiple comparisons test (as appropriate) was performed to identify time points that differed from each other. If a significant effect of drug treatment (DEX or MC) was identified, a parametric Student's *t*-test or a non-parametric Mann-Whitney test (as appropriate) was performed to compare drug-treated vs. vehicle-treated groups. In all cases, a result was considered statistically significant if $p < 0.05$.

3. Results

3.1. *A single dose of DEX induces hepatic ARNT and TAT mRNA in SHAM and ADX rats*

The levels of AHR, ARNT and TAT mRNAs were assessed by RT-PCR in ADX rats treated with one dose of DEX, 6 h before euthanasia (acute) or with a daily dose of DEX for seven consecutive days (subacute) (Fig. 1). The level of AHR mRNA was unchanged by ADX or by DEX in either the acute or subacute protocol. While ARNT mRNA was unchanged in the subacute protocol, expression of the established glucocorticoid-inducible gene *TAT* also was unchanged. To assess the effect of DEX on gene expression under conditions in which *TAT* was induced, the acute protocol was used, where 2-fold *TAT* induction was observed at 6 h after DEX treatment. Under these conditions, the level of ARNT mRNA was significantly induced by 3 to 4-fold at 6 h post-dosing in both SHAM and ADX rats. ADX did not alter ARNT or *TAT* mRNA levels following either the acute or subacute protocol.

3.2. *Hepatic AHR protein is decreased in ADX rats four days after surgery*

AHR protein was measured in hepatic cytosol from SHAM and ADX rats treated with either vehicle or DEX by immunoblotting using a rabbit polyclonal antibody generated against the N-terminal region of the mouse AHR protein that has cross-reactivity to the rat protein. Representative protein samples from each treatment group are shown in Fig. 2A, where the single band observed represents immunoreactive AHR protein migrating at approximately 106 kDa. Relative quantitation of immunoreactive AHR protein (Fig. 2B) demonstrated that there were no differences in the level of AHR protein at three weeks post-surgery, whereas the level of AHR protein four days post-ADX was reduced by 50-60% in livers from both vehicle- and DEX-treated rats. DEX treatment did not alter the level of AHR protein in SHAM or ADX rat liver following either the acute or subacute protocol. The molecular weight of the observed immunoreactive AHR does not appear to be altered by ADX or DEX. Measurements of AHR protein in whole liver homogenate yielded similar results to liver cytosol (data not shown).

3.3. *ADX and DEX do not affect ARNT protein level*

Given the 3- to 4-fold induction of ARNT mRNA by DEX at 6 h post-dosing in the acute protocol, we measured the level of ARNT protein in hepatic cytosol from SHAM and ADX rats

treated with either vehicle or DEX (Fig. 3). ARNT protein was measured in hepatic cytosol by immunoblotting using a rabbit polyclonal antibody generated against the C-terminal region of the human ARNT protein that has cross-reactivity to the rat protein. Representative rat liver cytosol samples from each treatment group are shown in Fig. 3A, where the most abundant band observed represents immunoreactive ARNT protein migrating at approximately 88 kDa that corresponds to an abundant band in Hepa-1 cytosol run on each gel at approximately 87 kDa (data not shown). Although ARNT mRNA was induced by DEX in the acute study, there was no observed change in the level of ARNT protein at 6 h post-dosing in SHAM or ADX rat liver cytosol. Similar to what was observed with ARNT mRNA with repeated DEX dosing three weeks after ADX, there was no effect of ADX or DEX on ARNT protein in the subacute protocol. Since ARNT is a nuclear protein that redistributes to the cytosol during subcellular fractionation of liver under basal conditions, it is important to point out that the measurements of ARNT protein in whole liver homogenate yielded similar results to liver cytosol (data not shown).

3.4. Hepatic AHR ligand-binding is decreased in vehicle-treated ADX rats four days after surgery

The level of TCDD binding sites was determined using a HAP assay. A saturating concentration of [³H]TCDD (2 nM) was used to measure AHR ligand-binding in hepatic cytosol from each individual rat. The average level of [³H]TCDD binding in SHAM-vehicle-treated rats was 71.4 ± 5.7 fmol/mg protein (subacute) or 77.7 ± 25.0 fmol/mg protein (acute) (Fig. 4). This level of TCDD binding sites corresponds to reported values measured by HAP assay in rat liver cytosol of 20-140 fmol/mg protein [27, 33, 34]. In the acute study, the number of TCDD binding sites was reduced in vehicle-treated ADX rats relative to SHAM. In DEX-treated ADX rats, the number of TCDD binding sites was also reduced relative to SHAM, but did not achieve statistical significance. In the subacute study, the level of TCDD binding sites was similar to the findings for the concentration of AHR protein, in that there was no effect of ADX or DEX.

3.5. *The marked induction of ARNT mRNA by DEX occurs rapidly with no apparent change in ARNT protein levels*

To assess the time course of ARNT mRNA induction by DEX (acute study) and whether there was a change in the level of ARNT protein at a time point before or after the 6 h measurement previously employed, intact rats received a single dose of DEX (1.5 mg/kg) followed by euthanasia at 3, 6, 12 or 27 h. Levels of ARNT and TAT mRNA were measured using real-time RT-PCR and the results are shown in Fig. 5. Corresponding to what was previously observed in the acute study, ARNT mRNA is significantly induced 8.7-fold by DEX treatment at 6 h post-dosing, and a 7.1-fold induction also was observed at 3 h post-dosing; induction was not sustained 12 or 27 h post-dosing. With respect to TAT mRNA, maximal 8.7-fold induction by DEX was seen at 6 h post-dosing, which corresponds to previous published reports [35, 36] and confirms glucocorticoid responsiveness.

The level of AHR (Fig. 6) and ARNT (Fig. 7) protein was measured by immunoblotting at all four time points after DEX treatment. There were no differences between vehicle-control and DEX-treated rats at any time point for the level of AHR protein. Similarly, DEX did not induce ARNT protein at any time point despite the substantial, although transient, induction of mRNA observed at 3 and 6 h post-dosing. Measurements of AHR and ARNT protein in whole liver homogenate yielded results similar to those in cytosol (data not shown).

3.6. *The induction of hepatic CYP1B1 mRNA by MC is reduced in ADX rats*

ADX and SHAM rats were treated with a single MC dose (20 mg/kg) by gavage at 4 days after surgery to assess whether the response to an aromatic hydrocarbon was altered, given the depleted level of hepatic AHR protein following ADX. As a measure of the AHR-dependent adaptive response to MC, the level of CYP1B1 mRNA was measured using real-time RT-PCR and the results are shown in Fig. 8. The level of hepatic CYP1B1 mRNA was induced 23.9-fold and 5.5-fold by MC in SHAM and ADX rats, respectively, relative to the surgery-matched, vehicle-treated control groups. The level of MC-induced hepatic CYP1B1 mRNA following ADX was 50% less than the level of MC-induced hepatic CYP1B1 mRNA in SHAM rats.

4. Discussion

In our current study, we showed that by removing the adrenal glands, the endogenous source of glucocorticoids and other adrenal-dependent factors, the level of rat hepatic AHR protein was reduced four days after surgery. By administering a single acute dose of DEX, a synthetic glucocorticoid with a longer half-life than CORT, we demonstrated that exogenous glucocorticoid treatment could not alone reverse this AHR protein depletion. While the level of AHR was not altered by DEX treatment, interestingly, hepatic ARNT mRNA was induced, with no apparent corresponding change in the level of ARNT protein. To determine whether the observed depletion of AHR protein was sufficient to alter the response to an aromatic hydrocarbon, we measured the level of CYP1B1 mRNA, one example of a transcriptional target of AHR activation, following MC treatment. We found that hepatic CYP1B1 mRNA induction by MC was reduced in ADX rats. This finding indicates that this *in vivo* adaptive response to MC is altered when AHR protein is depleted following ADX, providing evidence that aromatic hydrocarbon responsiveness is affected by AHR levels in rat liver.

A number of recent publications support the existence of cross-talk between the rodent AHR and GR pathways in primary and continuous cell cultures. In particular, a positive modulatory effect of glucocorticoids has been observed on the level of AHR expression in cultured rodent cells [19, 21, 22]. The nature of the interaction between these pathways in the whole animal had not yet been characterized. The evidence provided by our study suggests that: (1) the adrenal status of rats affects the level of the hepatic AHR protein acutely following surgery, (2) the adaptive response to MC is affected by adrenal status and AHR levels, but (3) acute activation of the GR by exogenous DEX treatment is not sufficient to overcome the loss of adrenal-dependent factors involved in the maintenance of AHR levels.

Our findings, of reduced hepatic AHR protein levels and reduced induction of CYP1B1 mRNA in ADX rats, may provide an explanation for previous studies of PAH responsiveness. Nebert and Gelboin [37] reported that MC-induced aryl hydrocarbon hydroxylase activity (a measure of CYP1A function) was significantly decreased in liver from ADX and HYPX rats, yet the nature of this difference in enzyme activity was not apparent. MC-induced metabolism of benzo[*a*]pyrene, a PAH that binds to and activates the AHR [1], was 52% lower in ADX rats compared to MC-treated intact rats [38]. These two previous studies demonstrated that ADX

reduced MC-induced enzyme activity. A lower level of AHR and reduced induction of an AHR target gene, *CYP1B1*, is consistent with reduced AHR-mediated induction of enzyme activity since the AHR mediates the effects of the aromatic hydrocarbons used in the two previous reports.

With respect to direct effects of exogenous glucocorticoid treatment on the AHR, a single DEX treatment did not affect AHR mRNA, nor did DEX return the level of AHR protein to SHAM levels in ADX rats four days after surgery. We show here that AHR regulation does not occur by a direct transcriptional mechanism of GR activation leading to increased levels of AHR mRNA. This observation is consistent with the observation that AHR protein levels, but not mRNA levels, are decreased in rat liver following HYPX [8]. It is not yet known whether the DEX dosing regimen was insufficient to reverse the depletion of AHR protein. The dose of DEX (1.5 mg/kg) and timing (6 h) before measurement were sufficient to induce the GR-activated marker, TAT, and ARNT mRNA in our study. Others have reported that the induction of TAT enzyme activity, and hence function, by methylprednisolone, a synthetic glucocorticoid, follows shortly after mRNA induction with a one-hour lag time [36]. Our results suggest that direct glucocorticoid activation of the GR and subsequent transcriptional activation via direct DNA binding, as is the mechanism for TAT induction [39], cannot account for the decreased hepatic AHR protein levels caused by ADX. It is more likely that other early endocrine perturbations caused by ADX, such as changes in insulin or growth hormone [40, 41], or other GR molecular mechanisms, such as protein-protein interactions [39], contribute to the effect of ADX on AHR protein levels.

Given that AHR mRNA levels remained unchanged following ADX, we showed that hepatic AHR protein depletion in ADX rats was not mechanistically related to transcriptional regulation of the *AHR* gene. However, we do not yet know the mechanism for ADX-dependent AHR protein depletion. The AHR protein is stabilized by components of the cytosolic protein complex in which it resides [42, 43] and AHR protein turnover is mediated by ubiquitin-dependent proteasomal degradation [44]. These are both possible avenues for mechanistic investigation of the observed ligand-independent regulation of AHR protein. Since AHR protein depletion was only observed at the earlier time point after ADX, the mechanism could be related to early perturbations of various adrenal-dependent factors that are compensated for over a longer post-surgical recovery. For these reasons, differences in DEX responsiveness between our

acute and subacute studies may relate to both differences in the dosing regimen and the time allowed for recovery from ADX.

We report here that when the level of the hepatic AHR is depleted following ADX, the response to MC treatment, as measured by hepatic CYP1B1 mRNA, is reduced. While there is a body of evidence regarding the effects of AHR ligands on the level of AHR expression in rodents, the net effect of changes to the magnitude of the response to aromatic hydrocarbons is not clearly understood because of the on-going presence of the ligand [30, 45, 46]. Our novel finding demonstrates a relationship between receptor levels and the response to aromatic hydrocarbons *in vivo*. While studying HYPX rats, our laboratory previously observed that the significant reduction of AHR protein and ligand-binding in HYPX rats did not correspond to a change in CYP1A1 inducibility [8]. In cell culture, it has been demonstrated repeatedly that the level of AHR expression can modulate sensitivity to aromatic hydrocarbons. For example, leukocytes express low basal levels of AHR. Sensitivity to the inhibitory effects of TCDD on these immunocompetent cells was directly related to a marked increase in AHR expression and function observed during leukocyte activation [47]. Taken together, these studies suggest that the target gene selected can be important in determining the impact of altered AHR levels on the response to aromatic hydrocarbon treatment.

Another finding of our study is that the level of hepatic ARNT mRNA was dramatically induced *in vivo* at early time points following a single dose of DEX in intact, SHAM and ADX rats. This observation is in line with supplemental data from a microarray analysis of liver showing that the level of ARNT mRNA was induced following methylprednisolone infusion, although only measured in ADX rats [48]. Given that induction of ARNT protein by DEX was not apparent in our study, it remains unclear whether the induction of ARNT mRNA alters ARNT function or the response to aromatic hydrocarbons or hypoxia. In order to determine the mechanism of the induction of ARNT mRNA and whether this translates to a change in ARNT protein using various doses of glucocorticoids and time points, additional studies in cell culture will be required. Teleologically, it would not be surprising if ARNT protein expression was under tight regulatory control, such as that by microRNA inhibition of translation, given that this gene is essential for survival [49]. This type of regulatory control could explain the apparent disconnect between the observed levels of ARNT mRNA and protein.

While the regulation of the AHR by physiological and xenobiotic agents has been reported [50], the evidence to support alteration of hepatic ARNT expression by endogenous or exogenous factors is minimal. Abbott et al. [51] showed that the level of ARNT mRNA expression increases developmentally in the embryonic mouse palate and that this effect can be suppressed by exposure to TCDD or hydrocortisone. The loss of ARNT has also been implicated in human pancreatic islet dysfunction associated with diabetes [52]. A recent paper showed that the level of human ARNT protein decreased with increasing glucose concentrations and increased with reduced calcium flux in MIN6 cultured pancreatic islet cells [53]. The elevation of mouse ARNT mRNA by the hepatotoxin pyrazole was recently demonstrated [54]. We conducted a preliminary analysis of the rat *ARNT* gene and identified two putative GREs within the first 10 kb of the transcription start site (data not shown). Our novel findings with respect to the regulation of ARNT mRNA by DEX in rat liver form the basis for continuing studies on the mechanism of ARNT mRNA regulation by glucocorticoids.

In conclusion, we have demonstrated that: (1) adrenal-dependent factors contribute to the physiological maintenance of hepatic AHR protein levels; (2) in ADX rats, suppressed induction of hepatic CYP1B1 mRNA by MC accompanies the depletion of AHR protein; and (3) exogenous glucocorticoid treatment increases hepatic ARNT mRNA levels regardless of adrenal status. Future studies will focus on further characterization of the molecular mechanisms underlying these effects and the functional impact of ADX or glucocorticoid alterations on the response to aromatic hydrocarbons or hypoxia. The current and follow-up studies serve to enhance our understanding of the physiological and pharmacological effects of hormones on the *in vivo* expression and function of the AHR and ARNT, mediators of the adaptive and toxic responses to dioxin-like environmental contaminants.

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Conflict of Interest Statement

The authors declare that there are no conflicts of interest.

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Figure Legends

Fig. 1. AHR, ARNT and TAT hepatic mRNA levels in SHAM and ADX male rats following subacute and acute DEX treatments. Total RNA was isolated from liver of male rats treated with DEX (D) or corn oil vehicle (V) once daily for seven days, starting two weeks after surgery (subacute) or once 6 h before euthanasia, four days after surgery (acute). Liver RNA samples were then subjected to semiquantitative RT-PCR analysis. **(A)** Representative images of Vistra Green-stained polyacrylamide gels showing PCR products for AHR, ARNT, and TAT relative to β -actin, showing a sample from each treatment group (subacute: n=6 per group; acute: n=4-5 per group). All samples for a particular study were run on the same gel. Dotted lines between lanes indicate non-adjacent wells run on the gel. **(B)** Semiquantitative image analysis of AHR, ARNT and TAT mRNA levels relative to β -actin. Results are presented as a percentage of the mean for the SHAM vehicle-treated group, and each bar represents the mean $\pm$ S.D. of determinations from 4-5 (acute) or 6 (subacute) rats. *Significantly different ($p < 0.05$) from surgical intervention-matched vehicle control; **significantly different ($p < 0.01$) from surgical intervention-matched vehicle control; ***significantly different ($p < 0.001$) from surgical intervention-matched vehicle control, based on two-way ANOVA followed by Student's *t*-test or Mann-Whitney test.

Fig. 2. Hepatic cytosolic AHR protein in SHAM and ADX male rats following subacute and acute treatment with DEX (D) or corn oil vehicle (V). Liver cytosol was collected after dosing once daily for seven days, starting two weeks after surgery (subacute) or once 6 h before euthanasia, four days after surgery (acute). Cytosolic protein was then subjected to immunoblot analysis. **(A)** Representative immunoblot analysis of cytosolic protein (30 μ g) from each rat using polyclonal AHR antibody, showing two samples from each treatment group (subacute: n=6 per group; acute: n=4-5 per group). Dotted 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, and each bar represents the mean $\pm$ S.D. of determinations from 4-5 (acute) or 6 (subacute) rats. [†]Significantly different ($p < 0.05$) from SHAM-D control; ^{††}significantly different ($p < 0.01$) from SHAM-V control, based on two-way ANOVA followed by Student's *t*-test or Mann-Whitney test.

Fig. 3. Hepatic cytosolic ARNT protein in SHAM and ADX male rats following subacute and acute treatment with DEX (D) or corn oil vehicle (V). Liver cytosol was collected after dosing once daily for seven days, starting two weeks after surgery (subacute) or once 6 h before euthanasia, four days after surgery (acute). Cytosolic protein was then subjected to immunoblot analysis. **(A)** Representative immunoblot analysis of cytosolic protein (40 μ g) from each rat using polyclonal ARNT antibody, showing two samples from each treatment group (subacute: n=6 per group; acute: n=4-5 per group). Dotted lines between lanes indicate non-adjacent wells run on the gel. **(B)** Semiquantitative image analysis of ARNT protein levels. Results are presented as a percentage of the mean for the SHAM vehicle-treated group, and each bar represents the mean $\pm$ S.D. of determinations from 4-5 (acute) or 6 (subacute) rats. There were no statistically significant differences ($p > 0.05$).

Fig. 4. TCDD binding to hepatic cytosolic AHR in SHAM and ADX male rats following subacute and acute treatment with DEX (D) or corn oil vehicle (V). Cytosolic protein (2 mg/ml) from rat liver was incubated with 2 nM [3 H]TCDD in the absence and presence of 100-fold molar excess of non-radioactive TCDF (200 nM) with shaking for 2 h at room temperature. Cytosol was then incubated with HAP slurry for 30 min on ice, washed and radioactivity was counted. Cytosol from the SHAM-V group had a mean concentration of 71.4 ± 8.0 fmol/mg protein (subacute) and 77.6 ± 25.0 fmol/mg protein (acute). Results are presented as a percentage of the mean for the SHAM vehicle-treated group, and each bar represents the mean $\pm$ S.D. of determinations from 4-5 (acute) or 6 (subacute) rats. † Significantly different ($p < 0.05$) from SHAM-V control, based on two-way ANOVA followed by Student's t -test or Mann-Whitney test.

Fig. 5. Time course of the effects of DEX (D) administration to intact male rats on hepatic ARNT and TAT mRNA levels quantified by real-time RT-PCR. Results are presented as a percentage of the mean for the 3 h-V group, and each bar represents the mean $\pm$ S.D. of determinations from 4 or 5 rats. *Significantly different ($p < 0.05$) from time-matched vehicle control; **significantly different ($p < 0.01$) from time-matched vehicle control, based on two-way ANOVA followed by Student's t -test or Mann-Whitney test. † Significantly different

($p < 0.05$) from 6 h-D group; †† significantly different ($p < 0.01$) from 6 h-D group, based on two-way ANOVA followed by a one-way ANOVA / post-hoc Newman-Keuls test or two-ANOVA followed by Kruskal-Wallis test / post-hoc Dunn's multiple comparisons test.

Fig. 6. Time course of the effects of DEX (D) administration to intact male rats on hepatic AHR protein levels. **(A)** Representative immunoblot analysis of cytosolic protein (30 μ g) from each rat using polyclonal AHR antibody, showing two samples from each treatment group ($n=4$ or 5 per group). Dotted 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 3 h-V group, and each bar represents the mean $\pm$ S.D. of determinations from 4 or 5 rats. There were no statistically significant differences ($p > 0.05$).

Fig. 7. Time course of the effects of DEX (D) administration to intact male rats on hepatic ARNT protein levels. **(A)** Representative immunoblot analysis of cytosolic protein (25 μ g) from each rat using polyclonal ARNT antibody, showing two samples from each treatment group ($n=4$ or 5 per group). Dotted lines between lanes indicate non-adjacent wells run on the gel. **(B)** Semiquantitative image analysis of ARNT protein levels. Results are presented as a percentage of the mean for the 3 h-V group, and each bar represents the mean $\pm$ S.D. of determinations from 4 or 5 rats. † Significantly different ($p < 0.05$) from 3 h-D group, based on two-way ANOVA followed by a one-way ANOVA / post-hoc Newman-Keuls test.

Fig. 8. Hepatic CYP1B1 mRNA levels in SHAM and ADX rats following MC administration, as quantified by real-time RT-PCR. Results are presented as a percentage of the mean for the SHAM-V group, and each bar represents the mean $\pm$ S.D. of determinations from 4 (vehicle- and MC-treated SHAM), 6 (vehicle-treated ADX) or 7 (MC-treated ADX) rats. *Significantly different ($p < 0.05$) from SHAM-V group; **significantly different ($p < 0.01$) from ADX-V group, based on two-way ANOVA followed by Student's t -test or Mann-Whitney test. † Significantly different ($p < 0.05$) from MC-treated SHAM rats, based on two-way ANOVA followed by Student's t -test or Mann-Whitney test.

Table 1. Primer sequences and thermal cycling conditions used for measurement of steady-state mRNA levels in rat liver by quadruplex semiquantitative RT-PCR

Target	Forward and Reverse PCR Primer Sequences	PCR product size (bp)	Thermal Cycling Conditions (Denature; Anneal; Extend) x Cycles	Reference
AHR	5' -AGGGAGGTTAAAGTATCTTCATGGAC-3' 5' -TCCCTAGGTTTCTCATGATGCTATAC-3'	917		[45]
ARNT	5' -CTTGGCTCTGTGAAGGAAGG-3' 5' -CGGAATCGGAACATGACAG-3'	413		[45]
TAT ^a	5' -GCCAATCCTGGACA <u>A</u> AACAT-3' 5' -TTCTGAAGGTG <u>T</u> CGCTTACT-3'	230	(30s/95°C; 30s/55°C; 40s/72°C) x 22	[55]
β-actin	5' -ACCGTGAAAAGATGACCCAG-3' 5' -GAGCCACCAATCCACACAG-3'	688		[45]

^a The TAT primer sequences were originally designed based on the mouse cDNA sequence. The underlined and bolded bases were changed to match the rat cDNA sequence.

Table 2. Primer sequences and final concentration used for measurement of steady-state mRNA levels in rat liver by real-time quantitative RT-PCR

Target	Forward and Reverse PCR Primer Sequences	PCR product size (bp)	Final concentration (nM)	Reference
ARNT	5' -GATGAGGTGTGGCACCAGCT-3' 5' -GTGAGGCTCTCCTTCCTTCACA-3'	109	200	New design
TAT	5' -CAGCAACGTGCTTCGAGTACC-3' 5' -CTCCTCCTGGCTGCCTTCAG-3'	137	100	New design
CYP1B1	5' -CTCATCCTCTTTACCAGATACCCG-3' 5' -GACGTATGGTAAGTTGGGTGGTC-3'	117	200	[56]
β -actin	5' -GACCCAGATCATGTTTGAGACCTTC-3' 5' -GGAGTCCATCACAATGCCAGTG-3'	109	100	[2]

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

A. **B.** **Figure 6**

Figure 7

Figure 8