Role of glucocorticoid receptor and pregnane X receptor in
dexamethasone induction of rat hepatic
aryl hydrocarbon receptor nuclear translocator and
NADPH-cytochrome P450 oxidoreductase

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ABBREVIATIONS: ADX, adrenalectomy or adrenalectomized; AHR, aryl hydrocarbon receptor; ANOVA, analysis of variance; ARNT, aryl hydrocarbon receptor nuclear translocator; DEX, dexamethasone; GR, glucocorticoid receptor; GRE, glucocorticoid-responsive element; MC, 3-methylcholanthrene; P450, cytochrome P450; PAH, polycyclic aromatic hydrocarbon; PCN, pregnenolone-16α-carbonitrile; POR, NADPH-cytochrome P450 oxidoreductase; PXR, pregnane X receptor; RT-PCR, reverse transcriptase-polymerase chain reaction; RU486, mifepristone; TA, triamcinolone acetonide; TAT, tyrosine aminotransferase; UAARP, unidentified ARNT antibody-reactive protein.
Abstract

The aryl hydrocarbon receptor (AHR) nuclear translocator (ARNT), as the AHR’s heterodimerization partner, and NADPH-cytochrome P450 oxidoreductase (POR), as the key electron donor for all microsomal P450s, are independent and indispensable components in the adaptive and toxic responses to polycyclic aromatic hydrocarbons. Expression of both ARNT and POR in rat liver is induced by dexamethasone (DEX), a synthetic glucocorticoid known to activate both the glucocorticoid receptor (GR) and the pregnane X receptor (PXR). To better understand the role of GR and PXR in the in vivo DEX induction of rat hepatic ARNT and POR at the mRNA and protein levels, we studied: (1) the effects of DEX doses that activate GR (≥ 0.1 mg/kg) or PXR (≥ 10 mg/kg); (2) responses produced by GR- and PXR-selective agonists; (3) the impact of GR antagonism on DEX’s inducing effects; and (4) whether biological responses to DEX are altered in PXR-knockout rats. Our findings are consistent with a role for GR as a key mediator of the induction of rat hepatic ARNT expression by glucocorticoids; a role for PXR in the modulation of ARNT protein levels could not be excluded. Although GR activation may contribute to POR mRNA induction, regulation of POR expression and function by DEX is primarily PXR-mediated. This work suggests that the hepatic expression and function of ARNT and POR may be modulated by exposure to exogenous PXR activators and/or conditions that alter glucocorticoid levels such as stress, steroidal therapies, and diseases of excess or deficiency.
Introduction

The aryl hydrocarbon receptor (AHR) has important physiological functions and mediates both adaptive and toxic responses to halogenated and polycyclic aromatic hydrocarbons (PAH) (Tian et al., 2015). The AHR nuclear translocator (ARNT) participates in most, if not all, of these responses as the AHR’s nuclear dimerization partner (Labrecque et al., 2013). Adaptive homeostatic responses to AHR agonists like 3-methylcholanthrene (MC) involve induction of drug-metabolizing enzymes, including microsomal cytochromes P450 (P450) (Nebert et al., 2004), which receive electrons from NADPH-cytochrome P450 oxidoreductase (POR) (Riddick et al., 2013). Thus, ARNT and POR are independent and indispensable components of the AHR response pathway.

Understanding physiological factors (e.g. hormones) that regulate the expression and function of the AHR itself, as well as other components of the AHR response system, provides insight into conditions that modulate an organism’s responsiveness to endogenous and exogenous AHR ligands (Harper et al., 2006). The level of hepatic AHR protein is decreased in hypophysectomized (Timsit et al., 2002) and adrenalectomized (ADX) (Mullen Grey and Riddick, 2009) male rats, and ADX rats have selectively impaired CYP1B1 induction by MC (Mullen Grey and Riddick, 2009; Mullen Grey and Riddick, 2011). Hence, our recent focus is adrenal glucocorticoids as regulators of the expression and function of AHR as well as AHR response pathway components like ARNT and POR.

Dexamethasone (DEX), a potent synthetic glucocorticoid, potentiates the AHR-mediated induction of hepatic CYP1A1 by aromatic hydrocarbons in rodents and cultured rodent cells (Mathis et al., 1986; Sherratt et al., 1989; Lai et al., 2004), a response possibly related to
increased AHR expression by DEX (Wiebel and Cikryt, 1990; Sonneveld et al., 2007; Bielefeld et al., 2008). AHR expression is induced by DEX in Hepa-1 mouse hepatoma cells via a glucocorticoid receptor (GR)-dependent transcriptional mechanism and this augments AHR-mediated transcriptional responses (Bielefeld et al., 2008). Species-specificity is an issue as AHR levels are decreased by DEX in cultured human cells (Dvorak et al., 2008; Vrzal et al., 2009) and this is reflected in diminished CYP1A1 induction by aromatic hydrocarbons (Monostory et al., 2005; Sonneveld et al., 2007; Vrzal et al., 2009; Wang et al., 2009).

Treatment of rats with a single low GR-activating dose of DEX (~ 1 mg/kg) had no effect on hepatic AHR mRNA or protein levels (Mullen Grey and Riddick, 2009); however, the same study suggested that rat hepatic ARNT and POR are DEX-inducible genes of interest.

Regarding ARNT regulation by glucocorticoids, hepatic ARNT mRNA levels are increased in ADX rats treated with the GR agonist methylprednisolone (Almon et al., 2005). Rat hepatic ARNT expression is not affected by ADX; however, treatment of intact, sham-operated, and ADX male rats with a single low GR-activating dose of DEX (~ 1 mg/kg) caused marked induction of hepatic ARNT mRNA levels, which peaked at 6 h post-treatment, with no accompanying change in ARNT protein levels (Mullen Grey and Riddick, 2009).

As for glucocorticoid control of POR, decreased hepatic POR activity in ADX rats is rescued by cortisone acetate (Castro et al., 1970). In our acute ADX model, hepatic POR protein and activity were decreased at 4 days post-ADX, with no change in POR mRNA levels, and decreased POR activity was implicated in the compromised adaptive response of ADX rats to MC (Mullen Grey and Riddick, 2011). Most rodent studies of POR regulation by DEX use high doses (~ 10 to 80 mg/kg) expected to activate both GR and the pregnane X receptor (PXR). DEX (10 mg/kg) increased rat hepatic POR activity (Sherratt et al., 1989; Linder and Prough,
1993) and a 80 mg/kg dose increased rat hepatic POR mRNA levels via mRNA stabilization (Simmons et al., 1987). Induction of hepatic POR protein levels by DEX (50 mg/kg) in wild-type and Gr-null mice (Schuetz et al., 2000) and diminished induction of POR mRNA levels by pregnenolone-16α-carbonitrile (PCN), a rodent PXR activator, in Pxr-null mice (Maglich et al., 2002) suggest PXR involvement.

The role of GR and PXR in the in vivo DEX induction of rat hepatic ARNT and POR at the mRNA and protein levels requires clarification. The objectives of this study were to determine whether: (1) rat hepatic ARNT and POR are induced by DEX doses that activate GR (≥ 0.1 mg/kg) or PXR (≥ 10 mg/kg); (2) selective GR or PXR agonists induce rat hepatic ARNT and POR expression; (3) rat hepatic ARNT and POR induction by DEX (0.5 and 50 mg/kg) is altered by GR antagonism; and (4) hepatic ARNT and POR induction by DEX (1 and 50 mg/kg) is altered in PXR-knockout rats.
Materials and Methods

Animals and treatment. Experimentation was guided by the principles of the Canadian Council on Animal Care and the University of Toronto Animal Care Committee approved all animal use protocols. All rats were 7 weeks of age at the time of procurement and underwent a one-week period of acclimatization to housing conditions (two rats per cage, 12-h light/12-h dark cycle with lights on at 7 am, ad libitum access to chow and water) in the Division of Comparative Medicine, University of Toronto. Male Fischer 344 rats were obtained from Charles River Laboratories Canada (St. Constant, QC). Male PXR-knockout rats (SD-Nriizm1sage, model TGRS4130) and their wild-type Sprague-Dawley controls were purchased from Horizon Discovery (Boyertown, PA). The PXR-knockout rats are homozygous for a 20-bp deletion created by zinc finger nuclease technology within the DNA-binding domain (exon 2) of the PXR gene, leading to multiple premature stop codons and lack of hepatic CYP3A induction following PCN treatment (https://www.horizondiscovery.com/pxr-knockout-rat-tgrs4130).

Bilateral ADX and corresponding sham operations were performed on 8-week old male Fischer 344 rats by a Division of Comparative Medicine surgical technician. In our subacute ADX model (Mullen Grey and Riddick, 2009), rats recovered for 13 days following surgery and ADX rats received 0.9% sodium chloride in drinking water for the remainder of the study. ADX and sham-operated rats were treated with DEX (1 mg/kg) or corn oil vehicle by i.p. injection daily at 3 pm for 7 days. At 10 am on the following day, rats were euthanized by decapitation.

For the DEX time-course study (Mullen Grey and Riddick, 2009), intact 8-week old male Fischer 344 rats received a single i.p. injection of DEX (1.5 mg/kg) or corn oil vehicle at 4 am.
followed by euthanasia by decapitation at 7 am, 10 am, 4 pm, or 7 am the following day, times corresponding to 3, 6, 12, or 27 h after dosing.

For the DEX dose-response study, intact 8-week old male Fischer 344 rats received a single i.p. injection of DEX (0.1, 1, 10, or 50 mg/kg) or corn oil vehicle at 10 am. For the study of GR- and PXR-selective agonists, intact 8-week old male Fischer 344 rats received a single i.p. injection of triamcinolone acetonide (TA) (5 mg/kg), PCN (50 mg/kg), or corn oil vehicle at 10 am. For the study of GR antagonism, intact 8-week old male Fischer 344 rats received an i.p. injection of mifepristone (RU486) (50 mg/kg) or corn oil vehicle at 9:30 am followed by a second i.p. injection of DEX (0.5 or 50 mg/kg) or corn oil vehicle at 10 am. Finally, 8-week old male PXR-knockout rats and wild-type Sprague-Dawley controls received a single i.p. injection of DEX (1 or 50 mg/kg) or corn oil vehicle at 10 am. For these studies, rats were euthanized by decapitation at 4 pm (6 h post-dosing) or 10 am the following day (24 h post-dosing).

Immediately following euthanasia, each liver was perfused in situ with ice-cold HEGD buffer (25 mM HEPES / 1.5 mM EDTA / 10% glycerol / 1 mM dithiothreitol, pH 7.4). The liver was excised and several individual pieces (each ~ 0.1 g) were frozen by immersion in liquid nitrogen and stored at -70°C for subsequent RNA isolation. The remaining liver was homogenized in HEGD buffer and cytosolic and microsomal fractions were isolated by differential centrifugation. Aliquots of liver homogenate, cytosol, and microsomes were frozen by immersion in liquid nitrogen and stored at -70°C until subsequent use. Cytosolic and homogenate protein concentrations were determined by the method of Bradford (1976) and microsomal protein concentrations were determined by the method of Lowry et al. (1951).
Analysis of mRNA levels by real-time quantitative RT-PCR. Hepatic mRNA levels for ARNT, POR, tyrosine aminotransferase (TAT) and CYP3A23, normalized to β-actin as the internal reference standard, were determined according to previously described relative standard curve (Mullen Grey and Riddick, 2011) or comparative threshold cycle (Lee and Riddick, 2012) methods. Primers were detailed previously as follows: ARNT, TAT, β-actin (Mullen Grey and Riddick, 2009), and POR (Mullen Grey and Riddick, 2011). Newly designed CYP3A23 primers had the following sequences: forward, 5’-TGGGTCCCTCTGGGACGTG-3’ and reverse, 5’-GTGTGCCTTGCCATCCGT-3’. The CYP3A23 product size was 55 bp.

Immunoblot analysis. Relative quantitation of hepatic protein levels for ARNT and POR, normalized to β-actin as the internal reference standard, was performed according to previously described methods (Mullen Grey and Riddick, 2009; Lee et al., 2013b). Polyacrylamide gels were loaded with liver homogenate (2 µg protein), cytosol (30 µg), or microsomes (6 µg) and the resulting blots were probed with the following primary antibodies: goat polyclonal against a C-terminal peptide of human ARNT (sc-8076; Santa Cruz Biotechnology, Santa Cruz, CA) used at a 1:200 dilution; rabbit polyclonal against amino acid 1-300 of human POR (sc-13984; Santa Cruz Biotechnology, Santa Cruz, CA) used at 1:1,000 or 1:5,000 dilutions; mouse monoclonal against amino acid 1-14 of β-actin (ab6276; Abcam, Cambridge, UK) used at a 1:100,000 dilution. The following secondary antibodies were used: rabbit anti-goat IgG-horseradish peroxidase conjugate (A5420; Sigma-Aldrich, St. Louis, MO) used at a 1:2,000 dilution for ARNT detection; donkey anti-rabbit IgG-horseradish peroxidase conjugate (NA934; GE Healthcare, Buckinghamshire, UK) used at a 1:5,000 dilution for POR detection; sheep anti-mouse IgG-horseradish peroxidase conjugate (NB120-6808; Novus Biologicals, Littleton, CO)
used at 1:5,000 or 1:20,000 dilutions for β-actin normalization of POR and ARNT blots, respectively.

**POR activity.** Hepatic microsomal POR catalytic activity was assessed as the rate of cytochrome c reduction according to previously described methods (Mullen Grey and Riddick, 2011; Lee et al., 2013a).

**Statistical analysis.** Data are shown as the mean ± S.D. of determinations from the number of rats specified in the figure legends. Statistical tests were performed on raw data rather than data derived as percent of controls. A result was considered statistically significant at $P < 0.05$.

For the subacute ADX study, DEX time-course study, DEX dose-response study, and selective agonists study, data were analyzed initially using a randomized-design two-way analysis of variance (ANOVA) to identify effects of the two independent variables and their interaction (DEX treatment or DEX dose or agonist; surgery or time; two-factor interaction). For the GR antagonism study and PXR-knockout rat study, data were analyzed initially using a randomized-design three-way ANOVA to identify effects of the three independent variables and their interactions (DEX dose; time; antagonist or genotype; all pair-wise two-factor interactions and three-factor interaction). Post tests, Bonferroni corrected for multiple comparisons and based on the mean square residual and degrees of freedom from the ANOVA, were performed for the planned comparisons to discern the effects of specific independent variables on the measured experimental outcomes. If Bartlett’s test showed significant heterogeneity of variance, specific comparisons of interest were based on the Welch-corrected unpaired $t$ test.
Results

Subacute ADX study. We showed previously that ADX has no effect on rat hepatic ARNT mRNA or protein levels at 4 days (acute model) and 3 weeks (subacute model) after surgery (Mullen Grey and Riddick, 2009). In the acute model, ADX caused a 70% decrease in rat hepatic POR protein levels and a 50% decrease in POR catalytic activity, with no accompanying change in POR mRNA levels (Mullen Grey and Riddick, 2011). In the subacute model, ADX had no effect on hepatic POR mRNA levels (Fig. 1A), but caused a 62% decrease in POR protein levels (Fig. 1B) and a 34% decrease in POR activity (Fig. 1C). Daily treatment of rats with a low GR-activating dose of DEX (1 mg/kg) for a week increased hepatic POR mRNA levels in both sham-operated and ADX rats (Fig. 1A), whereas this treatment selectively restored the depleted POR protein levels seen in ADX rats (Fig. 1B). The trend for this treatment to also restore POR activity in ADX rats did not achieve statistical significance (Fig. 1C). This DEX dosing regimen was previously shown to have no effect on hepatic ARNT mRNA or protein levels in sham-operated and ADX rats (Mullen Grey and Riddick, 2009).

DEX time-course study. Similar to what we found previously for ARNT regulation (Mullen Grey and Riddick, 2009), treatment of intact rats with a single low GR-activating dose of DEX (1.5 mg/kg) induced hepatic POR mRNA levels, with a maximum response at 6 h post-treatment (Fig. 2A), with no accompanying change in POR protein levels (Fig. 2B).

DEX dose-response study. To gain insight into the role of GR and PXR in the in vivo DEX induction of hepatic ARNT and POR expression, we first took advantage of the differential dose-
dependent activation of GR vs. PXR by DEX (Pascusi et al., 2001). All DEX doses used in this study (0.1, 1, 10, 50 mg/kg) were expected to cause GR activation, whereas only the highest DEX doses (10 and 50 mg/kg) were expected to activate the lower affinity xenosensor PXR.

ARNT mRNA levels were induced at the 6-h time point by 7.5- to 10-fold at DEX doses of 1, 10, and 50 mg/kg, a response not seen at the 0.1 mg/kg DEX dose (Fig. 3A). Induction of ARNT mRNA was no longer observed at 24 h. Hepatic TAT mRNA levels were assessed as a positive control to confirm induction of a known GR target gene. TAT mRNA levels were increased at the 6-h time point by approximately 2- to 3-fold at all DEX doses tested, with no induction seen at 24 h (Fig. 3B). Hepatic CYP3A23 mRNA levels were assessed as a positive control to confirm induction of a known PXR target gene. CYP3A23 mRNA levels were induced by approximately 5-fold at the 50 mg/kg dose at the 6-h time point, whereas the 10 and 50 mg/kg doses caused approximately 5- and 30-fold induction, respectively, at the 24-h time point (Fig. 3C). Finally, POR mRNA levels were induced at the 6-h time point by all DEX doses; the 0.1 and 1 mg/kg doses caused approximately 3-fold increases, whereas the magnitude of induction by the 10 and 50 mg/kg doses was approximately 4- and 5-fold, respectively (Fig. 3D). With only the 10 and 50 mg/kg doses, a lower magnitude induction persisted at 24 h.

There was a non-statistically significant trend for increased ARNT protein levels (~ 88 kDa) at the 24-h time point, particularly at the highest DEX doses (Fig. 4A). In rats administered 50 mg/kg of DEX, ARNT protein levels were higher at 24 h compared to 6 h. Surprisingly, an additional lower molecular mass band appeared on ARNT immunoblots under certain treatment conditions. The level of this unidentified ARNT antibody-reactive protein (UAARP) was increased at the 6-h time point, most notably by the 1 and 10 mg/kg DEX doses (Fig. 4A). POR protein levels (~ 78 kDa) were increased by 2-fold by the 50 mg/kg DEX dose at 24 h (Fig. 4B).
POR catalytic activity was increased at the 24-h time point by approximately 1.5-fold by the 10 and 50 mg/kg doses of DEX (Fig. 4C).

The effects described above occurred under conditions in which the liver to body weight ratio was increased by approximately 20 to 30% in rats treated with 10 and 50 mg/kg DEX at the 24-h time point (Supplemental Fig. 1A).

**GR- and PXR-selective agonists study.** The next strategy was to determine whether ARNT or POR mRNA/protein levels were induced by TA, a selective GR agonist (Runge-Morris et al., 1996), and PCN, a selective PXR agonist (Hartley et al., 2004).

TA caused a 7.5-fold induction of ARNT mRNA levels at the 6-h time point, whereas PCN treatment had no effect on ARNT mRNA levels (Fig. 5A). TAT mRNA levels, assessed as a confirmation of GR activation, were induced by TA by approximately 3-fold at the 6-h time point, with no observed effect of PCN (Fig. 5B). CYP3A23 mRNA levels, assessed as a confirmation of PXR activation, were induced by PCN by approximately 70-fold at the 24-h time point, with no observed effect of TA (Fig. 5C). TA alone was unable to induce POR mRNA levels; however, there appears to be a trend at 6 h for increased POR mRNA corroborated by the fact that TA-treated rats showed higher POR mRNA levels at 6 h compared to 24 h (Fig. 5D). PCN induced POR mRNA levels by approximately 3-fold at the 6-h time point and this inductive effect was no longer apparent at 24 h.

There was a trend for increased ARNT protein levels at 24 h following TA treatment, corroborated by the fact that TA-treated rats showed higher ARNT protein levels at 24 h compared to 6 h (Fig. 6A). PCN treatment did not alter ARNT protein levels. UAARP levels were increased by TA treatment at the 6-h time point (Fig. 6A). Neither TA nor PCN treatment
altered POR protein levels (Fig. 6B) or POR catalytic activity (Fig. 6C). Trends for PCN inductive effects at 24 h did not achieve statistical significance.

TA treatment caused an approximately 30% increase in the liver to body weight ratio at the 24-h time point, whereas PCN treatment did not alter this parameter (Supplemental Fig. 1B).

**GR antagonism study.** Pharmacological antagonism of the GR with RU486 (Gagne et al., 1985) was next used to further examine the role of GR in the in vivo regulation of ARNT and POR by DEX. Two DEX doses were selected: a low dose (0.5 mg/kg) that selectively activates GR and a high dose (50 mg/kg) that activates both GR and PXR. Use of RU486 as a GR antagonist in this context is complicated by the ability of this compound to act as a PXR agonist at high concentrations (Kliewer et al., 2002); the RU486 dose (50 mg/kg) was chosen to achieve effective antagonism of GR activation by low-dose DEX while minimizing PXR activation by RU486 alone.

The induction of ARNT mRNA levels at 6 h by the 0.5 mg/kg dose of DEX was eliminated by RU486, whereas the induction at this time point by the 50 mg/kg dose of DEX was augmented by RU486 (Fig. 7A). As a positive control for GR activation, the induction of TAT mRNA levels at 6 h by the 0.5 mg/kg dose of DEX was markedly attenuated by RU486, whereas the induction at this time point by the 50 mg/kg dose of DEX was not influenced by RU486 (Fig. 7B). As a positive control for PXR activation, the strong induction of CYP3A23 mRNA levels at 24 h by the 50 mg/kg dose of DEX was not affected by RU486 (Fig. 7C). The induction of POR mRNA levels at 6 h by the 0.5 mg/kg dose of DEX was not altered by RU486, whereas the induction at this time point by the 50 mg/kg dose of DEX was augmented by RU486 (Fig. 7D).
ARNT protein levels were increased at 24 h following treatment with 50 mg/kg DEX and this response was eliminated by RU486 (Fig. 8A). UAARP levels were increased at the 6-h time point by 0.5 and 50 mg/kg doses of DEX and the effect of the low DEX dose was eliminated by RU486 (Fig. 8A). The induction of POR protein levels at 24 h by the 50 mg/kg dose of DEX was augmented by RU486 (Fig. 8B). POR catalytic activity was induced at 24 h by the 50 mg/kg dose of DEX to a similar extent in the presence or absence of RU486 (Fig. 8C).

Treatment with the 50 mg/kg dose of DEX caused an approximately 30% increase in the liver to body weight ratio at the 24-h time point and this response was not altered by RU486 (Supplemental Fig. 1C).

**PXR-knockout rat study.** Finally, we used rats engineered via zinc finger nuclease technology to be devoid of functional PXR to further examine the role of PXR in the in vivo regulation of ARNT and POR by DEX. Two DEX doses were selected: a low dose (1 mg/kg) that selectively activates GR and a high dose (50 mg/kg) that activates both GR and PXR.

ARNT mRNA levels were induced at 6 h by the 1 mg/kg DEX dose in wild-type rats and the apparent induction at this time point by the 50 mg/kg DEX dose did not achieve statistical significance. Similarly, the apparent induction of ARNT mRNA levels at 6 h by either DEX dose in PXR-knockout rats did not achieve statistical significance (Fig. 9A). However, the induced levels of ARNT mRNA at 6 h following treatment with either DEX dose did not differ between wild-type and PXR-knockout rats. As a positive control for GR activation, the induction of TAT mRNA levels at 6 h by either DEX dose did not differ between wild-type and PXR-knockout rats (Fig. 9B). As a positive control for PXR activation, the strong induction of CYP3A23 mRNA levels at 24 h by the 50 mg/kg dose of DEX in wild-type rats was completely
absent in the PXR-knockout rats (Fig. 9C). The induction of POR mRNA levels at 6 h by either DEX dose in wild-type rats was present but significantly attenuated in PXR-knockout rats; the induction by 50 mg/kg DEX at 24 h was seen in wild-type but not PXR-knockout rats (Fig. 9D).

ARNT protein levels were increased at 24 h by the 50 mg/kg DEX dose in wild-type rats and this response was not observed in PXR-knockout rats (Fig. 10A). However, the induced levels of ARNT protein at 24 h following treatment with either DEX dose did not differ between wild-type and PXR-knockout rats. The increase in UAARP levels at 6 h in response to either DEX dose in wild-type rats was maintained in PXR-knockout rats (Fig. 10A). The induction of POR protein levels (Fig. 10B) and POR catalytic activity (Fig. 10C) at 24 h by the 50 mg/kg DEX dose in wild-type rats was completely absent in PXR-knockout rats.

The 20 to 30% increase in liver to body weight ratio at 24 h caused by either DEX dose did not differ between wild-type and PXR-knockout rats (Supplemental Fig. 1D).
Discussion

Adaptive responses to PAHs are influenced by adrenal status, which is commonly manipulated in rodent models via ADX and exogenous glucocorticoid administration. ADX rats display decreased MC-induced hepatic aryl hydrocarbon hydroxylase activity (Nebert and Gelboin, 1969) and benzo[a]pyrene metabolism (Bogdanffy et al., 1982), and a selectively impaired MC induction of CYP1B1 (Mullen Grey and Riddick, 2009; Mullen Grey and Riddick, 2011). We previously suggested a role for decreased hepatic POR protein levels and catalytic activity in the compromised adaptive response of ADX rats to MC exposure (Mullen Grey and Riddick, 2009; Mullen Grey and Riddick, 2011). Conversely, DEX treatment potentiates the induction by PAHs of rodent liver CYP1A1 and select other enzymes in vitro and in vivo (Mathis et al., 1986; Sherratt et al., 1989; Lai et al., 2004). Since ARNT and POR are established DEX-inducible genes (Simmons et al., 1987; Mullen Grey and Riddick, 2009) involved in adaptive responses to PAHs, this study aimed to clarify the role of GR and PXR in the in vivo DEX induction of rat hepatic ARNT and POR at the mRNA and protein levels.

Glucocorticoid regulation of ARNT has multiple layers of importance. First, ARNT is the shared dimerization partner for the AHR and hypoxia-inducible factor-1α, thus influencing responses to xenobiotics and low oxygen tension (Labrecque et al., 2013). Second, Arnt-null mice die during embryonic development due to abnormal vascularization in the yolk sac or placenta (Kozak et al., 1997; Maltepe et al., 1997). Finally, a decrease in ARNT levels mediates pancreatic islet dysfunction in human type 2 diabetes (Gunton et al., 2005).

Our data establish an important role for GR in the in vivo induction of rat hepatic ARNT expression by DEX, especially at the mRNA level. First, ARNT mRNA levels were induced
markedly by a low DEX dose (1 mg/kg) shown to activate GR but not PXR (Fig. 3). Second, ARNT mRNA levels were increased by TA, a GR-selective agonist, but not by PCN, a PXR-selective agonist (Fig. 5). Third, the GR antagonist RU486 prevented the induction of ARNT mRNA levels by low-dose DEX (Fig. 7). Finally, the induced levels of ARNT mRNA did not differ between wild-type and PXR-knockout rats, although the apparent DEX induction in PXR-knockout rats was not statistically significant (Fig. 9). The GR-mediated induction of ARNT mRNA levels may involve transcriptional or post-transcriptional mRNA stabilization mechanisms (Ishmael et al., 2011); differentiating these mechanisms will require additional in vivo investigations and primary rat hepatocyte studies. As potential sites for recruitment of activated GR, we have identified, but not yet functionally characterized, eight putative glucocorticoid-responsive elements (GREs) within the proximal 10 kb of the 5’-flanking region of the rat ARNT gene.

As revealed by genome-wide studies for numerous proteins (Schwanhausser et al., 2011), ARNT mRNA levels may not predict protein levels. There may be mechanisms limiting the magnitude of change in ARNT protein levels when mRNA levels are elevated markedly. Repression of ARNT protein levels by miRNAs such as miR-24 could be involved (Oda et al., 2012). Glucocorticoids can stimulate reactive oxygen species production (Sato et al., 2010), which is known to increase miR-24 levels (Yokoi and Nakajima, 2013) and decrease ARNT protein levels (Choi et al., 2008).

Although ARNT protein levels were not increased by a PXR-selective agonist (Fig. 6) and the induction of ARNT protein levels by high-dose DEX at 24 h was blocked by RU486 (Fig. 8), it may not be possible to exclude a role for PXR in the DEX induction of ARNT protein levels. Trends or statistically significant increases in ARNT protein levels were observed at the
highest PXR-activating DEX doses (Fig. 4, 8, 10). Induction of ARNT protein levels by high-dose DEX did not achieve statistical significance in PXR-knockout rats, although the induced levels of ARNT protein did not differ by genotype (Fig. 10). Although a high DEX dose may imply PXR involvement, ARNT protein induction may require maximal and sustained GR activation, which is only achieved at high DEX doses. The evidence for GR involvement in the induction of UAARP levels is clear. UAARP levels are increased at 6 h in response to a low DEX dose (1 mg/kg) shown to selectively activate GR (Fig. 4). UAARP levels were increased by TA, a GR-selective agonist, but not by PCN, a PXR-selective agonist (Fig. 6). The GR antagonist RU486 prevented the induction of UAARP levels by low-dose DEX (Fig. 8). The induction of UAARP levels by low- and high-dose DEX seen in wild-type rats was maintained in PXR-knockout rats (Fig. 10). The identity of UAARP remains unknown. Rather than a post-translationally modified or degraded form of ARNT, we hypothesize that UAARP is a closely related ARNT isoform such as ARNT2. Cross-reactivity with ARNT2 is a recognized possibility for the antibody used and our findings are consistent with ARNT2’s molecular mass (~ 79 kDa) and low basal expression in rodent liver (Hirose et al., 1996).

As the obligate electron donor for all microsomal P450s and several other acceptors (Riddick et al., 2013), POR has diverse physiological functions. Not surprisingly, Por-null mice experience multiple developmental defects and embryonic lethality (Shen et al., 2002; Otto et al., 2003). In humans, POR mutations are associated with disordered steroidogenesis and the Antley-Bixler skeletal malformation syndrome and isoform- and substrate-specific alterations in microsomal P450 activities (Pandey and Sproll, 2014).

Our findings support a requirement for PXR in the in vivo induction of rat hepatic POR expression by DEX, especially at the protein and catalytic activity levels. First, POR protein and
activity were induced only by the highest PXR-activating DEX doses (Fig. 4). Second, the GR antagonist RU486 did not inhibit the induction of POR protein and activity by DEX (Fig. 8). Finally, the increase in POR protein and activity by high-dose DEX was completely absent in PXR-knockout rats (Fig. 10). PXR also plays an important role in the regulation of POR expression at the mRNA level, shown most clearly by the induction of POR mRNA levels by PCN, a PXR-selective agonist (Fig. 5), and the attenuated induction of POR mRNA levels by low- and high-dose DEX in PXR-knockout rats (Fig. 9). However, GR also apparently contributes to the DEX induction of POR mRNA levels, as suggested by the modest increase in POR mRNA levels caused by low GR-activating DEX doses (Fig. 3) and the only partial attenuation of POR mRNA induction by DEX in PXR-knockout rats (Fig. 9). The inability of RU486 to block the induction of POR mRNA levels by low-dose DEX may be confounded by this compound’s weak PXR agonist activity (Kliwer et al., 2002).

Although both GR and PXR may contribute to the rat hepatic POR mRNA induction, our study with GR- and PXR-selective agonists suggests that activation of either receptor alone seems insufficient to elevate POR protein and activity (Fig. 6). The unique ability of high-dose DEX to induce POR protein and activity may involve simultaneous or sequential activation of GR and PXR under these conditions. A two-stage sequential GR-PXR cross-talk mechanism may be involved in POR regulation as has been established for the DEX induction of rat CYP3A23 and human CYP3A4; GR activation by low concentrations of DEX increases PXR expression via a transcriptional mechanism, making more PXR protein available for activation by high concentrations of DEX or other PXR agonists (Huss and Kasper, 2000; Pascussi et al., 2001). Under conditions of PXR activation, high DEX doses are reported to increase rat hepatic POR mRNA levels via transcript stabilization (Simmons et al., 1987); however, selective GR
activation at low DEX doses may induce POR expression at the level of transcription. We identified five putative GREs within the proximal 10 kb of the 5’-flanking region of the rat POR gene, but preliminary chromatin immunoprecipitation assays did not detect enhanced GR recruitment to these regions in rat liver tissue at 3 h following i.p. administration of DEX at 1.5 mg/kg (data not shown). Like we observed for ARNT, relatively large changes in POR mRNA levels result in comparatively small changes in POR protein and activity levels; this raises the possible involvement of miRNAs, such as miR-214 (Dong et al., 2014), in POR regulation, with multiple candidate miRNAs under control of nuclear receptors such as PXR (Ramamoorthy et al., 2013). We are exploring these mechanistic aspects of POR regulation in the H4IIE rat hepatoma cell line.

ARNT and POR have essential physiological functions and are independent and indispensable components in the AHR-mediated adaptive and toxic responses to PAHs. This work suggests that the hepatic expression and function of ARNT and POR may be modulated by exposure to exogenous PXR activators and/or conditions that alter glucocorticoid levels such as stress, steroidal therapies, and diseases of excess or deficiency. Our findings are consistent with a role for GR as a key mediator of the induction of rat hepatic ARNT expression by glucocorticoids; a role for PXR in the modulation of ARNT protein levels could not be excluded. Although GR activation may contribute to POR mRNA induction, regulation of POR expression and function by DEX is primarily PXR-mediated.
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Authorship Contributions

*Participated in research design:* Hunter, Vonk, Mullen Grey, and Riddick.

*Conducted experiments:* Hunter, Vonk, Mullen Grey, and Riddick.

*Performed data analysis:* Hunter, Vonk, Mullen Grey, and Riddick.

*Wrote or contributed to the writing of the manuscript:* Hunter, Vonk, Mullen Grey, and Riddick.
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Footnotes

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**Figure Legends**

**Fig. 1.** Effects of subacute DEX treatment in sham-operated (SHAM) and ADX male rats on hepatic POR mRNA levels (A), POR protein levels (B), and POR catalytic activity (C).

(B) Immunoblot of homogenate protein (2 µg) using polyclonal antibody against human POR, showing results for two vehicle (V)- or DEX (D)- treated rats per surgical category. Data represent the mean ± S.D. of determinations from six rats per group, expressed as a percentage of the mean for the SHAM vehicle group. Data were analyzed initially by two-way ANOVA and the P values for the ANOVA main effects are shown in Supplementary Table 1. Outcomes from Bonferroni-corrected post tests or Welch-corrected unpaired t tests were as follows: *significantly different (P < 0.05) from surgery-matched vehicle control; †significantly different (P < 0.05) from treatment-matched SHAM group.

**Fig. 2.** Time-course of the effects of DEX administration to intact male rats on hepatic POR mRNA (A) and protein (B) levels. (B) Immunoblot of homogenate protein (2 µg) using polyclonal antibody against human POR, showing results for one vehicle (V)- or DEX (D)-treated rat per time point. Data represent the mean ± S.D. of determinations from two to five DEX-treated rats per group, expressed as a percentage of the mean for the vehicle-treated controls at each time point. Data were analyzed initially by two-way ANOVA and the P values for the ANOVA main effects are shown in Supplementary Table 2. Outcomes from Bonferroni-corrected post tests were as follows: *significantly different (P < 0.05) from time-matched vehicle control; †significantly different (P < 0.05) from all other treatment-matched time points.
Fig. 3. Dose dependence of the effects of DEX administration to intact male rats on hepatic ARNT (A), TAT (B), CYP3A23 (C), and POR (D) mRNA levels. Data represent the mean ± S.D. of determinations from four rats per group, expressed as a percentage of the mean for the 6-h vehicle group. Data were analyzed initially by two-way ANOVA and the P values for the ANOVA main effects are shown in Supplementary Table 3. Outcomes from Bonferroni-corrected post tests were as follows: * significantly different (P < 0.05) from time-matched vehicle control; † significantly different (P < 0.05) from DEX dose-matched 6-h time point.

Fig. 4. Dose dependence of the effects of DEX administration to intact male rats on hepatic ARNT protein levels (A), POR protein levels (B), and POR catalytic activity (C). (A) Immunoblot of cytosolic protein (30 µg) using polyclonal antibody against human ARNT and monoclonal antibody against β-actin, showing results for one rat per treatment group. H = positive control, cytosol from the Hepa-1 mouse hepatoma cell line with abundant levels of ARNT protein; C = internal control, cytosol from an untreated rat loaded on all gels; UAARP = unidentified ARNT antibody-reactive protein. (B) Immunoblot of microsomal protein (6 µg) using polyclonal antibody against human POR and monoclonal antibody against β-actin, showing results for one rat per treatment group. Data represent the mean ± S.D. of determinations from four rats per group, expressed as a percentage of the mean for the 6-h vehicle group. Data were analyzed initially by two-way ANOVA and the P values for the ANOVA main effects are shown in Supplementary Table 3. Outcomes from Bonferroni-corrected post tests were as follows: * significantly different (P < 0.05) from time-matched vehicle control; † significantly different (P < 0.05) from DEX dose-matched 6-h time point.
**Fig. 5.** Effects of administration of TA, a selective GR agonist, and PCN, a selective PXR agonist, to intact male rats on hepatic ARNT (A), TAT (B), CYP3A23 (C), and POR (D) mRNA levels. Data represent the mean ± S.D. of determinations from four rats per group, expressed as a percentage of the mean for the 6-h vehicle group. Data were analyzed initially by two-way ANOVA and the $P$ values for the ANOVA main effects are shown in Supplementary Table 4. Outcomes from Bonferroni-corrected post tests were as follows: *significantly different ($P < 0.05$) from time-matched vehicle control; †significantly different ($P < 0.05$) from agonist-matched 6-h time point.

**Fig. 6.** Effects of administration of TA, a selective GR agonist, and PCN, a selective PXR agonist, to intact male rats on hepatic ARNT protein levels (A), POR protein levels (B), and POR catalytic activity (C). (A) Immunoblot of cytosolic protein (30 µg) using polyclonal antibody against human ARNT and monoclonal antibody against β-actin, showing results for two vehicle (V)-, TA-, or PCN-treated rats per time point. H = positive control, cytosol from the Hepa-1 mouse hepatoma cell line with abundant levels of ARNT protein; C = internal control, cytosol from an untreated rat loaded on all gels; UAARP = unidentified ARNT antibody-reactive protein. (B) Immunoblot of microsomal protein (6 µg) using polyclonal antibody against human POR and monoclonal antibody against β-actin, showing results for one vehicle (V)-, TA-, or PCN-treated rat per time point. Data represent the mean ± S.D. of determinations from four rats per group, expressed as a percentage of the mean for the 6-h vehicle group. Data were analyzed initially by two-way ANOVA and the $P$ values for the ANOVA main effects are shown in Supplementary Table 4. Outcomes from Bonferroni-corrected post tests were as follows: †significantly different ($P < 0.05$) from agonist-matched 6-h time point.
Fig. 7. Effects of low- and high-dose DEX in the absence and presence of the GR antagonist RU486 in intact male rats on hepatic ARNT (A), TAT (B), CYP3A23 (C), and POR (D) mRNA levels. Data represent the mean ± S.D. of determinations from four rats per group, expressed as a percentage of the mean for the 6-h vehicle/vehicle group. Data were analyzed initially by three-way ANOVA and the $P$ values for the ANOVA main effects are shown in Supplementary Table 5. Outcomes from Bonferroni-corrected post tests were as follows: * significantly different ($P < 0.05$) from time-matched, antagonist-matched vehicle (no DEX) control; † significantly different ($P < 0.05$) from DEX dose-matched, antagonist-matched 6-h time point; ‡ significantly different ($P < 0.05$) from DEX dose-matched, time-matched vehicle (no RU486) control.

Fig. 8. Effects of low- and high-dose DEX in the absence and presence of the GR antagonist RU486 in intact male rats on hepatic ARNT protein levels (A), POR protein levels (B), and POR catalytic activity (C). (A) Immunoblot of cytosolic protein (30 µg) using polyclonal antibody against human ARNT and monoclonal antibody against β-actin, showing results for one rat per treatment group. H = positive control, cytosol from the Hepa-1 mouse hepatoma cell line with abundant levels of ARNT protein; C = internal control, cytosol from an untreated rat loaded on all gels; UAARP = unidentified ARNT antibody-reactive protein. (B) Immunoblot of microsomal protein (6 µg) using polyclonal antibody against human POR and monoclonal antibody against β-actin, showing results for one rat per treatment group. Data represent the mean ± S.D. of determinations from four rats per group, expressed as a percentage of the mean for the 6-h vehicle/vehicle group. Data were analyzed initially by three-way ANOVA and the $P$ values for the ANOVA main effects are shown in Supplementary Table 5. Outcomes from Bonferroni-corrected post tests were as follows: * significantly different ($P < 0.05$) from time-
matched, antagonist-matched vehicle (no DEX) control; †significantly different ($P < 0.05$) from DEX dose-matched, antagonist-matched 6-h time point; ‡significantly different ($P < 0.05$) from DEX dose-matched, time-matched vehicle (no RU486) control.

Fig. 9. Effects of low- and high-dose DEX in intact wild-type and PXR-knockout (PXR-KO) male rats on hepatic ARNT (A), TAT (B), CYP3A23 (C), and POR (D) mRNA levels. Data represent the mean ± S.D. of determinations from three rats per group, expressed as a percentage of the mean for the 6-h vehicle/wild-type group. Data were analyzed initially by three-way ANOVA and the $P$ values for the ANOVA main effects are shown in Supplementary Table 6. Outcomes from Bonferroni-corrected post tests were as follows: *significantly different ($P < 0.05$) from time-matched, genotype-matched vehicle control; †significantly different ($P < 0.05$) from DEX dose-matched, genotype-matched 6-h time point; ‡significantly different ($P < 0.05$) from DEX dose-matched, time-matched wild-type group.

Fig. 10. Effects of low- and high-dose DEX in intact wild-type and PXR-knockout (PXR-KO) male rats on hepatic ARNT protein levels (A), POR protein levels (B), and POR catalytic activity (C). (A) Immunoblot of cytosolic protein (30 µg) using polyclonal antibody against human ARNT and monoclonal antibody against β-actin, showing results for one rat per treatment group. H = positive control, cytosol from the Hepa-1 mouse hepatoma cell line with abundant levels of ARNT protein; UAARP = unidentified ARNT antibody-reactive protein. (B) Immunoblot of microsomal protein (6 µg) using polyclonal antibody against human POR and monoclonal antibody against β-actin, showing results for one rat per treatment group. Data represent the mean ± S.D. of determinations from three rats per group, expressed as a percentage of the mean.
for the 6-h vehicle/wild-type group. Data were analyzed initially by three-way ANOVA and the

\(P\) values for the ANOVA main effects are shown in Supplementary Table 6. Outcomes from

Bonferroni-corrected post tests were as follows:  *significantly different \((P < 0.05)\) from time-

matched, genotype-matched vehicle control; †significantly different \((P < 0.05)\) from DEX dose-

matched, genotype-matched 6-h time point; ‡significantly different \((P < 0.05)\) from DEX dose-

matched, time-matched wild-type group.
Figure 1

A  POR mRNA

B  POR protein

C  POR activity
Figure 2

A  POR mRNA

B  POR protein

Time after DEX exposure (h)
Figure 3

A) ARNT mRNA

B) TAT mRNA

C) CYP3A23 mRNA

D) POR mRNA

[Graphs showing the effect of DEX dose on mRNA levels of ARNT, TAT, CYP3A23, and POR at 6h and 24h.]
Figure 4
Figure 5
Figure 8

A  ARNT protein

B  POR protein

C  POR activity
**Figure 9**

(A) ARNT mRNA

(B) TAT mRNA

(C) CYP3A23 mRNA

(D) POR mRNA

*Note: The figure shows the expression levels of ARNT, TAT, CYP3A23, and POR mRNAs under different conditions.

**Legend:**
- **ARNT / β-actin mRNA (% of 6h vehicle/wild-type):**
  - DEX (mg/kg): 0, 1, 50
  - 6h: open bars, 24h: filled bars

- **TAT / β-actin mRNA (% of 6h vehicle/wild-type):**
  - DEX (mg/kg): 0, 1, 50
  - 6h: open bars, 24h: filled bars

- **CYP3A23 / β-actin mRNA (% of 6h vehicle/wild-type):**
  - DEX (mg/kg): 0, 1, 50
  - 6h: open bars, 24h: filled bars

- **POR / β-actin mRNA (% of 6h vehicle/wild-type):**
  - DEX (mg/kg): 0, 1, 50
  - 6h: open bars, 24h: filled bars

*Significance:
- *: p < 0.05
- †: p < 0.01
- ‡: p < 0.001

**Comparison Groups:**
- **WILD-TYPE**
- **PXRY-KO**