Induction of the Rat Hepatic Aryl Hydrocarbon Receptor Nuclear Translocotor by Glucocorticoids

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science
Graduate Department of Pharmacology and Toxicology
University of Toronto

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2014

ABSTRACT

The aryl hydrocarbon receptor (AHR) nuclear translocator (ARNT), a partner in the hypoxia and AHR signaling pathways, is induced in rat liver by dexamethasone (DEX), a steroid that activates the glucocorticoid (GR) and pregnane X (PXR) receptors. I conducted in vivo rat studies to examine the roles of GR and PXR in ARNT regulation. A selective GR agonist or a low DEX dose activating GR but not PXR increased hepatic levels of ARNT mRNA and an unidentified ARNT antibody-reactive protein (UAARP). The trend for increased ARNT protein levels required high DEX doses activating GR and PXR. A GR antagonist prevented the induction of ARNT mRNA and UAARP levels by low-dose DEX and the increased ARNT protein levels by high-dose DEX. DEX-induced ARNT mRNA and protein levels did not differ between wild-type and PXR-knockout rats. These results support a role for GR in the DEX induction of rat hepatic ARNT expression.
ACKNOWLEDGEMENTS

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Finally, I must express my utmost gratitude to my thoughtful and supportive husband Jeremy. Thank you for all of your help: academic, spiritual, and even the mundane. My success is your success; I am so glad to share this with you.
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<th>Description</th>
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<tbody>
<tr>
<td>ACTH</td>
<td>adrenocorticotropic hormone</td>
</tr>
<tr>
<td>ADM</td>
<td>adrenomedullin</td>
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<tr>
<td>ADPF</td>
<td>AHR degradation-promoting factor</td>
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<tr>
<td>ADX</td>
<td>adrenalectomized</td>
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<tr>
<td>AF-1</td>
<td>activation function domain</td>
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<td>AH</td>
<td>aromatic hydrocarbon</td>
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<td>AHH</td>
<td>aryl hydrocarbon hydroxylase</td>
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<td>aryl hydrocarbon receptor</td>
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<td>aryl hydrocarbon receptor repressor</td>
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<td>AINP</td>
<td>ARNT-interacting peptide</td>
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<td>AHR-interacting protein</td>
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<td>α-naphthoflavone</td>
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<td>ANOVA</td>
<td>analysis of variance</td>
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<td>ARNT</td>
<td>aryl hydrocarbon receptor nuclear translocator (HIF-1β)</td>
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<tr>
<td>B[a]P</td>
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<td>βNF</td>
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<tr>
<td>BHLH</td>
<td>basic helix-loop-helix</td>
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<tr>
<td>Bim</td>
<td>Bcl-2 interacting mediator of cell death</td>
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<td>circadian locomotor output cycles kaput</td>
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<td>COCl₂</td>
<td>cobalt II chloride</td>
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<td>CoCoA</td>
<td>coiled-coil activator</td>
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<td>CORT</td>
<td>corticosterone</td>
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<td>CRF₁</td>
<td>corticotrophin-releasing factor 1</td>
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<td>CRH</td>
<td>corticotrophin-releasing hormone</td>
</tr>
<tr>
<td>CRY</td>
<td>cryptochrome</td>
</tr>
<tr>
<td>Ct</td>
<td>threshold cycle</td>
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<td>Abbreviation</td>
<td>Description</td>
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<td>-------------</td>
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<tr>
<td>CYP</td>
<td>cytochromes P450 (P450)</td>
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<td>DBP</td>
<td>D-element binding protein</td>
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<tr>
<td>DEX</td>
<td>dexamethasone</td>
</tr>
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<td>DHEA</td>
<td>dehydroepiandrosterone</td>
</tr>
<tr>
<td>DME</td>
<td>drug-metabolizing enzymes</td>
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<td>DRB</td>
<td>5,6-dichlorobenzimidazole riboside</td>
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<td>DRE</td>
<td>dioxin response element (AHRE/XRE)</td>
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<td>ER</td>
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<td>7-ethoxyresorufin O-deethylation</td>
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<td>FKBP</td>
<td>FK506 binding protein</td>
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<td>GAC63</td>
<td>GRIP1-associated co-activator 63</td>
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<td>GILZ</td>
<td>glucocorticoid-induced leucine zipper</td>
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<td>glucose transporter 2</td>
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<tr>
<td>GR</td>
<td>glucocorticoid receptor (NR3C1)</td>
</tr>
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<td>GRE</td>
<td>glucocorticoid response element</td>
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<td>GST</td>
<td>glutathione S-transferase</td>
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<tr>
<td>HAH</td>
<td>halogenated aromatic hydrocarbon</td>
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<td>HC</td>
<td>hydrocortisone</td>
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<td>HIF</td>
<td>hypoxia-inducible factor</td>
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<td>Hop</td>
<td>hsp70/hsp90 organizing protein</td>
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<td>HPA</td>
<td>hypothalamus-pituitary-adrenal</td>
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<td>HRE</td>
<td>hypoxia response element</td>
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<td>HSD3B</td>
<td>3β-hydroxysteroid dehydrogenase/Δ5-Δ4 isomerase</td>
</tr>
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<td>hsp</td>
<td>heat shock protein</td>
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<tr>
<td>I3C</td>
<td>indole-3-carbinol</td>
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<td>IARC</td>
<td>International Agency for Research on Cancer</td>
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<td>indolo[3,2-b]carbazole</td>
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<td>IkB</td>
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<td>interleukin</td>
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<td>MC2R</td>
<td>melanocortin-2-receptor</td>
</tr>
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<td>mGR</td>
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<tr>
<td>miRNA</td>
<td>microRNA</td>
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<tr>
<td>MMLV</td>
<td>Moloney murine leukemia virus</td>
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<tr>
<td>MR</td>
<td>mineralocorticoid receptor</td>
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<tr>
<td>MRP2</td>
<td>multi-drug resistance-associated protein 2</td>
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<td>MT2</td>
<td>metallothionein</td>
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<tr>
<td>NF-κB</td>
<td>nuclear factor kappa-light-chain-enhancer of activated B cells</td>
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</table>
nGRE  negative GRE
NLS   nuclear localization signal
NPAS  neuronal PAS domain protein
NQO1  NAD(P)H: quinone oxidoreductase 1
NR    nuclear receptor
p300  E1A binding protein p300
PAGE  polyacrylamide gel electrophoresis
PAH   polycyclic aromatic hydrocarbon
PAL-1  peroxisomal ABC transporter-like protein
PARP  poly (ADP-ribose) polymerase
PAS   PER-ARNT-SIM
PCN   pregnenolone-16α-carbonitrile
PCR   polymerase chain reaction
PEPCK phosphoenolpyruvate carboxykinase
PER   period
POMC  proopiomelanocortin
POR   NADPH-cytochrome P450 oxidoreductase
PR    progesterone receptor
PXR   pregnane-X-receptor (NR1I2)
PXRE  PXR response element
PXRKO PXR knockout
RNAi  RNA interference
ROS   reactive oxygen species
RT    relative fold change
RT-PCR reverse transcriptase-polymerase chain reaction
RU486 mifepristone
RXR   retinoid-X-receptor
SCN   suprachiasmatic nucleus
SD    standard deviation
SDS   sodium dodecyl sulphate
SIM   single-minded protein
SNP   single-nucleotide polymorphism
SRC   steroid receptor co-activator (NCOA)
StAR  steroidogenic acute regulatory protein
STAT  signal transducer and activator of transcription
SUMO-1 small ubiquitin-like modifier 1
SWI/SNF SWI/Sucrose NonFermentable
TA    triamcinolone acetonide
TAD   transactivation domain
TAT   tyrosine aminotransferase
TCDD  2,3,7,8-tetrachlorodibenzo-p-dioxin
TCR   T-cell receptor
TiPARP 2,3,7,8-tetrachlorodibenzo-p-dioxin–inducible poly (ADP-ribose) polymerase
TNF-α tumor necrosis factor-alpha

xiii
UAARP  unidentified ARNT antibody-reactive protein
UGT    uridine diphosphate-glucuronosyl transferase
VEGF   vascular endothelial growth factor
VEH    vehicle
VHL    Von Hippel Lindau
WT     wild-type
XAP-2  hepatitis B virus X-associated protein 2 (Ara9/AIP)
LIST OF THESIS PUBLICATIONS

Abstracts


S.R. Hunter and D.S. Riddick. (2013). Glucocorticoid induction of the aryl hydrocarbon receptor nuclear translocator in rat liver. *Drug Metabolism Reviews, 45*(S1), 140. [10th International ISSX Meeting; Toronto, Ontario; September-October 2013]

SECTION 1: INTRODUCTION

1.1 STATEMENT OF THE RESEARCH PROBLEM

The aryl hydrocarbon receptor nuclear translocator (ARNT, HIF-1β) is a promiscuous member of the basic-helix-loop-helix Per-ARNT-Sim (bHLH-PAS) family of gene regulatory proteins. It is important in xenobiotic metabolism, development, and hypoxic response. As a heterodimeric partner of transcription factors, it primarily functions in two significant signaling pathways: the dioxin-responsive aryl hydrocarbon receptor (AHR) signaling pathway, and the hypoxia signaling pathway. There is evidence to suggest crosstalk between these pathways and the glucocorticoid signaling pathway. The glucocorticoid signaling pathway can work in concert with the activated transcription factors in these pathways, AHR and hypoxia-inducible factor-1α (HIF-1α), to co-regulate certain genes, some of which are AHR or HIF targets. The molecular mechanisms for crosstalk among these signaling pathways remain unclear. These signaling pathways may interact due to shared chaperone and co-regulatory proteins. Glucocorticoids modulate AHR and HIF-1α expression, but this modulation is cell- and species-dependent. This thesis examined the regulation of rat hepatic ARNT expression by glucocorticoids, with a specific emphasis on characterizing the role of the glucocorticoid receptor (GR, NR3C1) and the pregnane X receptor (PXR, NR1I2) in this process. This research may provide insight into how signaling pathways that depend on ARNT may be modulated in conditions involving altered glucocorticoid levels, e.g. stress, diseases of excess or deficiency, and pharmacotherapy.
1.2 ARYL HYDROCARBON RECEPTOR NUCLEAR TRANSLOCATOR

1.2.1 Dimerization Partners of ARNT

ARNT heterodimerizes with AHR, HIF-1α, and single-minded (SIM) proteins to regulate gene expression. The expressed genes from each pathway participate in xenobiotic metabolism, hypoxia response, and development, respectively. Although these are the most significant signaling pathways involving ARNT, ARNT also binds to other bHLH-PAS proteins involved in embryogenesis, neurogenesis, and the negative regulation of the dioxin-responsive signaling pathway. ARNT can homodimerize and subsequently bind the E-box, inducing transcription of target genes such as the cytochrome P450, Cyp2a5 (Arpiainen et al., 2007). However, this interaction was found to be weak or absent in experiments using the yeast-two hybrid systems (Reisz-Porszasz et al., 1994; Hirose et al., 1996)

1.2.2 Health Issues Involving ARNT

There are serious health issues associated with ARNT dysregulation. ARNT-knockout mice die during embryonic development due to abnormal vascularization in the yolk sac (Maltepe et al., 1997) or placenta (Kozak et al., 1997). ARNT has been implicated in diabetes, cancer, and inflammation (Gunton et al., 2005; Shi et al., 2010; Eltzshig and Carmeliet, 2011). Loss of ARNT in vitro and in vivo decreased insulin secretion and altered islet gene expression, similar to what is seen in pancreatic islet cells of humans with type 2 diabetes (Gunton et al., 2005). ARNT’s role in the AHR signaling pathway has implicated it in bladder, lung, and colorectal cancer and leukemia (Salomon,-Nguyen et al., 2000; Figueroa et al., 2008; Shi et al., 2010; Wang
et al., 2011). ARNT’s association with some cancers involves single nucleotide polymorphisms (SNPs) of the ARNT gene. A SNP in the promoter region of the ARNT gene, rs7517566, was associated with increased risk of bladder cancer in humans (Figeuroa et al., 2008), while another SNP in the ARNT gene, rs12410394, was significantly inversely associated with colorectal cancer (Wang et al., 2011). ARNT’s role in inflammation will be discussed in more detail in the section on crosstalk with the nuclear factor kappa-light-chain- enhancer of activated B cells (NF-κB) signaling pathway (Section 1.5.3) and the GR signaling pathway (Section 1.5.4). Dysregulation of ARNT expression and function can have adverse effects on the health of mammalian species including humans.

1.2.3 Molecular Structure of ARNT

The ARNT protein is constitutively and ubiquitously expressed in mammalian tissues (Brooks et al., 1989; Carver et al., 1994, Jain et al., 1998). The rat ARNT protein is 88 kilodaltons (kDA). In human hepatoma cells (Hep3B), ARNT protein has a half life of 4.84 hours (Choi et al., 2006). It is localized in the nucleus, although it can be found in the cytoplasm of embryonic cells and routinely distributes to the cytosolic fraction when tissue or cell homogenates are centrifuged (Pollenz et al., 1994; Abbott et al., 1995). As a β-class bHLH-PAS protein, it is an obligatory, heteromdimeric partner of α-class bHLH-PAS transcription factor proteins (Gu et al., 2000). A representation of these proteins is shown in Fig.1.1. The nuclear localization signal (NLS) that maintains ARNT’s position in the nucleus is in the amino terminal region.
Figure 1.1. Domain organization of the bHLH-PAS proteins. Simplified diagrammatic representation of key bHLH-PAS proteins. Coloured blocks indicate regions of homology. The functions of conserved regions are noted on the AHR protein, and apply, in general, to the other members shown. Of the proteins shown here, only AHR is known to bind ligand. Please refer to text for details. NLS, nuclear localization signal; bHLH, basic helix-loop-helix; A and B: PAS A and PAS B; TAD, transactivation domain; Q: glutamine rich (Adapted from Dougherty and Pollenz, 2010; Mullen Grey, 2011).
The PAS A and PAS B domains, along with the HLH domain, are required for ARNT dimerization (Murre et al., 1989; Reisz-Porszasz et al., 1994). The basic portion of the bHLH region is the DNA binding domain (Ferré-D’Amaré et al., 1994). The glutamine-rich transactivation domain (TAD) is located towards the C-terminus. There is 96% amino acid sequence homology between the rat protein and the human protein in the bHLH domain, and 86% homology between the two species in the PAS domains. It is uncertain how the ARNT protein is degraded, but studies suggest degradation may be through the ubiquitin-proteasome pathway (Choi et al., 2006; Wang et al., 2009a).

**1.2.4 ARNT Isoforms**

The *ARNT* gene is 65 kilobases (kb) in length with 22 exons. In rat and mouse, there is an ARNT transcript missing exon 5, and it is expressed approximately half to equal amounts compared to the full length wild-type transcript (Korkalainen et al., 2003). The difference in function is unknown between the two splice variants in mice and rat (Wang et al., 1998). The variant and the wild-type ARNT are expressed in almost all tissues in Long–Evans (L-E) rats (Jana et al., 1998). In Han Wistar and L-E rats, several variants were found: a deletion at the 3’ end of exon 6, at the 5’ end of exon 11, and an insertion at the 5’ end of exon 20 (Korkalainen et al., 2003). Although the function of each variant is unknown, it is possible that the deletions in exon 5 and 6 may impair protein function in rats, as this is the location of the bHLH domain (Korkalainen et al., 2003). In breast cancer cells, the ratio of a novel splice variant (missing amino acids 330-789) to wild-type ARNT may be used as a positive prognostic indicator (Qin et al., 2001). In 101 samples from human lymphoma blast cell lines there
are 5 unique SNPs at the ARNT locus (chromosome1q21). Three were non-synonymous, but found at low frequencies. *In silico* analysis suggests that one of the non-synonomous SNPs could potentially affect the function of the encoded ARNT protein (Urban et al., 2011).

Other ARNT isoforms from the β-class of bHLH-PAS proteins are ARNT2 (HIF-2β), ARNT3 (BMAL1, MOP3, ARNTL), and ARNT4 (BMAL2, MOP9, CLIF, ARNTL2). Hirose and colleagues cloned ARNT2 in 1996 (Hirose et al., 1996). Of the 13 amino acids in the basic region, 12 are identical to ARNT. There is an 80% amino acid sequence homology over the HLH and PAS regions (Hirose et al., 1996). ARNT2 binds HIF-1α, HIF-2α and neuronal PAS domain protein 4 (NPAS4), which means it is involved in hypoxia response and neurogenesis (Maltepe et al., 2000, Ooe et al., 2004; Chavez et al., 2006). It is controversial whether or not ARNT2 is a heterodimeric partner of the AHR. Hirose et al. (1996) found that ARNT2 bound AHR and activated transcription as efficiently as ARNT. However, following this study, Dougherty and Pollenz (2008) found that ARNT2•AHR heterodimer did not initiate transcription of CYP1A1 in murine hepatocellular carcinoma, Hepa-1 cells that were ARNT-deficient. The Fujii-Kuriyama group had similar results. They found that ARNT2•AHR complex did not induce transcription of CYP1A1 (Sekine et al., 2006).

ARNT3 and its paralog ARNT4 are important regulators of circadian rhythm. The mammalian suprachiasmatic nucleus (SCN) in the anterior hypothalamus is the site of the master clock regulating these rhythms. ARNT3 and ARNT4 are ubiquitously expressed [reviewed in: (McIntosh et al., 2010)]. Environmental signals cue ARNT3 or
ARNT4 to heterodimerize with the circadian locomotor output cycles kaput (CLOCK) or NPAS2 (MOP4) and, along with co-activators, initiate transcription of hundreds of genes that are involved in physiological, behavioural, and metabolic processes on a 24-hour cycle. CLOCK•ARNT3 and CLOCK•ARNT4 induce their own negative regulation by transcribing genes that function in a negative feedback loop. Period (PER1, PER2, and PER3) and cryptochrome (CRY1 and CRY2) proteins repress the CLOCK•ARNT3 complex and cause a necessary delay in transcription of target genes [reviewed in: (McIntosh, 2010; Labrecque et al., 2013)]. Transcription of the nuclear receptor Rev-Erba/NR1D1 also inhibits this pathway by binding to the promoter region on the ARNT3 gene and attenuating transcription (reviewed in: [McIntosh et al., 2010; Labrecque et al., 2013]). ARNT3 is also important in the hypoxia signaling pathway as it is a heterodimeric partner of HIF-1α and HIF-2α (Hogenesch et al., 1997, Labrecque et al., 2013). Among disorders associated with a dysfunctional circadian rhythm, ARNT3 has been associated with type II diabetes and cancer (Mullenders et al., 2009; Rome, 2009; Zheng et al., 2010). ARNT4 has been implicated in type I diabetes, cancer, and Parkinson’s disease (Hung et al., 2006; Ding et al., 2011; Mazzoccoli et al., 2012).

1.2.5 ARNT Modulation

Our understanding of the modulators of ARNT expression and function is still in its formative stages. ARNT expression is modulated at the post-transcriptional level by microRNAs (miRNAs) such as miR-24 (Oda et al., 2012), miR-107 (Yamakuchi et al., 2010), and miR-221 (Yuan et al., 2013). At a post-translational level, ARNT protein is modified by the small ubiquitin-like modifier 1 (SUMO-1) protein in vitro and in vivo,
augmenting ARNT’s transcriptional activity (Tojo et al., 2002). Phosphorylation of ARNT is crucial for AHR dimerization and DNA binding [reviewed in: (Swanson, 2002)]. A recent study showed that an ARNT-interacting peptide (Ainp-1) reduced ARNT•HIF-1α dimerization and ultimately reduced hypoxia signaling (Wang et al., 2012a). Ainp-2 interacts with ARNT and enhances AHR signaling (Li et al., 2005). The ARNT interacting protein (AINT) also binds to ARNT and represses its function by localizing it to the cytoplasm (Sadek et al., 2000). β-tubulin also interacts with ARNT and shifts its localization to the cytoplasm, thereby reducing the function of ARNT (Zhang et al., 2010). Additionally, curcumin, the common spice and colouring agent, induces the ubiquitination and proteasomal degradation of ARNT via an oxidative stress mechanism (Choi et al., 2006; 2008). Finally, ARNT protein decreased with increasing glucose concentrations in the mouse pancreatic beta cell line MIN6 transfected with the human ARNT gene (Dror et al., 2008).

At a transcriptional level there are a few known modulators of ARNT expression. The carbohydrate-responsive element binding protein (ChREBP) is a repressor of ARNT expression in pancreatic islet β-cells (Nordeen et al., 2010). Kainic acid induces ARNT expression in hippocampus glial cells in rats (Kitamura, 2000). The clock-controlled gene, D-element binding protein (Dbp), binds to the promoter region of the Arnt gene in Min6 cells and when DBP is overexpressed in these cells it upregulates ARNT protein expression (Nakabayashi et al., 2013). Activated NF-κB increases ARNT mRNA and protein levels in Drosophila, and in cultured cells of mouse and human origin (van Uden et al., 2011). Also, mouse ARNT mRNA was induced in
response to the hepatotoxin pyrazole (Nichols et al., 2008). Hydrocortisone (HC) reduced ARNT mRNA expression in the developing embryonic mouse palate (Abbott et al., 1999). Two other studies showed that glucocorticoids increased ARNT mRNA levels in rats treated with either methylprednisolone (Almon et al., 2005) or dexamethasone (DEX) (Mullen Grey and Riddick, 2009). These studies will receive further consideration in the development of my research hypothesis.

1.3 AHR SIGNALING

1.3.1 Signal Transduction Pathway

The AHR•ARNT heterodimer binds the cognate enhancer sequence and initiates the transcription of genes involved in metabolism, development, homeostasis, and functions of the immune system ([reviewed in: (Beischlag et al., 2008)]. The transcriptional pathway is shown in Fig 1.2. The AHR resides in the cytoplasm as a multimeric complex with a 90 kDa heat shock protein (hsp) dimer (Denis et al., 1988; Perdew, 1988), the hsp90 co-chaperone p23 (Nair et al., 1996), and the AHR-interacting protein (AIP), also named AHR-associated protein 9 (Ara9) or hepatitis B virus X-associated protein 2 (XAP2) (Meyer et al., 1998). Upon activation with a ligand, the AHR translocates to the nucleus, sheds the chaperone complex, and heterodimerizes with ARNT. The heterodimer binds to dioxin response elements (DREs) and with the help of co-activators initiates transcription of target genes such as CYP1A1 (Watson and Hankinson, 1992), CYP1A2 (Quattrochi et al., 1994), CYP1B1 (Zhang et al., 1998), and the AHR repressor (AHRR) (Baba et al., 2001).
Figure 1.2 AHR and hypoxia signaling pathways. Please refer to the text for details. Abbreviations are defined in the text.
1.3.2 Regulatory Proteins in AHR signaling

The transcriptional activity of AHR is inhibited by the AHRR protein. Inhibition of AHR-mediated target gene expression by AHRR may involve sequestering ARNT as a heterodimeric partner and preventing the binding of AHR•ARNT to DREs by the DNA-bound AHRR•ARNT complex (Mimura et al., 1999). AHRR orthologs have been found in mammalian, avian, and piscine species (Korkalainen et al., 2004; Lee et al., 2011). In rats, AHRR is ubiquitously expressed, mirroring the human expression pattern with highest concentration in the testis and low concentration in the liver (Korkalainen et al., 2004; Nishihashi et al., 2006). Polymorphisms in the AHRR gene are associated with infertility and cancer (Fujita et al., 2002; Cauchi et al., 2003; Watanabe et al., 2004; Brokken et al., 2013). The AHRR could possibly play a role in inflammation. Baba and colleagues found a functional NF-κB regulatory element in the promoter region of the murine Ahrr gene (Baba et al., 2001). NF-κB is a key regulator of multiple cytokines that play important roles in immune and inflammatory responses. Also in the 5’-flanking region of the rat Ahrr is a putative glucocorticoid response element (GRE) (Nishihashi et al., 2006), and glucocorticoids have well-known anti-inflammatory properties.

A labile repressor protein may regulate the AHR signaling pathway by stimulating the degradation of the AHR. 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) induces AHR degradation through the ubiquitin 26S proteasome pathway and this degradation is inhibited by the proteasome inhibitor MG132 in mouse hepatoma Hepa-1c1c7 cells (Ma et al., 2000a). The inhibition of AHR proteasomal degradation
increases the amount of AHR•ARNT complex and superinduces the AHR•ARNT target gene Cyp1a1 in the Hepa cells (Ma et al., 2000a). In a second study by the Ma team, co-treatment of Hepa cells with TCDD and cycloheximide (CHX) resulted in superinduction of Cyp1a1 and inhibition of AHR degradation, suggesting that an AHR degradation-promoting factor (ADPF) is a labile repressor protein involved in the proteasomal degradation of AHR (Ma et al., 2000b).

Another group of researchers demonstrate that 2,3,7,8-tetrachlorodibenzo-p-dioxin–inducible poly (ADP-ribose) polymerase (TiPARP, ARTD14, PARP-7), a member of the PARP family known for ribosylating proteins or histones, may be an ADPF candidate protein (MacPherson et al., 2013). In human hepatoma HuH-7 cells, TiPARP was knocked down by RNA interference (RNAi) and this showed a greater induction of CYP1A1 and CYP1B1 mRNA, a greater recruitment of AHR•ARNT to the enhancer regions of these genes, and a reduction in AHR degradation (MacPherson et al., 2013). Overexpression of TiPARP resulted in an increase in repression of TCDD-induced AHR transactivation, and this TiPARP-dependent repression was independent of ARNT (MacPherson et al., 2013). Cyp1a1 expression increases in mouse embryonic fibroblast (MEF) cells co-treated with TCDD and CHX (MacPherson et al., 2013). TiPARP may be an ADPF protein, regulating the AHR signaling pathway by promoting the degradation of the AHR.

Several proteins may function as co-activators in the AHR signaling pathway. Most of the co-activators promote transcription in three ways. First, they can acetylate histones resulting in less condensed chromatin structure, thus allowing the RNA
polymerase and the transcriptional machinery to bind and assert their affect. The E1A binding protein p300 (p300) and the CREB-binding protein (CBP) are histone acetyltransferases that bind to ARNT and increase transcription (Kobayashi et al., 1997). E1A and TWIST inhibit this acetyltransferase activity (Hammamori et al., 1999).

Secondly, certain co-activator proteins can provide energy as part of an ATPase subunit. Brahma/SWI2-related gene-1(Brg-1) is the ATPase subunit of the SWIItch/Sucrose NonFermentable (SWI/SNF) chromatin-remodeling complex. It associates with AHR to promote transcription (Wang et al., 2002). Thirdly, other co-activators are adapter proteins facilitating an interaction between transcription factors and the transcriptional machinery. Steroid receptor coactivaor (SRC)-1 and SRC-2 bind to the N-terminal of ARNT and act as adapters for other co-activators to bind, such as coiled-coil activator (CoCoA) and GRIP1-associated co-activator 63 (GAC63) [reviewed in: (Labrecque et al., 2013).

### 1.3.3 AHR Ligands

Chemicals that modulate AHR activity can be classified as: 1) agonists, 2) selective AHR modulators, 3) antagonists, and 4) indirect regulators (Fig.1.3). The agonists are anthropogenic, natural, or endogenous. The high-affinity, exogenous agonists created by combustion or as industrial by-products are polycyclic aromatic hydrocarbons (PAHs) and halogenated aromatic hydrocarbons (HAHs). TCDD is the most notorious HAH, made infamous in the poisoning of former Ukrainian President Victor Yushchenko, and as the key toxic contaminant in Agent Orange. 3-Methylcholanthrene (3-MC) and benzo[a]pyrene (B[a]P) are representative PAHs.
HAH (Agonist)  

![Structure of HAH (Agonist)](image1)

2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)

Indirect Regulator

![Structure of Omeprazole](image2)

omeprazole

PAHs (Agonists)

![Structure of 3-Methylcholanthrene](image3)

3- methylcholanthrene (3-MC)

![Structure of Benzo[a]pyrene](image4)

benzo[a]pyrene (B[a]P)

Natural Ligands (Agonists)

![Structure of Indole-3-carbinol](image5)

indole-3-carbinol (I3C)

![Structure of 6-Formylindolo[3,2-b]carbazole](image6)

6-Formylindolo[3,2-b]carbazole (FICZ)

Selective AHR Modulator

![Structure of Substituted Diindolylmethane](image7)

substituted diindolylmethane

Antagonist/Partial Agonist

![Structure of α-Naphthoflavone](image8)

α-naphthoflavone (αNF)

Figure 1.3 Structures of selected chemical modulators of AHR activity.
Both HAHs and PAHs activate the AHR signaling pathway, ultimately inducing a broad range of target genes. Although HAHs and PAHs induce many of the same drug metabolizing enzymes (DMEs), PAHs such as 3-MC are generally less potent than HAHs such as TCDD (Poland and Knutson 1982). Whereas TCDD is poorly metabolized, 3-MC is metabolized readily by the DMEs it induces, thus reducing its apparent biological potency (Okey et al., 1994; Riddick et al., 1994; Okino and Whitlock, 2000; Parkinson, 2001). High doses of the high affinity agonists are associated with thymic involution, hepatotoxicity, tumour promotion, epithelial hyperplasia, teratogenesis, wasting, and death in experimental animals [reviewed in: (Nguyen and Bradfield, 2008)]. The skin disorder, chloracne, is a hallmark sign of high dose dioxin exposure in humans [reviewed in: (Nguyen and Bradfield, 2008)]. Whether effects occur in humans at low chronic exposure levels remains controversial (Paarzefall, 2002; Longnecker et al., 2003; and Rogan and Ragan, 2003). The International Agency for Research on Cancer (IARC) classifies TCDD as a human carcinogen (International Agency for Research on Cancer, 1997).

Some AHR agonists are naturally derived. They may come from plants, such as the flavonoids quercetin and galangin, and the weak agonist indole-3-carbinol (I3C) found in cruciferous vegetables (Bjeldanes et al., 1991; Ashida et al., 2000; Zhang et al., 2003). I3C is thought to be metabolized during digestion into a potent AHR ligand, indolo[3,2-b]carbazole (ICZ) (Bjeldanes et al., 1991). The AHR may have originally regulated the metabolism of endogenous compounds and evolved to regulating metabolism of exogenous compounds (Hahn et al., 2002). Mammalian endogenous compounds that activate the AHR include the heme degradation product bilirubin,
metabolites of arachidonic acid such as lipoxin A4 (i.e. Lipoxin A4), and derivatives of tryptophan such as 6-formylindolo[3,2-b]carbazole (FICZ) (Denison and Nagy, 2003). FICZ is the most potent endogenous agonist with the highest affinity among the naturally occurring ligands (Wincent et al., 2009).

Selective AHR modulators bind to AHR in the ligand binding domain and initiate a biological response; however, the responses are not mediated by a canonical DRE-dependent pathway (Safe et al., 1999; Patel et al., 2009; Murray et al., 2010). Ligands such as Way-169916 and substituted diindolylmethanes activate the AHR, which translocates to the nucleus, and binds ARNT, but the result is preferential induction or repression of the transcription of non-canonical responsive genes (Safe et al., 1999; Murray et al., 2010). Selective AHR modulators may offer the promise of separating beneficial aspects of AHR activation from toxic aspects.

The third kind of modulator includes the AHR antagonists (or partial agonists). Antagonists such as α-naphthoflavone (αNF) bind to the ligand binding domain and inhibit the agonist-induced conformational change of AHR, thus preventing it from translocating to the nucleus and initiating transcription of target genes [reviewed in: (Murray et al., 2011)]. Like many AHR antagonists, αNF is more correctly termed a partial agonist since it displays weak agonist activity at high concentrations (Santostefano et al., 1993). A pure AHR antagonist with no detectable agonist activity is 2-methyl-2H-pyrazole-3-carboxylic acid (CH-223191) (Kim et al., 2006).

Unlike the other modulators, indirect regulators do not bind to the ligand binding domain. Instead, they affect the expression of AHR target genes either by inhibiting
phosphorylation of the AHR or by binding the chaperone proteins. For example, chemicals such as omeprazole can indirectly activate the AHR by increasing phosphorylation of the AHR; this is important for shuttling the AHR between the nucleus and the cytoplasm (Kikuchi et al., 1998). Indirect regulators with antagonistic properties include epigallocatechin gallate (EGCG), which is capable of binding to the hsp90 component of cytoplasmic AHR complex. This still allows a ligand to bind and the complex to translocate to the nucleus; however, the newly formed complex is unable to shed the chaperone complex in order to bind DNA (Palermo et al., 2005).

1.4 HYPOXIA SIGNALING

1.4.1 Signal Transduction Pathway

A state of hypoxia occurs when oxygen levels are insufficient to meet metabolic demands. The body responds by increasing the amount of HIF-1α protein. This protein translocates to the nucleus and heterodimerizes with ARNT. This heterodimeric complex binds hypoxia response elements (HREs) and initiates transcription of target genes, many of which function to restore a normal oxygen supply or to enhance glucose utilization (Fig 1.2). Two examples of such genes are the vascular endothelial growth factor (VEGF) involved in angiogenesis, and erythropoietin (EPO), which controls red blood cell production. Under normoxic conditions HIF-1α is hydroxylated on proline residues and therefore recognizable by the Von Hippel Lindau (VHL) tumor suppressor E3-ligase complex. This complex targets HIF-1α for ubiquitination and proteasomal degradation (Maxwell et al., 1999; Kallio et al., 1999). The HIF-1α homologue, HIF-2α and paralog, HIF-3α are able to bind ARNT (Hogenesch et al.,
1997; Gu et al., 1998). HIF-2α initiates transcription of many of the same genes as HIF-1α during hypoxia and it is proteasomally degraded in a similar fashion [reviewed in: (Labrecque et al., 2013)]. A key distinction between the two is that HIF-1α is ubiquitously expressed and HIF-2α is cell and tissue-specific (Tian et al., 1997; Jain et al., 1998). HIF-3α negatively regulates the hypoxia signaling pathway, by inhibiting the induction of HIF target genes (Hara et al., 2001; Maynard et al., 2005). It has been suggested that the inhibition of induction is due to HIF-3α competitively binding to ARNT, HIF-1α, or both (Hara et al., 2001, Maynard et al., 2005). Other mechanisms to regulate this pathway are being investigated for possible cancer treatments [reviewed in: (Yang et al., 2013)]. This is because HIF-1α is upregulated in solid tumours and HIF target genes such as VEGF may promote the progression of cancer through angiogenesis (Zhong et al., 1999).

1.5 CROSSTALK IN RELEVANT SIGNALING PATHWAYS

1.5.1 AHR versus Hypoxia Signaling

ARNT may be a limiting factor because it is shared between the AHR and hypoxia signaling pathways. In 1996, Gradin and colleagues found that ARNT has a higher affinity for HIF-1α than for AHR (Gradin et al., 1996). Since then, studies have produced conflicting results as to whether ARNT is limiting due to its role in these two major signaling pathways. In transfected human hepatocellular carcinoma HepG2 101L cells, DRE-driven transcription of a reporter gene was inhibited by hypoxia and HRE-driven transcription of a reporter gene was inhibited by AHR agonists (Chan et al., 1999). Surprisingly, when they looked at the endogenously expressed EPO gene they
found that its induction under hypoxia was enhanced by AHR activation, possibly due to a DRE located in the promoter region of this gene (Chan et al., 1999). Similar to the reporter gene results found by Chan and colleagues, hypoxia inhibited TCDD-driven transcription in human hepatoma B-1 and Hepa-1 cells, and TCDD inhibited hypoxia-driven transcription in B-1 cells (Nie et al., 2000). However, these results were not replicated in rat hepatoma H4IIE cells (Nie et al., 2000). In chicken embryos, there was a decrease in hypoxia-induced HIF-1α levels and VEGF expression when embryos were exposed to TCDD (Ivinitski-Steele et al., 2004).

A plethora of evidence shows that under hypoxic conditions AHR activity is reduced. Under hypoxic conditions the expression of AHR responsive genes is reduced in Hepa-1 cells (Gassman et al., 1997; Kim and Sheen, 2000), in zebrafish (Prasch et al., 2004), and in primary mouse hepatocytes (Allen et al., 2005). Induction of CYP1A1 and its enzymatic activity are reduced by hypoxia in primary mouse hepatocytes lacking HIF-1α, indicating a HIF-independent mechanism (Allen et al., 2005). Similarly, in H4IIE and Hepa-1 cells, TCDD induction of CYP1A1 was reduced under hypoxic conditions, but only 15% of the ARNT pool was sequestered to the hypoxia signaling pathway. This further suggests an indirect effect of HIF (Pollenz et al., 1999). Proteins regulated by HIF-1 under hypoxic conditions may contribute to the reduction in AHR responsiveness (Kahn et al., 2007). It appears that the hypoxia signaling pathway inhibits the AHR pathway, with less evidence for the reciprocal, but the results differ between species, cells, and genes measured, and they depend on AHR agonists used, and ways to induce hypoxia. It is inconclusive whether ARNT is a
limiting factor in these two signaling pathways because of the amount of conflicting evidence.

1.5.2 AHR versus ER Signaling

Much like the crosstalk between hypoxia and AHR signaling pathways, interaction of the AHR pathway (and indeed ARNT itself) with the estrogen receptor (ER) signaling pathway is complex. Studies have found that activated AHR can either inhibit or enhance expression of 17β-estradiol (E2)-responsive genes. Reciprocally, there is evidence to suggest that activation of the ER signaling pathway can inhibit, enhance, or have no effect on the transcription of AHR•ARNT target genes. As the heterodimeric partner of AHR, HIF-1α or on its own, ARNT interacts with the ER signaling pathway as discussed below.

1.5.2.1 AHR Signaling Inhibits ER Signaling

Many diverse studies suggest an inhibitory impact of the AHR signaling pathway on E2-mediated responses [reviewed in: (Safe and Wormke, 2003)]. Early research indicated a reduction in spontaneous mammary and uterine tumour growth by chronic ingestion of TCDD in rats (Kociba et al., 1978). Further association between the pathways was considered when rats, co-treated with TCDD and E2, showed a decrease in E2-induced transcription and uterine wet weight gain (Romkes et al., 1987; Astroff et al., 1991). A number of studies then indicated that TCDD inhibited numerous E2-induced genes/proteins and their related activities such as cathepsin D (Krishnan et al., 1995), c-fos (Duan et al., 1999), and pS2 (Gillesby et al., 1997). The inhibition may
be a result of: i) accelerated E2 metabolism by TCDD-induced DMEs (Spink et al., 1990), ii) ERα proteasomal degradation (Wormke et al., 2003), iii) competition between AHR•ARNT and ERα for co-regulatory proteins (Kobayashi et al., 1997, 2000; Kumar and Perdew, 1999; Beischlag et al., 2002, Nguyen et al., 1999), iv) an inhibitory DRE in the ERα target genes, and v) indirect inhibition by gene products upregulated by activated AHR•ARNT [reviewed in: (Safe and Wormke, 2003)]

1.5.2.2 AHR Signaling Augments ER Signaling

Some studies suggest that AHR activation can have estrogenic effects. The ligand-activated AHR•ARNT complex was shown to associate with the unliganded ER, leading to recruitment of ER and transactivation of its target genes in cultured cells and the uterus of ovariectomized mice (Ohtake et al., 2003). This estrogenic response may be AHR-independent, as this effect was not attenuated in Ahr-null mice and likely explained by the direct binding and activation of ER by PAHs and metabolites (Fertuck et al., 2001; Abdelrahim et al., 2006). A subset of ER target genes was induced by TCDD in the uterus of ovariectomized mice (Boverhof et al., 2006).

1.5.2.3 ER Signaling Augments AHR Signaling

Under some conditions, ER signaling enhances AHR signaling. When human breast adenocarcinoma MDA-MB-231 (AHR non-responsive) cells were transfected with hER, this restored AHR responsiveness (Thomsen et al., 1994). In human breast adenocarcinoma MCF-7 cells, E2 increased expression of TCDD-induced CYP1A1 and CYP1B1 mRNA, protein, and activity (Spink et al., 2003). Similarly, TCDD stimulated
ERα recruitment to CYP1A1 and CYP1B1 promotor regions and this recruitment was
enhanced by E2 co-treatment (Matthews et al., 2005). Other studies have found that ER signaling has no effect on AHR signaling (Hoivik et al., 1997; Wormke et al., 2000).

1.5.2.4 ER Signaling Represses AHR Signaling

There are some studies suggesting the opposite: that ER signaling inhibits AHR signaling. The TCDD-induced CYP1A1 mRNA expression in MCF-7 cells and human endometrial ECC-1 cells decreases after co-treatment with E2 (Ricci et al., 1999). In E2-treated ovariectomized rats, mRNA levels for AHR, ARNT, ARNT2, and CYP1A1 were downregulated in the uterus (Kluxon et al., 2012). A further inhibitory mechanism is suggested by a study using MCF-7 cells, where E2 stimulates ERα recruitment to the promotor region of CYP1A1 and CYP1B1 in a complex with AHR•ARNT that represses expression of these genes (Beischlag and Perdew, 2005). These conflicting results complicate our understanding of the relationships between these important signaling pathways, especially considering other studies have found that ER signaling has no effect on AHR signaling (Hoivik et al., 1997; Wormke et al., 2000). Our understanding of the mechanisms governing AHR versus ER crosstalk is incomplete and more research is needed to provide a greater understanding of this complex interaction.

1.5.2.5 A Direct Role for ARNT in ER Signaling

ARNT can also affect ER signaling via mechanisms that are independent of the AHR. In two separate studies ARNT was found to be a co-activator for ERα and ERβ (Brunnberg et al., 2003; Rüegg et al., 2008). This is perhaps because ARNT stabilizes
the interaction between the ER and the cofactor p300 to enhance transcription (Kobayashi et al., 1997; 2000). Interaction of ARNT in the promoter regions of ER target genes is through the C-terminal portion of the protein containing the transactivation domain (Rüegg et al., 2008). This is different from how ARNT interacts with its obligate heterodimerization partners (through its HLH and PAS domains). Furthermore, an ARNT isoform missing most of the C-terminal portion of the protein was the predominant form in ER-negative, AHR non-responsive MDA-MB-231 cells (Wilson et al., 1997). These cells were compared to the ER-positive, AHR responsive MCF-7 cells predominantly expressing the wild-type ARNT protein (Wilson et al., 1997).

Activation of the AHR or hypoxia signaling pathways downregulate ER transcriptional activity, indicating that ARNT may be a limiting factor shared between these pathways (Rüegg et al., 2008). Sequestering ARNT to the AHR pathway could possibly explain the anti-estrogenic effects of TCDD (Rüegg et al., 2008). An interesting experiment that sequestered ARNT to the AHR signaling pathway arrested hypoxia and ER signaling (Jensen et al., 2006). When ARNT is knocked down in MCF-7 cells, TCDD-mediated repression of ER transcriptional activity is not attenuated (Labrecque et al., 2012). However, loss of ARNT in E2-activated MCF-7 cell lines attenuates ER transcriptional activity, but enhances it in ECC-1 endometrial cells, indicating the cell-specific co-activator versus co-repressor properties of ARNT (Labrecque et al., 2012). In addition, knocking down ARNT in human cervical carcinoma HeLa cells using siRNA showed a reduction in ER transcriptional activity, most strongly through ERβ (Rüegg et al., 2008). ARNT’s role as a co-activator may be cell-specific and depend on the modulation of ARNT by other factors. The ARNT-
interacting-peptide 2 (Ainp-2) mostly found in human liver cells and Jurkat cells, suppresses ER signaling by interacting with ARNT (Li et al., 2010). There is also a correlation between ERβ and the proteasomal degradation of ARNT through the ubiquitination pathway (Lim et al., 2011). ARNT2 may be redundant to ARNT, and it is also downregulated by xenoestrogen-activated ER in breast cancer cells (Qin et al., 2011).

1.5.3 Pathways Involving ARNT versus NF-κB Signaling

1.5.3.1 ARNT Influences NF-κB Signaling Pathways

There is crosstalk involving ARNT, either indirectly or directly, in the NF-κB signaling pathway. NF-κB consists of 5 members, p50, p52, p65 (Rel A), c-Rel, and Rel B. Normally, NF-κB is sequestered in the cytoplasm attached to an inhibitor, IκB (inhibitor of kappa B). Inducible degradation of IκB leads to NF-κB translocation to the nucleus, where it acts as a transcription factor inducing genes important in immune response, inflammation, cell growth and survival, and development. ARNT may indirectly influence NF-κB signaling via its role in the AHR-signaling pathway. One interesting finding was that the AHR was involved in the production of T helper 17 cells (T_{h17}) and in the differentiation of regulatory T cells (T_{reg}) depending on the type of AHR ligand (Quintana et al., 2008; Veldhoen et al., 2008). These two types of cells have opposing functions. T_{reg} cells are involved in immunosuppression and T_{h17} cells promote the immune response and clearance of antigens (Quintana et al., 2008; Veldhoen et al., 2008). At the molecular level these different functions may be the result of an interaction between NF-κB and AHR (Vogel and Matsumura, 2009). AHR
has been shown to interact with two different NF-κB family members, Rel A and Rel B, to either initiate or inhibit NF-κB-dependent transcription (Tian et al., 1999; Jensen et al., 2003; Thatcher et al., 2007; Vogel et al., 2007; 2009; Hollingshead et al., 2008). AHR has been shown to interact with Rel B at a response element distinct from the DRE, and increase expression of an interleukin (IL-8) independently of ARNT (Vogel and Matsumara, 2009). Some studies show that TCDD-activated AHR can also bind Rel A and inhibit transcription of NF-κB target genes (Tian et al., 1999; Jensen et al., 2003). However, the interaction of AHR and Rel A may actually have an enhancing effect on transcription of target genes, such as c-myc (Kim et al., 2000). Additionally, TCDD and IL-1β were shown to induce IL-6 expression through the interaction of AHR with Rel A in MCF-7 cells (Hollingshead et al., 2008). The role of ARNT was not investigated in this.

ARNT may directly interfere with NF-κB signaling. Tumor necrosis factor-alpha (TNF-α) activates NF-κB and its receptor CD30 interacts with ARNT and modulates Rel B transcriptional activity in human lymphoma cells and embryonic kidney cells (Wright and Duckett, 2009). After ligand activation, CD30 associates with cytoplasmic ARNT. ARNT translocates to the nucleus and interacts with Rel B, which binds the NF-κB response element and inhibits Rel-A-mediated transcription of the target gene (Wright and Duckett, 2009).

1.5.3.2 NF-κB Influences Signaling Pathways Involving ARNT

NF-κB affects both AHR and hypoxia signaling pathways. The AHR•Rel A complex can bind to the promoter region of CYP1A1 and inhibit transcription (Tian et
TNF-α has been shown to suppress CYP1A1 and CYP1B1 mRNA and protein levels in human primary hepatocytes (Muntané-Relat et al., 1995). NF-κB activation upregulates ARNT and AHRR (Baba et al., 2001; van Uden et al., 2011). Subsequently, AHRR can interact with ARNT to inhibit AHR signaling (Baba et al., 2001; van Uden et al., 2011). It appears that NF-κB has an inhibitory impact on the AHR signaling pathway.

1.5.4 GR Signaling versus Pathways Involving ARNT

GR signaling modifies pathways involving ARNT. The hypoxia signaling pathway and the GR signaling pathway affect each other through regulation of key proteins, co-regulated genes, hormones, and red blood cell production. The dioxin-responsive AHR signaling pathway and GR signaling pathway interact with each other in a myriad of ways: i) the activated AHR disrupts endocrine function related to the hypothalamic-pituitary-adrenal (HPA) axis, ii) the AHR and GR co-regulate genes, iii) the activated GR modulates AHR-inducible enzyme activity, iv) the AHR signaling pathway regulates and is regulated by insulin and glucose, v) the AHR and the GR share chaperone proteins, co-regulatory proteins, and a similar mechanism of action. This last point and the fact that AHR expression may be regulated by GR indicate that factors other than ARNT may be pivotal in the crosstalk. However, ARNT is also upregulated by glucocorticoids, which I will discuss further as I introduce my hypothesis. Most importantly, glucocorticoid signaling crosstalks with the two signaling pathways in which ARNT is a key factor: the hypoxia-signaling pathway and the AHR-signaling pathway.
1.5.4.1 Hypoxia Signaling versus GR Signaling

The hypoxia signaling pathway affects and is affected by the GR signaling pathway. Under hypoxic conditions both GR expression and glucocorticoid levels are increased. GR mRNA and protein levels are increased via a transcription mechanism under hypoxic conditions in human renal epithelial cells (Leonard et al., 2005). Additionally, at high altitudes and low oxygen, glucocorticoids are released in humans and when they are used as a pharmacological agent they alleviate mountain sickness (Basu et al., 2002). Adrenomedullin (ADM), a HIF-1 target gene, influences the glucocorticoid secretory pathway (the HPA-axis); hence cortisol release from the adrenal cortex in humans is perturbed (Nussdorfer, 2001). Plasma corticosterone (CORT) levels increased in rats under hypoxic conditions, while corticotrophin-releasing hormone (CRH)-induced adrenocortropic hormone (ACTH) secretion decreased (Raff and Jacobson, 2007). Altogether, it appears that under hypoxic conditions the GR is upregulated and glucocorticoid levels are increased. This activates the GR signaling cascade, and results in the transcription of genes crucial in alleviating mountain sickness.

Certain genes are upregulated in vivo and in vitro after simultaneous exposure to glucocorticoids and hypoxia. Stomatin, an integral membrane protein in red blood cells, is upregulated in hypoxic human lung adenocarcinoma epithelial A549 cells and primary alveolar epithelial AEC cells of rats treated with glucocorticoids (Chen et al., 2013). Bcl2/E1B 19 kDa interacting protein (Bnip3), a pro-apoptotic protein, is upregulated in the cortex of rat pups treated with glucocorticoid and deprived of oxygen.
Hypoxia had an additive effect on DEX-induced glucocorticoid-induced leucine zipper (GILZ) expression in macrophages and the spleen of rats (Wang et al., 2012b). Lastly, peroxisomal ABC transporter-like protein (PAL-1) mRNA and protein are increased in glucocorticoid-treated human epithelial cells under hypoxic conditions (Kimura et al., 2011).

Finally, the activated GR signaling pathway modulates HIF-1α and VEGF expression and it exerts a physiological response under hypoxic conditions. In two studies, VEGF transcription was downregulated in hypoxic human cells treated with DEX (Leonard et al., 2005; Wagner et al., 2008). Surprisingly, in the study done by Wagner and colleagues HIF-1α was upregulated (Wagner et al., 2008). In the study by Leonard’s group, HIF-DNA binding was not affected (Leonard et al., 2005). On the contrary, another study showed an increase in VEGF transcription in DEX-treated HeLa cells under hypoxic conditions and no change in HIF-1α protein levels (Kodama et al., 2003). Wagner states that differences may be due to a substance they used that may affect HIF-1α stability (Wagner et al., 2008). What is significant to note is that none of these three studies measured the amount of ARNT to see if it played a role in the DEX modulation of VEGF expression. Although we are unsure how the GR signaling pathway modulates VEGF expression, it does have an effect on erythropoiesis. Stress induces erythropoiesis under hypoxic conditions (Bauer et al., 1999). The GR is implicated, as the numbers of erythrocytes do not increase under hypoxic conditions in mice with a GR deficiency (Bauer et al., 1999). These GRdim mice have GRs that can be ligand-activated; however, these receptors are unable to homodimerize or bind to DNA (Bauer et al., 1999). There has been a great deal of work done to show the
responses to simultaneous activation of the GR and hypoxia pathways, but more work is needed to explain the interplay at a molecular level.

1.5.4.2 AHR Signaling Affects GR Signaling

AHR ligands influence the GR signaling pathway by modulating ACTH production, endogenous glucocorticoid levels, the GR protein, and GR target genes. ACTH and the ACTH precursor, proopiomelanocortin (POMC), are increased in the pituitary of TCDD-treated rats and mice, respectively (Bestervelt et al., 1998; Huang et al., 2002). The results vary when measuring effects of AHR ligands on levels of endogenous glucocorticoids in rodents. Plasma CORT levels increase in male Sprague-Dawley rats and female C57BL/6J mice treated with TCDD (Gorski et al., 1988; Lin et al., 1991). However, Pitt and colleagues found no change in CORT levels in the adrenal glands of male and pregnant female Sprague-Dawley rats treated with TCDD (Pitt et al., 2000). Mixed results were also found in fish. The AHR ligand β-naphthoflavone (βNF) abolished the stress-induced production of cortisol in rainbow trout (Wilson et al., 1998). Conversely, plasma cortisol levels increased in non-stressed rainbow trout treated with βNF (Aluru and Vijayan, 2004).

The AHR ligand TCDD can modulate the expression of GR. The increase in AHR and GR protein levels in the craniofacial tissues of mouse embryos treated with TCDD and HC was related to the formation of cleft palate (Abbott et al., 1994). When these mice were solely exposed to TCDD the AHR was downregulated and the GR was upregulated. When the mice were solely exposed to HC, AHR expression increased while GR expression decreased (Abbott et al., 1994). Contrary to this, the
GR was found to be downregulated in the thymus of neonatal rats treated with TCDD (Csaba et al., 1991). Confusingly, in HeLa cells, the GR was downregulated when the cells were incubated with TCDD, DEX or a combination of DEX and TCDD (Vrzal et al., 2007). TCDD also decreased the binding capacity of hepatic GR, and GR-regulated tyrosine aminotransferase (TAT) activity in C57BL/6J mice (Lin et al., 1991).

Metallothionein (MT2) is a known GR target gene and co-treatment with 3-MC and DEX caused a synergistic induction of this gene in HepG2 cells (Sato et al., 2013). Also, the AHR activator cyprodinil enhanced DEX-induced gene expression (Fang et al., 2013).

Although the mechanism by which AHR ligands affect the GR signaling pathway is uncertain, it is clear that AHR activation can disrupt endocrine pathways involving the HPA axis.

1.5.4.3 Reciprocal Modulation between AHR Signaling and the Gluconeogenesis Pathway

The AHR signaling pathway regulates and is regulated by insulin and glucose. The decrease in glucocorticoid-induced insulin resistance by berberine was mediated through the AHR (Dvorák et al., 2011; Zhao et al., 2011). Berberine is an AHR ligand. Echoing the study by Abbott and colleagues, the Dvorák team suggested that the AHR ligand may alter GR expression (Dvorák et al., 2011). Consequently, reduction in GR protein would decrease the induced insulin resistance (Dvorák et al., 2011). Although ARNT is probably not a key factor in reducing the insulin resistance response to glucocorticoids, it is implicated in diabetes as its expression is modulated by glucose levels. ARNT is induced by low glucose in human islet and Min6 cells (Dror et al.,
2008). When glucose levels were high, ARNT was downregulated (Nordeen et al., 2010). ChREBP binds to the ARNT promoter region in a glucose-dependent manner and downregulates ARNT (Nordeen et al., 2010). When ChREBP is knocked down using siRNA, ARNT mRNA levels are increased (Nordeen et al., 2010). ARNT levels are decreased in pancreatic islet cells of diabetics (Gunton et al., 2005). A case-control study associated two SNPs of the ARNT gene with acute insulin response (Das et al., 2008). When ARNT was knocked out in the liver of mice, gluconeogenesis was increased (Wang et al., 2009c). Clearly, one manifestation of the interaction between AHR signaling and glucocorticoid signaling is seen in the context of gluconeogenesis and glucose control.

1.5.4.4 GR Signaling Alters AHR Signaling

GR has an effect on AHR signaling that varies from species to species and from cell to cell. The activated GR and AHR co-regulate some DMEs. It appears that the regulation of these DMEs is different in humans compared to rats, fish, and pigs. Traditional end-points have often been the induction of CYP1A1-mediated 7-ethoxyresorufin O-deethylation (EROD) and aryl hydrocarbon hydroxylase (AHH) activity. The influence of one signal on the other may be due to shared chaperone and co-regulatory proteins. Also, modulated expression of AHR target genes may be due to changes in AHR or ARNT levels caused by glucocorticoids. This will be discussed in more detail as I prepare to discuss my hypothesis below.
1.6 GLUCOCORTICOIDS

1.6.1 The HPA-Axis and the Production of Glucocorticoids

Glucocorticoids are produced primarily in the adrenal cortex. Their production is stimulated by ACTH from the pituitary gland. CRH from the hypothalamus stimulates the release of ACTH. Negative feedback mechanisms regulate this process at each step; they are vital in controlling the output of a sensitive system with pleiotropic effects (Fig. 1.4). Glucocorticoids are responsible for metabolic and anti-inflammatory effects. The HPA-axis is activated by stresses such as an immune response or nerve impulses along the sympathetic noradrenergic fibres originating in the locus ceruleus in the brain stem. The immune response releases cytokines that signal to the hypothalamus to release CRH. CRH is also released in response to the neurotransmitters norepinephrine, acetylcholine, and serotonin (Martino et al., 2012). Although the HPA-axis is the classic system by which glucocorticoids are produced, the skin and hair also produce glucocorticoids in an analogous fashion including a negative feedback mechanism (Slominski et al., 2007; Zeigler et al., 2007; Paus et al., 2008). Recent evidence suggests that glucocorticoids are also produced in the primary lymphoid organs, intestine, and brain (Taves et al., 2011)

Upon stimulation, CRH and vasopressin are released from the paraventricular nucleus of the hypothalamus. CRH is delivered to the anterior pituitary gland via the hypophyseal portal system. CRH binds a G protein-coupled receptor, corticotrophin-releasing factor 1 (CRF₁), and stimulates the production of ACTH from POMC. This large protein is proteolytically cleaved by convertase 1 enzyme to form ACTH and other
Figure 1.4. The hypothalamic-pituitary-adrenal axis. Please refer to the text for the details. [CRH, corticotropin-releasing hormone; ACTH, adrenocorticotropic hormone]
important hormones. Vasopressin can also be co-released from CRH neurons as described above, although most vasopressin undergoes axonal transport to the posterior pituitary. Along with CRH, vasopressin stimulates the release of ACTH, which travels to the adrenal glands and binds a G protein-coupled receptor, melanocortin-2-receptor (MC2R), to exert its effects (Schimmer and Funder, 2011). ACTH activates signaling cascades which stimulate transcription of genes encoding steroidogenic enzymes, resulting in enhanced de novo synthesis of glucocorticoids, mineralocorticoids, and androgens from cholesterol.

Various steroidogenic proteins are responsible for the synthesis of glucocorticoids from cholesterol. Cholesterol is transferred from the outer mitochondrial membrane to the inner mitochondrial membrane by the steroidogenic acute regulatory protein (StAR) where it is converted into pregnenolone by the cholesterol side chain cleavage enzyme, CYP11A1 (Fig.1.5). Pregnenolone is sequentially acted upon by several cytochromes P450 and 3-β-hydroxysteroid dehydrogenase/Δ5-Δ4 isomerase (HSD3B) to ultimately form cortisol or CORT, depending on the presence of 17α-hydroxylase (CYP17A1). In humans CYP17A1 is present and cortisol is the predominant glucocorticoid synthesized. CYP17A1 is not produced in the adrenal glands of rodents and therefore corticosterone is the acting glucocorticoid (Chung et al., 2011).

Synthesis of glucocorticoids takes place in the adrenal cortex. The adrenal cortex is made up of three zones: zona glomerulosa, zona fasciculata, and the zona reticularis. Glucocorticoids are produced in the zona fasciculata. The androgen dehydroepiandrosterone (DHEA) is a sex steroid precursor and is produced in the zona
Figure 1.5. Synthesis of glucocorticoids. A depiction of the glucocorticoid biosynthetic pathway in the human adrenal cortex. CYP17A1 is not expressed in the rodent adrenal cortex. (CYP11A1, cholesterol side chain cleavage enzyme/cholesterol desmolase; CYP17A1, 17α-hydroxylase; HSD3B, 3β-hydroxysteroid dehydrogenase; CYP21A2, 21-hydroxylase; CYP11B1 and CYP11B2, 11β-hydroxylase; StAR, steroidogenic acute regulatory protein). (Adapted from Schimmer and Funder, 2011; Chung et al., 2011)
reticularis of humans, but not made in the rodent adrenal gland due to the absence of CYP17A1 (Kroboth et al., 1999). Mineralocorticoids are produced in the zona glomerulosa in response to ACTH signaling, but principally in response to angiotensin II, and extracellular potassium. Mineralocorticoids regulate concentrations of electrolytes, primarily sodium and potassium, in the extracellular fluid, ultimately having a significant impact on heart and kidney function (Hawkins et al., 2012). Mineralocorticoids regulate blood volume and pressure (Taves et al., 2011).

The levels of circulating glucocorticoids change due to food intake, physical activity, stress, and circadian rhythm. Environmental or physiological stress such as injury, infection, or disease, can activate the HPA-axis. On a daily cycle, glucocorticoid levels surge in the morning at approximately 8 am for humans. After this they continue to drop except under situations of duress, after eating, or after exercise. In rats, the glucocorticoid level increases during the dark cycle and decreases during the light cycle (Schimmer and Funder, 2011; Atkinson and Waddell, 1997).

Glucocorticoids exert a plethora of effects in the animal body and therefore they are under tight control. The negative feedback loop acts as a brake to decrease the amount of glucocorticoids released. An increase in glucocorticoids acts at each step in the HPA-axis to decrease the amount of CRH, ACTH, and ultimately, glucocorticoid secretion. Furthermore, glucocorticoids repress the expression of GR protein and mRNA in most tissues (Rosewicz et al., 1988; Freeman et al., 2004). An imbalance in glucocorticoid levels can lead to conditions of excess (Cushing’s syndrome) or deficiency (Addington’s disease).
1.6.2 Physiological and Pharmacological Roles of Glucocorticoids

The primary functions of glucocorticoids are energy metabolism and anti-inflammation/immunosuppressive responses. Glucocorticoids are produced for these purposes in response to circadian rhythms or stress. The activated GR increases blood glucose levels through metabolic responses such as lipid and protein catabolism, decreased glucose utilization, inhibition of insulin secretion, gluconeogenesis, and glycogen storage. As discussed in Section 1.6.5, the creation of Gr-null mice revealed that GR is essential for survival.

Glucocorticoids are used as anti-inflammatory and immunosuppressive therapeutics. They act through the GR to inhibit transcription of genes such as cytokines (i.e. IL-1β), that aid in the inflammatory/immune response (Herrlich et al., 2001). Additionally, the activated GR exerts its anti-inflammatory response through induction of apoptosis of lymphocytic cells (Herrlich et al., 2001). As discussed in Section 1.6.5, chronic use of glucocorticoids can be associated with important side effects and the development of resistance.

1.6.3 Signal Transduction Pathway

Glucocorticoids, secreted from the adrenal cortex, exert their effects primarily by binding to the GR. The GR is ubiquitously expressed in the body and only free glucocorticoids may bind to the GR. Approximately 90% of endogenous glucocorticoids are bound to corticosteroid binding globulin (CBG) in plasma with a small percentage bound to albumin (Lu et al., 2006.). Synthetic glucocorticoids are mostly bound to albumin (Lu et al., 2006). When glucocorticoids bind the cytoplasmic
GR, it sheds its chaperone proteins, translocates to the nucleus, and homodimerizes in order to bind glucocorticoid response elements (GREs) and affect gene transcription (Fig.1.6). This is a classical or canonical GR signaling pathway. Glucocorticoid-activated GR also exerts its effects by protein-protein interactions and non-genomic mechanisms.

The classical GR signaling pathway via GRE mostly regulates genes important in glucose metabolism (Fig.1.6). Lipophilic glucocorticoids pass through the plasma membrane by passive diffusion and bind to the cytoplasmic GR complex. Unliganded-GR resides in the cytoplasm as a complex with multiple chaperone proteins such as hsp60, hsp70, and hsp90, along with the co-chaperones p23, hsp70/hsp90 organizing protein (Hop), and hsp40 (Dittmar et al., 1997; Pratt et al., 2006). These chaperone proteins govern ligand binding, translocation, and the ubiquitination/proteasomal degradation of the GR (Pratt et al., 2006). Immunophilins are adapter proteins which can support the interaction of the chaperone proteins with the GR. The immunophilins FK506 binding protein of molecular weight 51 (FKBP51) and the related protein FKBP52 are involved in stabilizing and shuttling of the activated GR to the nucleus (Tatro et al., 2009). Ligand binding is followed by phosphorylation of the GR. Phosphorylation patterns of GR contribute to the myriad ways the activated GR can regulate different genes. That is to say, depending on where the GR is phosphorylated, gene transcription can be modulated in positive or negative directions in a gene-specific context (Chen et al., 2008). Acetylation, sumoylation, and ubiquitination of the GR protein also influences its effects within a cell [reviewed in:}
Figure 1.6. The canonical GR signal transduction pathway. Agonist binding to GR triggers translocation to the nucleus, dimerization, DNA-binding, and transcription of target genes. (ARNT, aryl hydrocarbon receptor nuclear translocator; DEX, dexamethasone; GR, glucocorticoid receptor; GRE, glucocorticoid response element; hsp90, heat shock protein 90; PXR, pregnane X receptor; TA, triamcinolone acetonide; TAT, tyrosine aminotransferase).
(Kadmiel and Cidlowski, 2013). Ligand-activated GR sheds the hsp90 complex, revealing the nuclear localization signal, which allows it to translocate to the nucleus.

In the nucleus, the GR homodimerizes, interacts with co-regulatory proteins, and binds the GRE in regulatory regions of target genes. These co-regulatory proteins are members of the p160 family such as SRC-1 (adaptor proteins mentioned in Section 1.3.2), CBP/p300 proteins with histone acetyltransferase activity, and the SWI/SNF chromatin remodeling proteins such as BRG1 (LaBeur and Holsboer, 2010). The GRE is an imperfect palindrome; the consensus sequence (5'-GGTACAnnnTGTTCT-3') consists of two imperfect inverted half-sites separated by a three-base pair spacer (Lu et al., 2006). The GR homodimer binds one GRE; each GR subunit attaches to a half site. Most GREs in GR target genes are located in intergenic or intragenic regions far removed from the transcription start site [reviewed in: (Oakley and Cidlowski, 2013)]. Conversely, only a small fraction of GREs are actually occupied by the activated GR (Oakley and Cidlowski, 2013). The chromatin landscape and epigenetic factors may play a role in GR binding (John et al., 2011; Oakley and Cidlowski, 2013). The activated GR binds preferentially to certain GREs at low glucocorticoid concentrations, whereas other GREs are bound by the GR at high glucocorticoid concentrations (Oakley and Cidlowski, 2013). Some target genes contain multiple GRE binding sites in their promoter region such as phosphoenolpyruvate carboxykinase (PEPCK), which encodes a key gluconeogenic enzyme. Additionally, there are negative GREs (nGREs) that mediate the repression of specific genes by glucocorticoids. The POMC and CRH genes are repressed in this manner (Drouin et al., 1998, van der Laan et al., 2009).
Other transcription factors may enhance or reduce GR-dependent transcription. Smad3 interacts with GRE-bound GR to enhance transcription of target genes [reviewed in: (Smoak and Cidlowski, 2004)]. The signal transducer and activator of transcription (STAT) proteins also bind at their DNA recognition sites and interact with the GR to increase or decrease transcription of target genes [reviewed in: (Smoak and Cidlowski, 2004)].

Transrepression through protein-protein interaction is how GRs mediate some of their anti-inflammatory action. The GR tethers to the p65 protein, a member of the NF-κB family, to repress transcription of NF-κB target genes such as IL-1β and TNF-α [reviewed in: (Smoak and Cidlowski, 2004)]. Curiously, it is these cytokines that are partly responsible for stimulating CRH production in the hypothalamus (Spinedi et al., 1992). The GR also exerts its effect on NF-κB by inducing transcription of IκBα (Auphan et al., 1995; Scheinman et al., 1995). IκBα binds to NF-κB and inhibits its activity. Activator-protein-1 (AP-1) is another transcription factor responsible for inducing cytokines that are important for mounting an immune response. The GR tethers to c-Jun to inhibit the transcriptional activity of AP-1 (Smoak and Cidlowski, 2004)

The activated GR also has non-genomic effects. The unliganded GR associates with T-cell receptor (TCR) kinases at the cell membrane and promotes TCR signaling (Löwenberg et al., 2006). When activated, the GR dissociates from TCR kinases, thus inhibiting this signaling pathway (Taves et al., 2011). The activated GR may also exert its effects by releasing components of its multiprotein-complex, which are then free to
inhibit the release of arachidonic acid [reviewed in: (Buttgereit and Scheffold, 2002)].

Another non-genomic mechanism of action involves the membrane bound GR (mGR),
which is possibly a variant of the cytosolic GR. mGRs have been detected in amphibian
neuronal membranes, rodent lymphoma, human blood mononuclear cells, and human
embryonic kidney (HEK293) cells (Strehl et al., 2011). Although the function of the
mGR is unknown, its existence has been associated with rheumatoid diseases and
human T-cells (Strehl et al., 2011). Glucocorticoids, independent of GR, can also have
non-genomic effects in the cell membrane of immune cells. Glucocorticoids intercalate
in the membrane and disrupt cation transport, ultimately suppressing the immune
system [reviewed in: (Buttgereit and Scheffold, 2002)].

1.6.4 Molecular Structure of GR

There is only one GR gene and it is conserved across many species. The GR is
classified as a nuclear receptor. It shares homology in the DNA- and ligand-binding
domains with other nuclear receptors such as the PXR and the ERα. There are two
trans-activating domains within the protein. One ligand-independent activation function
domain (AF-1) is found in the N-terminal region, and a ligand-dependent activation
function domain (AF-2) is found in the C-terminal region. Like other nuclear receptors,
the central DNA-binding domain of the GR contains two zinc finger motifs.

1.6.4.1 GR Isoforms

The pleiotropic effects of activated GR in different tissues can also be partly
explained by different GR isoforms. The human GR gene consists of 9 exons, while
the mouse gene consists of 11 exons. Although there is only one GR gene per se,
there are two dominant isoforms expressed within the body, the GRα and the GRβ. The GRα itself has 8 different initiation sites, which results in translation of 8 different isoforms; each isoform regulates a distinct set of genes depending on the tissue type (Lu and Cidlowski, 2005). Glucocorticoids affect apoptosis differently, depending on the activated isoform (Lu et al., 2007).

Additionally, there are four splicing variants of the GR gene, also with distinct signaling properties. The GRβ is one of these spliced variants. It resides mainly in the nucleus, it lacks the ligand-binding domain, and it acts as a dominant negative inhibitor of GRα activity [reviewed in: (Oakley and Cidlowski, 2013)]. The GRβ may competitively bind the GRE, sequester coregulatory proteins, or homodimerize with the GRα, ultimately blocking the action of GRα. Glucocorticoid resistance may be due to upregulation of GRβ. It is upregulated in neutrophils and it can be increased by pro-inflammatory cytokines (Strickland et al., 2001; Webster et al., 2001).

1.6.5 Role of Glucocorticoids in Disease Pathology and Treatment

GR signaling is both implicated in disease causation and important for treatment of a number of diseases because of its anti-inflammatory, immunosuppressive, pro-apoptotic, and anti-angiogenic roles. Glucocorticoid action via the GR affects embryonic development, metabolism of carbohydrates, proteins and lipids, and the nervous, cardiovascular, immune, musculoskeletal, visual, respiratory, reproductive, and integumentary systems. Mice carrying a homozygous deletion of the Gr gene die shortly after birth due to inadequate lung development (Cole et al., 1995). When the Gr is knocked out in the liver, 50% of homozygous mutants died, and those that survived
exhibited a severe growth deficit due to decreased length of bones and size of internal organs (Tronche et al., 2004). Irregular glucocorticoid secretion can lead to conditions of excess (Cushing’s syndrome) or deficiency (Addington’s disease) [reviewed in: (Kadmiel and Cidlowski, 2013)]. Glucocorticoids have been used to treat a variety of conditions such as conjunctivitis, arthritis, asthma, psoriasis and lupus. They are important therapeutic agents in treating cancer and in preventing rejection following organ transplantation [reviewed in: (Kadmiel and Cidlowski 2013)].

There is a risk of resistance and side effects when using glucocorticoids as therapeutic agents. Patients with inflammatory diseases or asthma may become resistant to treatment due to a variety of reasons. Some possible mechanisms include: GRβ upregulation, an nGRE binding site may be present in the GR gene, changes in GR phosphorylation, genetic susceptibility, hyperactivity of pro-inflammatory transcription factors and cytokines, or an overexpression of the multi-drug resistance gene [reviewed in: (Kadmiel and Cidlowski 2013)]. Side effects occur predominately from long-term use and may involve glucocorticoid activation of the mineralocorticoid receptor (MR). Some side effects include bone necrosis, osteoporosis, glaucoma, diabetes mellitus, hypertension, and life-threatening gastric bleeding (Schake et al., 2002). Upregulation of certain genes such as TAT has been implicated in some of the side effects, presumably because of its role in protein catabolism (Schake et al., 2002).
1.6.6 GR Ligands

1.6.6.1 GR Agonists

From a therapeutic perspective, the ideal glucocorticoid would have high GR activity coupled with low MR affinity and minimal side effects. DEX and triamcinolone acetonide (TA) are potent synthetic glucocorticoids with greater affinity for the GR than the MR (Fig. 1.7). They have similar structures, but interestingly regulate different subsets of genes (Nehmé et al., 2009). Mineralocorticoid activity is reduced by a substitution at carbon 16 and an extra double bond between carbons 1 and 2, which also impedes biotransformation (Schimmer and Funder, 2011; Gohel et al., 2013). The half-life of DEX is around 36-54 hours, whereas the half-life of TA is 18-36 hours (Jacobs and Bijlsma, 2013). The potency of DEX and TA, relative to endogenous glucocorticoids, is attributed to the fluorination on the steroid ring in the α-position of carbon 9. DEX is about 5 times more potent than TA, which itself is about 5 times more potent than cortisol (Jacobs and Bijlsma, 2013). The K_d for binding of TA to the rat hepatic GR is approximately 0.94 nM (Matić et al., 1989). A range of K_d values have been reported for the binding of CORT and DEX to rat liver GR. For CORT, the relevant K_d values range from 3 nM (Izawa et al., 1985) to 77 nM (Allera and Wildt, 1992) and for DEX the relevant K_d values range from 0.9 nM (Izawa et al., 1985) to 5.3 nM (Isohashi et al., 1979). In humans, DEX is approximately 25-30 times more potent as an anti-inflammatory than cortisol (Shimmer and Funder, 2011). The endogenous glucocorticoids cortisol and CORT are not ideal therapeutic tools as they interact with the MR and cause both transrepression and transactivation (Fig. 1.7).
Endogenous Glucocorticoids

- Cortisol
- Corticosterone (CORT)

Synthetic Glucocorticoids

- Dexamethasone (DEX)
- Triamcinolone Acetonide (TA)

Antagonist

- Mifepristone (RU486)

Figure 1.7 Structures of natural and synthetic GR ligands.
In a general sense, transrepression is key for the anti-inflammatory effects while the transactivation is responsible for the expression of genes involved in metabolism and implicated in side effects. Ligands that can selectively activate the transrepression pathway and not the transactivation pathway are known as dissociated compounds (Rosen and Miner, 2005; De Bosscher, 2010). Research using GRdim mice has cast doubt on the hypothesis that ligands only activating the transrepression pathway are the best therapeutic agents. GRdim mice have diminished transactivation capacity due to the inability of the GR to homodimerize. In these mice, not all of the GR target genes lose expression; there are even some genes that are more strongly upregulated [reviewed in: (Clark and Belvisi, 2012)]. When the transactivation signaling pathway is inhibited, mice are not protected from muscle atrophy or osteoporosis [reviewed in: (Clark and Belvisi, 2012)]. Lastly, there are genes that are classically activated by the GR that have anti-inflammatory functions [reviewed in: (Clark and Belvisi, 2012)]. The search is on for a novel glucocorticoid with improved therapeutic indices in vivo and less restriction of transactivation versus transrepression capabilities.

1.6.6.2 GR Antagonist

RU486, also known as mifepristone, is a GR and progesterone receptor (PR) antagonist (Fig. 1.7). At high concentrations, RU486 is effective at inhibiting the GR activation in vitro and in vivo and has been used effectively in treating depression (Johnson et al., 2007; Gallagher et al., 2008). RU486 binds to the rat hepatic and human placental GR with a Kd of 1.5 nM (Gagne et al., 1986) and 1.3 nM (Heikinheimo et al., 1987), respectively. As a high-affinity PR antagonist, RU486 is used as an
abortifacient and emergency contraceptive in certain countries [reviewed in: (Cadepond et al., 1997)]. RU486 is an effective antagonist because it binds with high affinity to the 11β pocket of the GR and the PR, causes a conformational change of the receptors, and inhibits the transcriptional activity of these receptors (Cadepond et al., 1997). The exact mechanisms of action have yet to be clarified.

1.6.7 The Pregnane X Receptor

The PXR is a steroid and xenobiotic-sensing, member of the nuclear receptor superfamily. As a nuclear receptor it is similar in structure and domain organization to the GR: a ligand-independent AF-1 domain in the N-terminal region, a central zinc finger-containing DNA-binding domain, a ligand-binding domain and associated ligand-dependent AF-2 domain, and a variable C-terminal region. PXR is a glucocorticoid-inducible gene (Pascussi et al., 2003). Glucocorticoid agonists such as DEX behave in a bi-phasic manner, upregulating PXR expression via the GR at low concentrations (pM to nM range) and activating the PXR at higher concentrations (µM range) (Pascussi et al., 2003). As an orphan receptor, the identity of physiologically relevant endogenous PXR ligands remains unclear. Pregnenolone-16α-carbonitrile (PCN) is an efficacious activator of PXR in mice and rats, and rifampicin is the prototypical agonist of the human PXR (Staudinger et al., 2011). RU486 can also act as a PXR agonist in mice, rats and humans at high (µM) concentrations [reviewed in: (Kliewer et al., 2002)]. Ligand activation of the PXR causes a conformational change whereby the PXR sheds its chaperone proteins, translocates to the nucleus, and binds the retinoid X receptor (RXR). Together, they interact with pregnane X response elements (PXREs) and
induce target genes such as CYP3A4 in the human or CYP3A23 in the rat (Fig. 1.8) (Kliwer et al., 2002). As well as its role in drug metabolism, the PXR is involved in bile acid homeostasis. It also plays a negative regulatory role in gluconeogenesis, lipid metabolism, and inflammation (Staudinger et al., 2011).

1.7 EFFECTS OF GLUCOCORTICOIDS ON SIGNALING PATHWAYS INVOLVING ARNT

The interplay between the AHR and GR signaling pathway is evident in the modulated induction of AHR target genes. The AHR target gene AHRR may be induced in rat liver by glucocorticoids due to the GRE located in its promoter region (Nishihashi et al., 2006). AHR-induced DMEs are modulated in rat hepatocytes treated with DEX and PAHs in combination. In rats, the induction of CYP1A1 and Uridine diphosphate-glucuronosyl transferase (UGT1A6) mRNA and protein by PAHs is enhanced by DEX, whereas the induction of NAD(P)H: quinone oxidoreductase 1 (NQO1), glutathione S-transferase A2 (GSTA2) and aldehyde dehydrogenase 3 (ALDH3) mRNA and protein by PAHs is inhibited by DEX (Xiao et al., 1995; Linder et al., 1999). In rat mammary fibroblasts the TCDD induction of CYP1B1 mRNA and protein is inhibited by DEX (Brake et al., 1998). In rodents, fish, and pigs, PAH or HAH induction of CYP1A1 is potentiated by glucocorticoids (Mathis et al., 1986; Sherratt et al., 1989; Linder and Prough, 1993; Xiao et al., 1995; Celander et al., 1996; 1997; Lai et al., 2004; Monostory et al., 2005; Sonneveld et al., 2007). CYP1A1 protein is upregulated in fish and in pigs when treated with an AHR ligand, and DEX potentiates this effect (Celander et al., 1996; 1997). In rats, CYP1A1 induction by AHR ligand is
Figure 1.8 The PXR signal transduction pathway. Agonist binding to PXR triggers translocation to nucleus, dimerization with RXR, DNA-binding and transcription of target genes such as rat CYP3A23. (PCN, pregnenolone 16α-carbonitrile; DEX, dexamethasone; hsp90, heat shock protein 90; RXR, retinoid X receptor; PXR, pregnane X receptor; CCRP, cytoplasmic constitutive active/adrostan receptor retention protein; PXRE, pregnane X response element)
potentiated by DEX \textit{in vitro} and \textit{in vivo} (Mathis et al., 1986; Sherratt et al., 1989; Linder and Prough, 1993; Xiao et al., 1995; Lai et al., 2004; Monostory et al., 2005; Sonneveld et al., 2007). In human hepatocytes, TCDD induction of CYP1A2 mRNA, but not CYP1A1 mRNA is potentiated by DEX (Vrzal et al., 2008; 2009). In contrast to the rodent, fish, and pig studies, most studies in human cells show that PAH or HAH induction of \textit{CYP1A1} is inhibited by DEX (Monostory et al., 2005; Sonneveld et al., 2007; Vrzal et al., 2009; Wang et al., 2009b). \textit{CYP1A1} induction by TCDD or 3-MC in human placental JEG 3 cells is suppressed by DEX, and this can be explained by the ability of DEX to accelerate degradation of the AHR protein (Stejoskalova et al., 2013a). Conversely, one study showed an enhanced induction of CYP1A1 mRNA in human placental trophoblasts treated with 3-MC and DEX (Stejoskalova et al., 2013b) and another study showed that DEX did not alter CYP1A1 mRNA induction by PAHs or HAHs in human hepatocytes (Monostory et al., 2005). A key GRE located in the first intron of the CYP1A1 gene is conserved in rat, mouse, and human. Therefore, the basis for the species difference in DEX modulation of CYP1A1 inducibility remains elusive (Linder et al., 1999). It appears that the GR-mediated effect on AHR target genes is cell-, species-, and gene-specific.

GR and AHR signaling also interact to control P450-mediated enzyme activity. First of all, 3-MC induction of AHH activity is decreased in liver of adrenalectomized (ADX) rats (Nerbert and Gelboin, 1969; Bogdanffy et al., 1982). Similar to \textit{CYP1A1} expression, the AHR-inducible EROD/AHH activity is augmented in rats or rat cells exposed to excess glucocorticoids (Wiebel and Cikryt, 1990; Konstandi et al., 2000; Lai et al., 2004). Likewise, EROD activity is increased in fish and pigs treated with
glucocorticoids and TCDD in combination (Celander et al., 1996; 1997). In human placental trophoblasts, there is potentiation of the AHR-induced EROD activity when the cells are exposed to DEX (Stejskolava et al., 2013b). However, this effect is not observed in human hepatocyte cultures. The induction of EROD activity by an AHR ligand was inhibited by DEX in human hepatocytes and hepatoma cells (Dvorák et al., 2008; Vrzal et al., 2009). An explanation is needed for the cell- and species-specific effects of GR activation on AHR-inducible enzyme activity.

The effect of activated GR on AHR signaling pathways can partially be explained by common chaperones, shared co-regulatory proteins, and the regulation of AHR expression by the GR. The AHR and the GR share the chaperone proteins p23 and hsp90 (Hollingshead et al., 2004; Pratt et al., 2006; Beischlag et al., 2008). These proteins stabilize the receptors, localize the unliganded receptors in the cytoplasm, and when ligand-activated, they help translocate the receptors to the nucleus (Kelley and Georgopoulos, 1992). If these proteins are limiting due to the sequestration by the other receptor, then this could have an effect on the stabilization, localization, and transport of the receptor.

Shared co-regulatory proteins may also be limited and affect AHR or GR signaling. The co-regulatory proteins SRC-1 and CBP/p300 are shared by both the GR and the AHR (Chen, 2000; De Bosscher et al., 2003). CBP/p300 is a histone acetyltransferase that increases transcription, and SRC-1 is an adaptor that helps co-activators bind to transcription factors [reviewed in: (Labrecque et al., 2013)]. If these
proteins are sequestered by the other receptor, then transcription of GR or AHR target genes may be effected.

GR agonists regulate the expression of AHR, providing a partial explanation of the effects on AHR target genes and enzyme activity. Hepatic AHR protein, not mRNA, levels decreased in ADX rats (Mullen Grey and Riddick, 2009), with selectively impaired induction of CYP1B1 by 3-MC (Mullen Grey and Riddick, 2011). Many studies in rodents and rodent cells show a potentiating effect of DEX on CYP1A1 induction by HAHS and PAHS, and this is matched by an increase in AHR expression (Wiebel and Cikryt, 1990; Abbott et al., 1994; Sonneveld et al., 2007; Bielefeld et al., 2008). Importantly, Ahr expression is induced by DEX via a GR-dependent transcriptional mechanism and relatively small magnitude changes in AHR levels can have a significant impact on AHR-mediated transcriptional responses in Hepa-1 mouse hepatoma cells (Bielefeld et al., 2008). TCDD-induced CYP1B1 expression is inhibited by DEX in rat mammary fibroblasts, and this coincides with a decrease in AHR mRNA following DEX treatment (Brake et al., 1998). AHR levels are decreased in human hepatocytes treated with DEX (Dvorák et al., 2008; Vrzal et al., 2009), and this is reflected in diminished induction of CYP1A1 protein and enzyme activity (Monostory et al., 2005; Sonneveld et al., 2007; Vrzal et al., 2009; and Wang et al., 2009b). However, one study using HeLa cells showed DEX reversed the decrease in AHR mRNA caused by TCDD (Vrzal et al., 2007). This does not help to explain the diminished CYP1A1 inducibility usually seen in human cells following DEX treatment. Additionally, the enhanced CYP1A1 inducibility by DEX in human placental trophoblasts was not accompanied by a change in AHR expression (Strejiskalova et al., 2013b). An in vivo
study in our lab showed DEX has no effect on hepatic AHR expression in rat (Mullen Grey and Riddick, 2009). It appears that GR ligands modulate the AHR signaling pathway via effects on expression of the AHR and potentially because the two pathways share co-regulatory proteins, and chaperone proteins. However, these factors taken together are not sufficient to account for the complexities of the species- and cell-specific aspects of GR•AHR crosstalk. There is more to this story.

ARNT is another important factor potentially involved in the modulation of the AHR pathway by glucocorticoids. Many of the studies thus far have measured GR and AHR levels, but few considered the AHR heterodimerization partner, ARNT. The studies that have assessed ARNT expression do not agree with each other. ARNT mRNA levels are unchanged in rat mammary fibroblasts and human hepatocytes treated with DEX (Brake et al., 1998; Vrzal et al., 2009). In mouse craniofacial tissue, ARNT mRNA and protein levels are downregulated following exposure to HC, or concomitant HC and TCDD treatment (Abbott et al., 1999). However, hepatic ARNT mRNA levels are increased in ADX male Wistar rats treated with the GR agonist methylprednisolone (Almon et al., 2005). Also, hepatic ARNT mRNA levels are higher in stressed versus control aged mice (Mikihailova et al., 2005). Lastly, work in our lab showed that hepatic ARNT mRNA levels are induced in both SHAM and ADX male Fischer 344 rats following acute DEX treatment (Mullen Grey and Riddick, 2009). In this study, the increase in ARNT mRNA levels was triggered by a relatively low dose of DEX (1.5 mg/kg), which was shown to activate GR, but not PXR. For this reason, the central focus of my thesis research was the role of GR in the regulation of rat hepatic
ARNT expression as a potential component in the complex crosstalk between GR and AHR signaling pathways.

1.8 RESEARCH HYPOTHESIS

The literature review discussed above strongly suggests that glucocorticoids modulate the AHR and hypoxia signaling pathways. Effects of glucocorticoids on levels of AHR or HIF-1α proteins and target genes are inconsistent, causing me to focus on the shared dimerization partner of AHR and HIF-1α, ARNT. Glucocorticoids induce hepatic ARNT mRNA levels in rodents (Almon et al., 2005; Mikhailova et al., 2005; Mullen Grey and Riddick 2009). In the study by Mullen Grey and Riddick (2009), the induction of ARNT mRNA by a relatively low DEX dose suggests a GR-mediated mechanism, but this needs to be further examined. I also wanted to determine whether increases in ARNT mRNA levels caused by DEX result in increases in ARNT protein levels, which would be an important aspect of discerning potential functional impacts of this response. These unmet needs provided the rationale for the formulation of the following research hypothesis:

Hypothesis: The in vivo induction of rat hepatic ARNT expression by glucocorticoids is mediated by the GR.

1.9 SPECIFIC AIMS/OBJECTIVES

This study has four main objectives:

1) To determine whether rat hepatic ARNT is induced by DEX doses that activate GR (≥ 0.1 mg/kg) or PXR (≥ 10 mg/kg).
2) To determine whether TA (a selective GR agonist) or PCN (a selective PXR agonist) cause induction of rat hepatic ARNT expression

3) To determine whether hepatic ARNT induction by DEX is altered in PXR-knockout rats.

4) To determine whether rat hepatic ARNT induction by DEX is altered by the GR antagonist RU486

1.10 RATIONAL FOR THE EXPERIMENTAL APPROACH

The effects of glucocorticoids on hepatic ARNT expression were investigated primarily in male Fischer 344 rats, as well as PXR-knockout rats (in relation to their wild-type Sprague-Dawley controls). ARNT protein and mRNA levels were assessed by immunoblot analysis and quantitative real-time PCR, respectively. In addition, as a positive control for GR activation, TAT mRNA levels were assessed by quantitative real-time PCR. CYP3A23 mRNA levels were also measured by quantitative real-time PCR as a positive control for PXR activation. The purpose of using an in vivo approach, rather than an in vitro approach, is to study the complex interactions between xenobiotics and hormonal regulatory pathways in intact animals with functional endocrine circuits. Also, male Fischer 344 rats were used in a previous study that showed an upregulation of hepatic ARNT mRNA levels when the rats were treated with DEX (Mullen Grey and Riddick, 2009). Hepatic TAT mRNA levels were also upregulated in these DEX-treated rats, suggesting a fully functional GR signaling pathway (Mullen Grey and Riddick 2009). These rats are also AHR-responsive, as
demonstrated by the modulated 3-MC induction of various hepatic AHR target genes following ADX (Mullen Grey and Riddick, 2011).

I chose rats and not mice because of the previous studies done in our lab with Fischer 344 rats and the availability of PXR-knockout rats. The question of whether hepatic ARNT expression is altered by exogenous glucocorticoids has not been addressed in mice. Although there are Pxr-null mice that are viable and fertile (Xie et al., 2000a), there are considerable health issues associated with Gr-null mice (Cole et al., 1995; Schuetz et al., 2000) and hepatocyte-specific conditional Gr-null mice (Tronche et al., 2004). However, pharmacological antagonism by RU486 blocks GR activity in rat liver (Gagne et al., 1985; Alexandrová, 1992; Honer et al., 2003). PXR-knockout rats created by SAGE Technologies via zinc finger nuclease technology are commercially available.

In the DEX Dose-Response Study, all DEX doses (0.1, 1, 10, 50 mg/kg) were expected to activate GR with PXR activation anticipated at only the highest DEX doses (10 and 50 mg/kg) (Mullen Grey, 2011; Zhang et al., 2012; Fujimara et al., 2012). The DEX Dose-Response Study showed an increase in hepatic ARNT mRNA levels in rats exposed to DEX doses of 1, 10, and 50 mg/kg. Based on this finding, I selected DEX doses of 1 mg/kg and 50 mg/kg for the PXR-Knockout Rat Study since the low 1 mg/kg dose activates GR selectively, whereas the high 50 mg/kg dose activates both GR and PXR. TA and PCN are prototypical ligands, selective in the receptor they activate; TA is a selective GR agonist (Runge-Morris et al., 1996; Duanmu et al., 2000) whereas PCN is a selective PXR agonist (Hartley et al., 2004; El-Sayed, 2011). Considering DEX is
approximately five times more potent than TA (Schimmer and Funder, 2011), and hepatic ARNT mRNA levels were upregulated by 1 mg/kg DEX, I used a TA dose of 5 mg/kg to examine effects on ARNT following GR activation. A standard PXR-activating and CYP3A-inducing dose of PCN 50 mg/kg was used (Amacher et al., 1998; Xie et al., 2000b). In the GR Antagonist Study, the dose of RU486 (50 mg/kg) was based on a pilot study we performed and on the published study by Gagne et al. (1985). This study showed that a ratio of 100:1 (RU486: DEX) is able to demonstrate in vivo GR antagonism (Gagne et al., 1985; Alexandrová, 1992). Thus, I examined the ability of RU486 (50 mg/kg) to block effects of a low 0.5 mg/kg DEX dose that activates GR selectively and a high 50 mg/kg DEX dose that activates both GR and PXR. Since RU486 can also activate PXR at high concentrations, the RU486 dose was selected to achieve in vivo GR antagonism without causing substantial PXR activation by RU486 alone.

In all in vivo rat studies, animals were euthanized at both 6 hours and 24 hours following glucocorticoid treatment. The 6-hour time-point was selected primarily to capture early effects on mRNA levels, as reflected by a previous time-course study of DEX effects on ARNT and TAT mRNA levels (Mullen Grey and Riddick, 2009). The 24-hour time-point was selected primarily to capture later effects on ARNT protein levels, which would likely require more time for changes to occur in comparison to mRNA levels.
SECTION 2: MATERIALS AND METHODS

2.1 ANIMALS, TREATMENT, AND LIVER PROCESSING

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. Four in vivo rat studies were performed to test my hypothesis: 1) DEX Dose-Response Study, 2) GR- and PXR-Selective Agonist Study, 3) PXR-Knockout Rat Study, and 4) GR Antagonist Study (Fig. 2.1).

Male Fischer 344 rats (7 weeks old at time of purchase) were used in the DEX Dose-Response Study, the Selective Agonist Study, and the GR Antagonist Study. These rats were purchased from Charles River Laboratories (St. Constant, QC). They were housed two to a cage and allowed to acclimatize for one week in the Division of Comparative Medicine at the University of Toronto. Rats were fed the Harlan Teklad 2018 diet and given water, ad libitum. The rats were on a 12-hour light and 12-hour dark cycle, with lights on at 7 am. For the DEX Dose-Response Study, the rats received an intraperitoneal (i.p.) injection of corn oil (vehicle), or DEX (Sigma Chemical Co., St. Louis, MO) at doses of 0.1, 1, 10, and 50 mg/kg at 10 am and then were housed in single cages (Fig. 2.1A). In the Selective Agonist Study, the rats received an i.p. injection of corn oil vehicle, TA (Sigma Chemical Co., St. Louis, MO) at 5 mg/kg, or PCN (Sigma Chemical Co., St. Louis, MO) at 50 mg/kg at 10 am and then were housed in single cages (Fig. 2.1B). In the GR Antagonist Study, the rats received an i.p. injection of corn oil vehicle or RU486 (Sigma Chemical Co., St. Louis, MO) at 50 mg/kg
Figure 2.1 Schematic representation of the four *in vivo* rat studies. Flow charts showing experimental protocols used for A) DEX Dose-Response Study, B) GR- and PXR-Selective Agonist Study, C) PXR-Knockout Rat Study, and D) GR Antagonist Study. See text for details.
at 9:30 am followed by a second i.p. injection at 10 am with corn oil vehicle or DEX at doses of 0.5 mg/kg or 50 mg/kg (Fig. 2.1D).

In the PXR-Knockout Rat Study, male PXR-knockout rats and wild-type Sprague-Dawley controls (7 weeks old at time of purchase) were obtained from SAGE Laboratories (Boyertown, PA). Upon arrival in the Division of Comparative Medicine at the University of Toronto, these rats were placed in quarantine for one week, followed by acclimatization to standard housing for an additional period of 3 to 10 days. Housing and diet were as described above. Rats received an i.p. injection of corn oil vehicle or DEX at doses of 1 mg/kg or 50 mg/kg at 10 am and then were housed in single cages (Fig. 2.1C). The PXR-knockout rats are homozygous for a 20-base pair (bp) deletion within exon 2 of the PXR gene created by zinc finger nuclease technology, leading to multiple premature stop codons, and non-functional PXR protein as evidenced by a lack of hepatic CYP3A induction following PCN treatment (www.sagresearchlabs.com/research-models/knockout-rats/pxr-knockout-rat).

Rats from all of the studies were handled daily during the acclimatization period and an experienced animal technician restrained the rats during injections to minimize stress and activation of the HPA axis. At 4:00 pm (6 h post-injection) or 10:00 am the following day (24 h post-injection), rats were lightly anesthetized by isoflurane inhalation (Abbott Laboratories, North Chicago, IL), weighed, and euthanized by decapitation.

Processing of liver for subsequent RNA and cytosol isolation followed established methods (Timsit et al., 2002). The liver was perfused in situ with 30 mL of
ice-cold HEGD (25 mM HEPES/1.5 mM EDTA/10% glycerol/1 mM DTT, pH 7.4), excised and weighed. Approximately 0.1 g liver pieces were frozen by immersion in liquid nitrogen and stored at -80°C for RNA isolation. Approximately 8 g of each liver were homogenized in 24 ml of HEGD using a motor-driven teflon-glass homogenizer (Caframo, Wiarton, ON). Aliquots of homogenate were frozen in liquid nitrogen and stored at -80°C while the remaining homogenate was centrifuged at 9000x g for 20 minutes at 4°C using a JA-17 rotor in a J2-21 M centrifuge (Beckman, Fullerton, CA). The postmitochondrial supernatant was removed and centrifuged in a 70Ti rotor at 106,000x g for 60 min at 4°C in an L-80 ultracentrifuge (Beckman, Fullerton, CA). Aliquots (1 mL per cryovial) of this supernatant (cytosol) were frozen in liquid nitrogen and stored at -80°C for subsequent protein quantification.

2.2 DETERMINATION OF CYTOSOLIC PROTEIN CONCENTRATION

The Bradford Assay was performed to determine the concentration of cytosolic protein (Bradford, 1976). This spectrophotometric method is based on the observation that the maximum absorbance of Coomassie Brilliant Blue G-250 changes from 465 nm to 595 nm when the dye binds to protein. A 5-μL aliquot of each cytosolic sample was pipetted in duplicate into 13x100mm culture tubes. A standard curve was prepared by pipetting 0, 10, 20, 30, 40 and 50 μL from a stock solution of bovine serum albumin (BSA) (Sigma Chemical Co., St. Louis, MO) prepared at 1 μg/μL into 13x100mm culture tubes. The Protein Assay Dye Reagent Concentrate (Biorad, Hercules, CA) was diluted in 4 volumes of water and 2.5 mL of this diluted dye was added to each tube. After 15 minutes at room temperature, the absorbance of standards and samples was
measured at 595 nm, with 465 nm as background reference, on a Beckman DU-65 Spectrophotometer (Beckman, Fullerton, CA). Cytosolic protein concentrations were determined by interpolation from the generated standard curve.

2.3 SDS-PAGE AND IMMUNOBLOTTING

Cytosol samples were diluted with 2X sample buffer (0.125 M Tris-HCL, pH 6.8/20% glycerol/ 4% SDS/ 10% β-mercaptoethanol/ 0.002% bromophenol blue) to yield an appropriate protein concentration. After this, the samples were boiled for 4 minutes to denature proteins. Cytosolic ARNT and β-actin protein levels were assessed by immunoblot analysis using sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE) procedures developed by Laemmli (1970) and blotting procedures developed by Towbin et al. (1979). The proteins were separated by gel electrophoresis, transferred to nitrocellulose and incubated in primary and secondary antibody in order to detect ARNT and β-actin protein. A standard curve relating the ARNT protein band intensity to the amount of loaded cytosolic protein suggested that 30 μg of cytosolic protein would permit relative comparisons between different rat cytosol samples (Fig 2.2). A similar standard curve was also constructed for β-actin protein detection as a reference protein loading control. All gels were also loaded with a protein molecular mass ladder (Thermo Scientific), a constant calibrator sample to allow comparisons across multiple gels, and cytosol from mouse Hepa-1 hepatoma cells as an abundant source of ARNT protein (positive control).

Proteins were resolved by SDS-PAGE employing a 4% stacking gel and 10% separating gel, with a 60-minute run time at 180 volts in a Mini-Protean II gel apparatus.
Figure 2.2 Representative immunoblot and standard curve analysis of hepatic ARNT protein levels in rat liver cytosol

A) Immunoblot analysis of liver cytosolic protein (5-120 µg) from an untreated rat using a polyclonal ARNT antibody. The first lane contains the Hepa-1 cytosol positive control. B) The standard curve shows a linear relationship between the amount of protein loaded and the immunoreactive ARNT signal intensity. The optimal quantity of input protein (30 µg) for a 5 and 10 minute exposure is highlighted. The equation of the line of best fit was generated by least-squares linear regression (shown along with $r^2$, the coefficient of determination).
(Biorad, Hercules, CA) filled with running buffer (0.025 M Tris-HCl, 0.192 M glycine, 0.1% SDS, pH 8.3) and using a Biorad model 1000/500 Power Supply. The proteins were electrophoretically transferred to the nitrocellulose (Hybond-ECL, GE Healthcare, Baie d’Urfé, QC) in a Mini Trans-Blot Electrophoretic Transfer Cell (Biorad, Hercules, CA) filled with Transfer buffer (25 mM Tris-HCl, 192 mM glycine, 20% methanol, pH 8.3) at 100 volts for 60 minutes and using a Biorad model 200/2.0 Power Supply. I checked the efficiency of the transfer by reversibly staining the membrane with Ponceau Stain (BioShop, Burlington, ON). Membranes were destained by rinsing with TNT buffer (20 mM Tris, 137 mM NaCl, 0.1 % Tween-20, pH 7.6) for 5 minutes and then incubated overnight in 5% blotto (a 5% solution of Carnation Instant Skim Milk Powder in TNT buffer with a pinch of thermasol) on an orbital shaker at 4°C. The following day the membranes were rinsed with TNT for 45 minutes. The nitrocellulose was bisected with the top part (high molecular mass range) incubated with a 1:200 dilution of a goat polyclonal antibody against the C-terminal of the human ARNT (Santa Cruz Biotechnology, Santa Cruz, CA) and the bottom half (low molecular mass range) incubated with a 1:100,000 dilution of a mouse monoclonal antibody against the N-terminal of the beta isoform of actin (Abcam, Cambridge, MA) for 1 hour at room temperature. Antibodies were diluted in the 5% blotto mentioned above. Membranes were once again rinsed with TNT for 45 minutes. The blots previously incubated in the ARNT antibody were next incubated in a 1: 2,000 dilution of a rabbit anti-goat Ig-horseradish peroxidase conjugate (Sigma Chemical, St. Louis, MO) for 1 hour at room temperature. The blots previously incubated in the β-actin antibody were next incubated in a 1:20,000 dilution of a sheep anti-mouse Ig-horseradish peroxidase
conjugate (Novus Biologicals, Littleton, CO) for 1 hour at room temperature. After a final rinse of the membranes in TNT, blots were treated with Amersham ECL Western Blotting Detection Reagents (GE Healthcare, Baie d'Urfé, QC) for 1 minute, followed by exposure of X-Ray film (Medlink Imaging, Terboro, NJ). The film was exposed to the ARNT blots for 5 and 10 minutes and the β-actin blots for 10, 30, and 60 seconds. Films were processed using an SRX-101A Developer (Konica Minolta, Toronto, ON). The film was scanned using an Epson Perfection V500 Scanner and band intensities were quantified by using IPLabGel software (Signal Analytics, Vienna, VA). Signal intensities for ARNT and the unidentified ARNT antibody-reactive protein (UAARP) were first normalized to the constant calibrator sample to adjust for inter-gel differences and then expressed relative to the β-actin signal intensity for the corresponding sample lane. Normalized signal intensity values were subject to statistical analysis to detect differences between treatment groups.

2.4 RNA ISOLATION

RNA was isolated from rat liver tissue using the acid guanidinium thiocyanante-phenol-chloroform extraction method (Chomczynski and Sacchi, 1987). Equipment was cleaned using RNase Zap (Ambion, Austin, TX) prior to isolation. Approximately 0.1 g of frozen rat liver tissue was homogenized in 1 mL of TriReagent (Sigma-Aldrich, St. Louis, MO) and stored at room temperature for 5 minutes. Chloroform (0.2 mL) was added and the mixture was shaken for 15 seconds and stored at room temperature for 5 minutes. Subsequently, the samples were centrifuged at 12,000x g for 15 minutes at 4°C in a 5415 centrifuge (Eppendorf), separating the RNA into the upper aqueous
phase. The aqueous phase (0.6 mL) was transferred to a microcentrifuge tube, and 0.5 mL of isopropanol was added to precipitate the RNA. The mixture was shaken for 15 seconds and stored for 5 minutes at room temperature. Samples were centrifuged at 12,000×g for 10 minutes at 4°C. The supernatant was discarded; the RNA pellet was washed with 1 mL of 75% ethanol and shaken for 5 seconds. All samples were centrifuged at 7,500×g for 5 minutes at 4°C. The supernatant was decanted with a pipette and the pellet was air dried for 10 minutes. RNA was dissolved in 0.1 mL of RNase-free water (Invitrogen, Grand Island, NY) and incubated for 10 minutes at 55°C to solubilize the RNA. Next, the samples were put on ice for 5 minutes and then 20 U of DNase I (Invitrogen) was added to the samples, followed by incubation at 37°C for 20 minutes. Samples were then incubated at 55°C for 20 minutes to inactivate the enzyme. The samples were stored at 4°C for 30 minutes. The RNA yield and purity were determined using a Biomate 3 Spectrophotometer (Thermo Scientific) to measure the absorbance at 260 nm and 280 nm. RNA was considered pure if the absorbance ratio $A_{260}/A_{280}$ was greater than or equal to 1.7. The yield was calculated using the absorbance at 260 nm and the samples were then stored at -80°C.

2.5 RNA INTEGRITY

The integrity of the RNA was assessed by running each sample on a 1% agarose gel, prepared in TAE buffer (40 mM Tris-acetate/1 mM EDTA, pH 8.0) with ethidium bromide (0.05 µL per mL of gel). RNA samples were diluted in 6x loading dye (Thermo Scientific, Ottawa, ON) and RNase-free water to yield a final concentration of 0.1 µg/µL. The gel was submerged in TAE running buffer in the Mini-Sub DNA Cell
(Biorad). The prepared sample (10 μL = 1 μg of RNA) was loaded onto the gel and run at 70 volts for 25-30 minutes using a BioRad Power Supply 200/2.0. The 28S and 18S ribosomal RNA bands were illuminated under ultraviolet light using a UV Transilluminator (UVP, Bio-Doc-It System). The RNA integrity was assessed by visual inspection of the relative intensity of the two bands, with high quality samples showing a 28S rRNA band of greater intensity (Fig. 2.3).

2.6 cDNA SYNTHESIS

The cDNA was synthesized by reverse transcription (RT) as described previously (Franc et al., 2001). First, total RNA (1 μg) was incubated with oligo(dT)$_{15}$ (2 μg, Fermentas, Ottawa, ON) at 60°C for 5 minutes in a DNA thermal cycler (Perkin Elmer). After this annealing step, samples were then incubated in a final volume of 40 μL with Moloney murine leukemia virus (MMLV)-reverse transcriptase (400 U; Invitrogen), Ribolock RNase inhibitor (80 U; Thermo Scientific), 1 mM of each dNTP (Invitrogen), 10 mM DTT, and 1x First Strand buffer containing 50 mM Tris-HCl, 75 mM KCl, and 3 mM MgCl$_2$. The primer extension occurred during the reaction at 37°C for 60 minutes and was terminated when the samples were incubated at 70°C for 10 minutes. The samples were centrifuged and stored at -80°C until used in the polymerase chain reactions.
Figure 2.3 Visualization of 28S and 18S rRNA in total RNA samples isolated from rats in the DEX Dose-Response Study. Total RNA (1µg) from rats treated with corn oil vehicle or DEX at the indicated doses for 6 h or 24 was run on a 1% agarose gel with ethidium bromide staining.
2.7 POLYMERASE CHAIN REACTION (PCR)

2.7.1 Primers

The design and optimization of PCR primers for the following rat targets were described in previous publications: ARNT (Mullen Grey and Riddick, 2009), TAT (Mullen Grey and Riddick, 2009), CYP3A23 (Mullen Grey, 2011), and β-actin (Tijet et al., 2006). The primers span an intron-exon boundary to avoid amplification from genomic DNA. Specificity within the rat genome of each primer pair was affirmed by BLAST search (www.ncbi.nlm.nih.gov/BLAST/) and Primer-Unigene Selectivity (PUNS) analysis (Boutros and Okey, 2004). Primers were purchased from Integrated DNA Technologies (Coralville, IA). The primer sequences, cycling parameters, and primer concentrations are shown in Table 2.1.

2.7.2 Conventional RT-PCR

Conventional RT-PCR with gel-based product detection was used to assess the specificity of PCR primers and the size of PCR products. Each PCR reaction contained input cDNA derived from 25 ng of RNA, Taq DNA polymerase (2.5 U; Invitrogen), 1x PCR buffer (20 mM Tris/50 mM KCl/3 mM MgCl₂), an optimized primer concentration (0.1 mM), and a 1.6 mM concentration of each dNTP. Denaturing, annealing, and extending temperatures were optimized to 94°C for 30 seconds, 58°C for 30 seconds, and 72°C for 40 seconds, respectively. This was repeated for 29 cycles. All PCR reactions began with a hot start phase, typically 5 minutes at 95°C and ended with a final extension at 72°C for 7 minutes. Samples were cooled at 4°C for at least 10 minutes and then diluted in 6X loading dye (Thermo Scientific) and run in ethidium
Table 2.1
Rat Primer Sequences, Cycling Parameters and Primer Concentrations, for Conventional RT-PCR and Quantitative Real-Time PCR

<table>
<thead>
<tr>
<th>Target Gene</th>
<th>Forward and Reverse Primer Sequences</th>
<th>Conventional PCR Cycling Conditions</th>
<th>Real-Time PCR Cycling Conditions</th>
<th>PCR Product Size (bp)</th>
<th>Real-time PCR Cycling Conditions</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>ARNT</td>
<td>5'-GATGAGGTGTGGGACCAGCT-3' 5'-GTGAGGCTCTCCTCCCTCACA-3'</td>
<td>50°C/2min 95°C/10min</td>
<td>STAGE 3 40 cycles: 95°C/15s 58°C/15s 72°C/45s</td>
<td>109</td>
<td>58°C/15s 72°C/45s</td>
<td>Mullen Grey and Riddick, 2009</td>
</tr>
<tr>
<td>TAT</td>
<td>5'-CAGCAACGTGCTTGAGTACCC-3' 5'-CTCCTCTTCGCTGCCTCCAG-3'</td>
<td>94°C/30sec 58°C/30sec 72°C/40sec For 29 cycles</td>
<td>STAGE 3 40 cycles: 95°C/15s 58°C/15s 72°C/45s</td>
<td>137</td>
<td>58°C/15s 72°C/45s</td>
<td>Mullen Grey and Riddick, 2009</td>
</tr>
<tr>
<td>CYP3A23</td>
<td>5'-TGGGTCTCTCTGGCAGTGTG-3' 5'-GTGAGGCGGTTCCCAAATCCGTG-3'</td>
<td>5'-GACCCAGATCATGTTTGAGACCTTC-3' 5'-GGAGTCCATCACAATGCCAGTG-3'</td>
<td>109</td>
<td>5'-GACCCAGATCATGTTTGAGACCTTC-3' 5'-GGAGTCCATCACAATGCCAGTG-3'</td>
<td>100</td>
<td>Tijet et al., 2006</td>
</tr>
</tbody>
</table>
bromide-stained 1% agarose gels (See Section 2.5) at 90 volts for 20 minutes. PCR products were visualized under UV light as shown in Fig.2.4.

2.7.3 Quantitative Real-Time PCR

Real-time PCR was performed for relative quantification of ARNT, TAT, and CYP3A23 mRNA levels using an ABI Prism 7500 Sequence Detection System (Applied Biosystems, Foster City, CA). Similar efficiencies for the target genes and the endogenous reference gene, β-actin, were observed, as required for accurate relative quantitation by the comparative threshold cycle (∆∆Ct) method (Fig. 2.5) Optimized primer concentrations are shown in Table 2.1. Each 25-µL PCR reaction was run in triplicate and included input cDNA derived from 10 ng of RNA, an optimized primer concentration, and 1x Power SYBR Green Master Mix (Applied Biosystem, Foster City, CA). Cycling conditions were: a first stage of 2 minutes at 50°C; a second stage of 10 minutes at 95°C; and a third stage consisting of 40 cycles at 95°C for 15 seconds, 58°C for 15 seconds, and 72°C for 45 seconds. Ct values for each sample were normalized to the Ct value for β-actin (∆Ct) and then normalized to the corresponding value for a common calibrator sample run on all plates (∆∆Ct). The change in expression of mRNA was calculated as a relative fold change (RQ) equal to \(2^{-\Delta\Delta C_t}\). Mean RQ values were subject to statistical analysis to detect differences between treatment groups.
Figure 2.4 Conventional RT-PCR analysis with gel-based product detection to characterize the specificity of the TAT primers and the size of the TAT product. Total RNA was isolated from the liver of an untreated male Fischer 344 rat and subjected to reverse transcription and PCR amplification to detect the presence of TAT transcript. PCR products were analyzed on ethidium bromide-stained 1% agarose gels. Lane 1, negative control demonstrating lack of exogenous DNA contamination in PCR reaction; Lane 2-3, negative controls demonstrating a requirement for TAT primers for product detection; Lane 4, in a complete reaction the TAT product migrates to a position consistent with its predicted size of 137 bp; Lane 5, negative control demonstrating lack of exogenous RNA contamination in RT reaction; Lane 6, negative control demonstrating lack of exogenous DNA contamination in RT reaction.
Figure 2.5 Representative efficiency curves for quantitative real-time PCR analysis of ARNT and β-actin mRNA levels. Rat hepatic ARNT and β-actin mRNA levels as quantified by real-time PCR with input cDNA derived from either 0.1, 1, 10, or 100 ng of RNA. The Ct value from each PCR reaction in triplicate is plotted against the amount of input cDNA in each reaction, displayed on a log scale. The lines of best fit were generated by least-squares linear regression. The efficiency of the ARNT reaction is 101.6% (slope = -3.285) and the efficiency of the β-actin reaction is 93.8% (slope = -3.480). The high amplification efficiencies and the parallel nature of the efficiency curves supported the use of the ΔΔCt method.
2.8 STATISTICS

NOTE: This section was prepared by Dr. David Riddick in consultation with Dr. José Nobrega. The same description also appears in the M.Sc. thesis of Alex Vonk, who studied the regulation of NADPH-cytochrome P450 oxidoreductase (POR) using tissues derived from the same experimental animals.

The DEX Dose-Response Study, the GR- and PXR-Selective Agonist Study, and the GR Antagonist Study utilized a sample size of four rats per treatment group. This decision was based on a formal sample size/power calculation. This sample size provided 80% power ($\beta = 0.20$), at a level of significance of $\alpha = 0.05$, to detect cases where the ratio of the estimated standard deviation (SD) to the minimum effect magnitude is 0.5. A typical example from previous and current studies of the regulation of constitutive rodent liver P450s performed in our lab would be a 50% increase or decrease in expression with a SD for the measured parameter equal to 25% of the mean. For the PXR-Knockout Rat Study, we were limited to a sample size of three rats per treatment group due to the high cost of these genetically-modified rats. This sample size provided 80% power ($\beta = 0.20$), at a level of significance of $\alpha = 0.05$, to detect cases where the ratio of the estimated SD to the minimum effect magnitude is 0.45. In cases where a study design offers inadequate power to detect a difference, potentially interesting data patterns are described conservatively as trends that did not achieve statistical significance.
All data are expressed as mean ± SD. All statistical analyses were performed on the original raw data and not on the percent control data presented in the figures. A result was considered statistically significant if $P < 0.05$.

For the DEX Dose-Response Study, data were analyzed initially using a randomized-design two-way analysis of variance (ANOVA) using Graphpad Prism 4 to identify significant influences of the two independent variables and their interaction (dose, time, dose x time interaction). Post test analyses for the planned comparisons (dose effect, time effect) were performed to assess whether there were significant differences between particular groups. Post tests were Bonferroni-corrected for multiple comparisons and used the mean square residual (pooled variance) and corresponding degrees of freedom from the two-way ANOVA and were performed using the online tool provided by Graphpad Software (www.graphpad.com).

For the GR- and PXR-Selective Agonist Study, data were analyzed initially using a randomized-design two-way ANOVA using Graphpad Prism 4 to identify significant influences of the two independent variables and their interaction (agonist, time, agonist x time interaction). Post test analyses for the planned comparisons (agonist effect, time effect) were performed to assess whether there were significant differences between particular groups. Post tests were Bonferroni-corrected for multiple comparisons and used the mean square residual (pooled variance) and corresponding degrees of freedom from the two-way ANOVA and were performed using the online tool provided by Graphpad Software (www.graphpad.com).
For the PXR-Knockout Rat Study, data were analyzed initially using a randomized-design three-way ANOVA using Statview 4.0.2 to identify significant influences of the three independent variables and their interactions (dose, time, genotype, dose x time interaction, dose x genotype interaction, time x genotype interaction, dose x time x genotype interaction). Post test analyses for the planned comparisons (dose effect, time effect, genotype effect) were performed to assess whether there were significant differences between particular groups. Post tests were Bonferroni-corrected for multiple comparisons and used the mean square residual (pooled variance) and corresponding degrees of freedom from the three-way ANOVA and were performed using the online tool provided by Graphpad Software (www.graphpad.com).

For the GR Antagonist Study, data were analyzed initially using a randomized-design three-way ANOVA using Statview 4.0.2 to identify significant influences of the three independent variables and their interactions (dose, time, antagonist, dose x time interaction, dose x antagonist interaction, time x antagonist interaction, dose x time x antagonist interaction). Post test analyses for the planned comparisons (dose effect, time effect, antagonist effect) were performed to assess whether there were significant differences between particular groups. Post tests were Bonferroni-corrected for multiple comparisons and used the mean square residual (pooled variance) and corresponding degrees of freedom from the three-way ANOVA and were performed using the online tool provided by Graphpad Software (www.graphpad.com).
SECTION 3: RESULTS

To gain insight into the roles of GR and PXR in the induction of rat hepatic ARNT expression by DEX, I performed four in vivo rat studies: 1) DEX Dose-Response Study, 2) GR- and PXR-Selective Agonist Study, 3) PXR-Knockout Rat Study, and 4) GR Antagonist Study.

3.1 DEX DOSE-RESPONSE STUDY

DEX is a potent synthetic glucocorticoid capable of activating both GR and PXR; however, the dose response curves for DEX activation of GR and PXR differ (Mullen Grey, 2011; Zhang et al., 2012). The high affinity of the GR for DEX means that GR can be activated in vivo by relatively low DEX doses, and I expected that all DEX doses used in this study (0.1, 1, 10, 50 mg/kg) would cause GR activation. On the other hand, only the highest doses of DEX used (10 and 50 mg/kg) were expected to cause PXR activation. I used this dose-dependent differential activation of GR and PXR to gain insight into ARNT regulation.

3.1.1 ARNT mRNA

ARNT mRNA levels were upregulated 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. 3.1A). The induction of ARNT mRNA was no longer observed at 24 hours.
Figure 3.1. Real-time PCR analysis of hepatic ARNT, TAT, and CYP3A23 mRNA levels in rats treated with varying doses of DEX. Adult male Fischer 344 rats received an i.p. injection of corn oil vehicle or DEX at doses of 0.1, 1, 10, or 50 mg/kg and were euthanized at 6 or 24 h following treatment. Quantitative analysis of ARNT (A), TAT (B), and CYP3A23 (C) mRNA levels, relative to β-actin. Data represent the mean ± SD of determinations from four rats per group, expressed as a percentage of the mean for the 6 h vehicle control. The P values for the two-way ANOVA main effects were: $P < 0.0001$ (dose), $P < 0.0001$ (time), $P < 0.0001$ (dose x time interaction) for ARNT mRNA; $P = 0.0073$ (dose), $P < 0.0001$ (time), $P < 0.0001$ (dose x time interaction) for TAT mRNA; $P < 0.0001$ (dose), $P < 0.0001$ (time), $P < 0.0001$ (dose x time interaction) for CYP3A23 mRNA. Outcomes of Bonferroni-corrected post tests for the planned comparisons were: *significantly different ($P < 0.05$) from timematched vehicle control; ‡significantly different ($P < 0.05$) from time-matched, 0.1 mg/kg DEX group; ◊significantly different ($P < 0.05$) from time-matched vehicle control, 0.1 mg/kg DEX, and 1 mg/kg DEX groups; †significantly different ($P < 0.05$) from DEX dose-matched opposite time-point.
3.1.2 TAT mRNA

The amount of TAT mRNA was measured in all studies to illustrate the induction of a GR target gene. Hepatic TAT mRNA levels were increased at the 6 h time-point by approximately 2- to 3-fold at all DEX doses tested (Fig. 3.1B). This increase in TAT mRNA was not observed at 24 hours. The DEX dose-responses for ARNT and TAT mRNA induction were somewhat similar, but two differences were noted: 1) TAT, but not ARNT, mRNA levels were induced at the lowest DEX dose, and 2) the magnitude of the mRNA induction response was greater for ARNT than TAT.

3.1.3 CYP3A23 mRNA

The induction of CYP3A23 mRNA by DEX was monitored in all studies as a classical PXR target gene. CYP3A23 mRNA levels were induced 7.5-fold in rats treated with DEX at 10 mg/kg and 35-fold in rats treated with DEX at 50 mg/kg, 24 hours post-dosing (Fig. 3.1C), whereas no induction was seen at the lower DEX doses. Unlike the ARNT and TAT mRNA responses, CYP3A23 mRNA levels were not increased by DEX at the 6 h time-point; there was a trend for CYP3A23 mRNA induction at the early time by DEX doses of 10 and 50 mg/kg, but this did not achieve statistical significance.

3.1.4 ARNT Protein

ARNT protein levels were measured to substantiate whether the increase in ARNT mRNA resulted in an increase in ARNT protein. A representative immunoblot is shown in Fig. 3.2A. Rat liver cytosolic ARNT was detected using a goat polyclonal
Figure 3.2. Immunoblot analysis of liver cytosolic ARNT and UAARP protein levels in rats treated with varying doses of DEX. Adult male Fischer 344 rats received an i.p. injection of corn oil vehicle or DEX at doses of 0.1, 1, 10, or 50 mg/kg and were euthanized at 6 or 24 h following treatment. (A) Immunoblot of cytosolic protein (30 µg) using a polyclonal antibody against ARNT and a monoclonal antibody against β-actin, showing results for one rat per treatment group. The main ARNT protein band migrates at approximately 88 kDa, with the secondary UAARP band at a slightly lower molecular mass; β-actin migrates at approximately 42 kDa. The positive control is cytosol from mouse Hepa-1 hepatoma cells and the internal control is the constant calibrator sample of cytosol from an untreated rat loaded on all gels. Quantitative analysis of ARNT (B) and UAARP (C) immunoreactivity levels, relative to β-actin. Data represent the mean ± SD of determinations from four rats per group, expressed as a percentage of the mean for the 6 h vehicle control. The P values for the two-way ANOVA main effects were: \( P = 0.4082 \) (dose), \( P = 0.0003 \) (time), \( P = 0.3117 \) (dose x time interaction) for ARNT immunoreactivity; \( P = 0.1346 \) (dose), \( P = 0.0077 \) (time), \( P = 0.2081 \) (dose x time interaction) for UAARP immunoreactivity. Outcomes of Bonferroni-corrected post tests for the planned comparisons were: *significantly different \((P < 0.05)\) from time-matched vehicle control; †significantly different \((P < 0.05)\) from DEX dose-matched opposite time-point.
antibody raised against the C-terminus of the human ARNT protein. The main ARNT protein band migrates at approximately 88 kDa, showing comparable mobility to the prominent band detected in cytosol from mouse Hepa-1 hepatoma cells, an abundant source of ARNT protein. There was a trend for increased ARNT protein levels at 24 hours post-dosing (Fig. 3.2A and B), particularly at the highest DEX doses; however, this effect of DEX dose did not achieve statistical significance. When administered 50 mg/kg DEX, rats had an increase in ARNT protein at 24 hours compared to 6 hours. There was no induction of ARNT protein levels at 6 hours for any of the DEX doses tested.

3.1.5 Unidentified ARNT Antibody-Reactive Protein (UAARP)

Surprisingly, a secondary band with a slightly lower molecular mass compared to ARNT appeared on ARNT immunoblots under certain DEX treatment conditions (Fig. 3.2A). The amount of this protein was quantified using the same methods used to measure the ARNT protein. At 6 hours post-dosing the level of UAARP was increased 6-fold in rats treated with 1 mg/kg DEX (Fig. 3.2C). There was also a trend for induced UAARP levels at the 6 h time-point by other DEX doses (particularly 10 mg/kg), but these effects did not achieve statistical significance. The induction of UAARP protein was no longer observed at 24 hours.

3.1.6 Liver Weight to Body Weight Ratio

I monitored the liver to body weight ratio in all of the in vivo rat studies as a general index of potentially harmful effects of chemical exposure. The ratio of liver to body weight increased by approximately 20 to 30% in rats treated with 10 and 50
Figure 3.3. Relative liver weight in rats treated with varying doses of DEX. Adult male Fischer 344 rats received an i.p. injection of corn oil vehicle or DEX at doses of 0.1, 1, 10, or 50 mg/kg and were euthanized at 6 or 24 h following treatment. The body weight was determined just prior to euthanasia and the wet liver weight was measured following in situ perfusion with HEGD buffer and excision. Quantitative analysis of the liver weight to body weight ratio. Data represent the mean ± SD of determinations from four rats per group, expressed as a percentage of the mean for the 6 h vehicle control. The P values for the two-way ANOVA main effects were: \( P = 0.0003 \) (dose), \( P = 0.0307 \) (time), \( P = 0.2550 \) (dose x time interaction). Outcomes of Bonferroni-corrected post tests for the planned comparisons were: *significantly different \( (P < 0.05) \) from time-matched vehicle control.
mg/kg of DEX, 24 hours post-dosing (Fig. 3.3). There was no change in the ratio in rats treated with DEX at 6 hours. However, the increased ratios mentioned above at 24 hours were not significantly different from the ratios measured at the 6 h time-point for the corresponding DEX doses.

3.2 GR- AND PXR-SELECTIVE AGONIST STUDY

My next strategy was to determine if rat hepatic ARNT expression was induced by chemicals that selectively activate GR or PXR. For this purpose, I used TA as a GR-selective agonist (Runge-Morris et al., 1996; Duanmu et al., 2000) and PCN as a selective PXR agonist (Hartley et al., 2004; El-Sayed, 2011).

3.2.1 ARNT mRNA

There was a 7.5 fold induction of ARNT mRNA levels at 6 hours post-dosing, when rats were treated with TA (Fig. 3.4A). The induction of ARNT mRNA levels by TA was no longer observed at 24 hours. No induction was observed in rats treated with PCN.

3.2.2 TAT mRNA

Similar to ARNT expression, TAT mRNA levels were induced at 6 hours post-dosing with TA (Fig.3.4B); the magnitude of this induction was approximately 3-fold. The induction of TAT mRNA levels by TA was no longer observed at 24 hours. PCN treatment did not alter the levels of TAT mRNA. ARNT and TAT mRNA showed notable similarity in their responses to TA and PCN treatment.
Figure 3.4. Real-time PCR analysis of hepatic ARNT, TAT, and CYP3A23 mRNA levels in rats treated with TA (GR agonist) or PCN (PXR agonist). Adult male Fischer 344 rats received an i.p. injection of corn oil vehicle, TA (5 mg/kg), or PCN (50 mg/kg) and were euthanized at 6 or 24 h following treatment. Quantitative analysis of ARNT (A), TAT (B), and CYP3A23 (C) mRNA levels, relative to β-actin. Data represent the mean ± SD of determinations from four rats per group, expressed as a percentage of the mean for the 6 h vehicle control. The P values for the two-way ANOVA main effects were: P = 0.0086 (agonist), P = 0.0154 (time), P = 0.0054 (agonist x time interaction) for ARNT mRNA; P = 0.0014 (agonist), P < 0.0001 (time), P = 0.0001 (agonist x time interaction) for TAT mRNA; P < 0.0001 (agonist), P = 0.0006 (time), P<0.0001 (agonist x time interaction) for CYP3A23 mRNA. Outcomes of Bonferroni-corrected post tests for the planned comparisons were: *significantly different (P < 0.05) from all other time-matched groups; †significantly different (P < 0.05) from agonist-matched 24 h time-point.
3.2.3 CYP3A23 mRNA

CYP3A23 mRNA levels were induced 75-fold at 24 hours following PCN treatment (Fig. 3.4C). The nearly 10-fold increase in CYP3A23 mRNA levels seen at 6 hours following PCN treatment did not achieve statistical significance, and the PCN effects at 6 and 24 hours differed significantly. There was no change in expression of CYP3A23 mRNA when rats were treated with TA.

3.2.4 ARNT Protein

There was a trend for increased ARNT protein levels at 24 hours following TA treatment (Fig. 3.5A and B); however, this TA effect did not achieve statistical significance. Nevertheless, ARNT protein levels in TA-treated rats were higher at 24 hours compared to 6 hours. PCN treatment did not alter ARNT protein levels.

3.2.5 UAARP

The levels of UAARP increased 4.5-fold at 6 hours in rats treated with TA as compared to the 6 hour vehicle (Fig. 3.5C). This induction was not observed at 24 hours in rats treated with TA or at either time-point following PCN treatment.

3.2.6 Liver Weight to Body Weight Ratio

TA treatment caused an approximately 30% increase in the liver to body weight ratio at the 24 h time-point (Fig. 3.6), and this increased ratio was also elevated compared to that for the 6 h TA group and the 24 h PCN group. PCN treatment did not alter the liver to body weight ratio.
Figure 3.5. Immunoblot analysis of liver cytosolic ARNT and UAARP protein levels in rats treated with TA (GR agonist) or PCN (PXR agonist). Adult male Fischer 344 rats received an i.p. injection of corn oil vehicle, TA (5 mg/kg), or PCN (50 mg/kg) and were euthanized at 6 or 24 h following treatment. (A) Immunoblot of cytosolic protein (30 µg) using a polyclonal antibody against ARNT and a monoclonal antibody against β-actin, showing results for one rat per treatment group (in duplicate). The main ARNT protein band migrates at approximately 88 kDa, with the secondary UAARP band at a slightly lower molecular mass; β-actin migrates at approximately 42 kDa. The positive control is cytosol from mouse Hepa-1 hepatoma cells and the internal control is the constant calibrator sample of cytosol from a 6 h vehicle-treated rat loaded on all gels. Quantitative analysis of ARNT (B) and UAARP (C) immunoreactivity levels, relative to β-actin. Data represent the mean ± SD of determinations from four rats per group, expressed as a percentage of the mean for the 6 h vehicle control. The P values for the two-way ANOVA main effects were: P = 0.0854 (agonist), P = 0.0011 (time), P = 0.5932 (agonist x time interaction) for ARNT immunoreactivity; P = 0.0073 (agonist), P = 0.1906 (time), P = 0.1621 (agonist x time interaction) for UAARP immunoreactivity. Outcomes of Bonferroni-corrected post tests for the planned comparisons were: *significantly different (P < 0.05) from time-matched vehicle control; †significantly different (P < 0.05) from agonist-matched 6 h time-point.
Figure 3.6. Relative liver weight in rats treated with TA (GR agonist) or PCN (PXR agonist). Adult male Fischer 344 rats received an i.p. injection of corn oil vehicle, TA (5 mg/kg), or PCN (50 mg/kg) and were euthanized at 6 or 24 h following treatment. The body weight was determined just prior to euthanasia and the wet liver weight was measured following in situ perfusion with HEGD buffer and excision. Quantitative analysis of the liver weight to body weight ratio. Data represent the mean ± SD of determinations from four rats per group, expressed as a percentage of the mean for the 6 h vehicle control. The P values for the two-way ANOVA main effects were: P = 0.0011 (agonist), P = 0.0090 (time), P = 0.0046 (agonist x time interaction). Outcomes of Bonferroni-corrected post tests for the planned comparisons were: *significantly different (P < 0.05) from time-matched vehicle control; ‡significantly different (P < 0.05) from time-matched PCN group; †significantly different (P < 0.05) from agonist-matched 6 h time-point.
3.3 **PXR-KNOCKOUT RAT STUDY**

A definitive strategy to test the involvement of a given receptor in a biological response is the use of genetically modified animals lacking the receptor of interest. To this end, I examined ARNT induction by two doses of DEX in PXR-knockout rats, created by SAGE Laboratories via zinc finger nuclease technology, and wild-type Sprague-Dawley controls. The low DEX dose (1 mg/kg) activates GR selectively, whereas the high DEX dose (50 mg/kg) activates both GR and PXR.

### 3.3.1 ARNT mRNA

ARNT mRNA levels were upregulated almost 18-fold at 6 hours in wild-type rats treated with 1 mg/kg of DEX (Fig. 3.7A). Although there was an apparent 7.5-fold increase in ARNT mRNA levels at 6 hours in wild-type rats treated with 50 mg/kg of DEX, this did not achieve statistical significance. Similarly, there were apparent 8.5-fold, and 5.5-fold increases in ARNT mRNA levels at 6 hours in PXR-knockout rats treated with 1 and 50 mg/kg DEX, respectively, responses that also did not achieve statistical significance. However, the induced levels of ARNT mRNA at 6 hours following treatment with either DEX dose did not differ between wild-type and PXR-knockout rats. This suggests that knocking out the PXR has no effect on basal or DEX-inducible ARNT mRNA levels. There was no induction of ARNT mRNA levels at 24 hours by either DEX dose in either rat strain.

### 3.3.2 TAT mRNA

As expected, knocking out the PXR had no effect on basal or DEX-inducible TAT mRNA expression. TAT mRNA levels were induced almost 8-fold in wild-type rats
Figure 3.7. Real-time PCR analysis of hepatic ARNT, TAT, and CYP3A23 mRNA levels in wild-type and PXR-knockout rats treated with low- or high-dose DEX. Adult male wild-type Sprague-Dawley rats or PXR-knockout rats (PXRKO) received an i.p. injection of corn oil vehicle or DEX at doses of 1 or 50 mg/kg and were euthanized at 6 or 24 h following treatment. Quantitative analysis of ARNT (A), TAT (B), and CYP3A23 (C) mRNA levels, relative to ß-actin. Data represent the mean ± SD of determinations from three rats per group, expressed as a percentage of the mean for the 6 h vehicle control. The P values for the three-way ANOVA main effects were: P = 0.0064 (dose), P = 0.0003 (time), P = 0.1811 (genotype), P = 0.0067 (dose x time interaction), P = 0.3889 (dose x genotype interaction), P = 0.1817 (time x genotype interaction), P = 0.3631 (dose x time x genotype interaction) for ARNT mRNA; P < 0.0001 (dose), P < 0.0001 (time), P = 0.2177 (genotype), P < 0.0001 (dose x time interaction), P = 0.1262 (dose x genotype interaction), P = 0.1891 (time x genotype interaction), P = 0.8438 (dose x time x genotype interaction) for TAT mRNA; P < 0.0001 (dose), P = 0.0008 (time), P = 0.0023 (genotype), P = 0.001 (dose x time interaction), P < 0.0001 (dose x genotype interaction), P = 0.0006 (time x genotype interaction), P < 0.0001 (dose x time x genotype interaction) for CYP3A23 mRNA. Outcomes of Bonferroni-corrected post tests for the planned comparisons were: *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 time-matched, DEX dose-matched wild-type group.
treated with 1 and 50 mg/kg DEX, and approximately 6-fold in PXR-knockout rats treated with either DEX dose (Fig. 3.7B). Similar to the two previous studies, there was no induction of TAT mRNA levels at the 24 hour time-point.

3.3.3 CYP3A23 mRNA

The main diagnostic criterion for successful PXR-knockout is the absence of CYP3A induction by PXR ligands such as PCN or high dose DEX. CYP3A23 mRNA levels were induced almost 60-fold in the wild-type rats treated with 50 mg/kg DEX at the 24 hour time-point (Fig. 3.7C). This effect was completely absent in the time-matched, DEX dose-matched, PXR-knockout rats. There was no change in CYP3A23 mRNA expression at 6 hours in wild-type or PXR-knockout rats treated with DEX.

3.3.4 ARNT Protein

When wild-type rats were treated with 1 and 50 mg/kg DEX there were apparent 4.5-fold and 6-fold increases in ARNT protein levels at the 24 hour time-point (Fig. 3.8A and B), but only the high dose DEX treatment effect achieved statistical significance. The DEX induction response was attenuated in the PXR-knockout rats such that ARNT protein levels did not differ between DEX-treated and vehicle-treated PXR-knockout rats. However, the induced levels of ARNT protein at 24 hours following treatment with either DEX dose did not differ between wild-type and PXR-knockout rats. There was no change in ARNT protein levels at 6 hours in wild-type or PXR-knockout rats treated with DEX.
Figure 3.8. Immunoblot analysis of liver cytosolic ARNT and UAARP protein levels in wild-type and PXR-knockout rats treated with low- or high-dose DEX. Adult male wild-type Sprague-Dawley rats (WT) or PXR-knockout rats (PXRKO) received an i.p. injection of corn oil vehicle or DEX at doses of 1 or 50 mg/kg and were euthanized at 6 or 24 h following treatment. (A) Immunoblot of cytosolic protein (30 µg) using a polyclonal antibody against ARNT and a monoclonal antibody against ß-actin, showing results for one rat per treatment group. The main ARNT protein band migrates at approximately 88 kDa, with the secondary UAARP band at a slightly lower molecular mass; ß-actin migrates at approximately 42 kDa. The positive control is cytosol from mouse Hepa-1 hepatoma cells. Quantitative analysis of ARNT (B) and UAARP (C) immunoreactivity levels, relative to ß-actin. Data represent the mean ± SD of determinations from three rats per group, expressed as a percentage of the mean for the 6 h vehicle control. The P values for the three-way ANOVA main effects were: P = 0.0170 (dose), P < 0.0001 (time), P = 0.9156 (genotype), P = 0.1708 (dose x time interaction), P = 0.3883 (dose x genotype interaction), P = 0.1746 (time x genotype interaction), P = 0.5835 (dose x time x genotype interaction) for ARNT immunoreactivity; P < 0.0001 (dose), P < 0.0001 (time), P = 0.7699 (genotype), P < 0.0001 (dose x time interaction), P = 0.7368 (dose x genotype interaction), P = 0.5808 (time x genotype interaction), P = 0.6428 (dose x time x genotype interaction) for UAARP immunoreactivity. Outcomes of Bonferroni-corrected post tests for the planned comparisons were: *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.
3.3.5 UAARP

UAARP levels were induced in DEX-treated wild-type and PXR-knockout rats 6 hours post-dosing. The amount of UAARP increased 27-fold in wild-type rats treated with 1 mg/kg DEX and 24-fold in wild-type rats treated with 50 mg/kg DEX at the 6 hour time-point (Fig. 3.8C). At this same time-point, UAARP levels were upregulated 34-fold in PXR-knockout rats treated with 1 mg/kg DEX and 23-fold in PXR-knockout rats treated with 50 mg/kg DEX. The induced levels of UAARP at 6 hours following treatment with either DEX dose did not differ between wild-type and PXR-knockout rats. There was no upregulation of UAARP levels at the 24 hour time-point in the wild-type or the PXR-knockout rats treated with DEX.

3.3.6 Liver Weight to Body Weight Ratio

The liver weight to body weight ratio increased by approximately 20 to 30% at 24 hours in both wild-type and PXR-knockout rats treated with 1 mg/kg and 50 mg/kg doses of DEX (Fig. 3.9). The small increase in liver to body weight ratio caused by DEX at 24 hours did not differ between wild-type and PXR-knockout rats. DEX had no effect on the liver to body weight ratio at 6 hours in wild-type and PXR-knockout rats.
Figure 3.9. Relative liver weight in wild-type and PXR-knockout rats treated with low- or high-dose DEX. Adult male wild-type Sprague-Dawley rats or PXR-knockout rats (PXRKO) received an i.p. injection of corn oil vehicle or DEX at doses of 1 or 50 mg/kg and were euthanized at 6 or 24 h following treatment. The body weight was determined just prior to euthanasia and the wet liver weight was measured following in situ perfusion with HEGD buffer and excision. Quantitative analysis of the liver weight to body weight ratio. Data represent the mean ± SD of determinations from three rats per group, expressed as a percentage of the mean for the 6 h vehicle control. The P values for the three-way ANOVA main effects were: \( P < 0.0001 \) (dose), \( P = 0.0019 \) (time), \( P = 0.7516 \) (genotype), \( P = 0.0019 \) (dose x time interaction), \( P = 0.4984 \) (dose x genotype interaction), \( P = 0.9412 \) (time x genotype interaction), \( P = 0.9154 \) (dose x time x genotype interaction). Outcomes of Bonferroni-corrected post tests for the planned comparisons were: *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.
3.4 GR ANTAGONIST STUDY

Since a viable rat model lacking GR does not exist, I used a pharmacological strategy to examine the affect of GR antagonism with RU486 on ARNT induction by two doses of DEX. The low DEX dose (0.5 mg/kg) activates GR selectively, whereas the high DEX dose (50 mg/kg) activates both GR and PXR. The RU486 dose of 50 mg/kg was selected in order to achieve a 100:1 ratio of RU486 to DEX in rats receiving low dose DEX for effective GR antagonism without PXR activation by RU486 alone.

3.4.1 ARNT mRNA

ARNT mRNA levels were induced 4-fold at 6 hours following treatment with 0.5 mg/kg of DEX (Fig. 3.10A). This effect was attenuated in rats co-treated with RU486 and the same dose of DEX. The higher dose of DEX (50 mg/kg) also induced ARNT mRNA levels at the 6 hour time-point in both the absence and presence of RU486 co-treatment, although the magnitude of this response seemed to be augmented by RU486. There was no upregulation of ARNT mRNA levels at 24 hours by either DEX dose in the absence or presence of RU486.

3.4.2 TAT mRNA

TAT mRNA levels were induced 3-fold in rats treated with either 0.5 mg/kg or 50 mg/kg of DEX (Fig. 3.10B). The induction of TAT mRNA levels at 6 hours by the low DEX dose, but not the high DEX dose, was attenuated by RU486 co-treatment. There was no induction of TAT mRNA levels at 24 hours by either DEX dose in the absence or presence of RU486. There was a tendency for RU486 to lower basal levels of TAT mRNA at both time-points, but this did not achieve statistical significance.
Figure 3.10. Real-time PCR analysis of hepatic ARNT, TAT, and CYP3A23 mRNA levels in rats treated with low- or high-dose DEX in the absence or presence of the GR antagonist RU486. Adult male Fischer 344 rats received an i.p. injection of corn oil vehicle or RU486 (50 mg/kg) followed 30 min later by an i.p. injection or corn oil vehicle or DEX at doses of 0.5 or 50 mg/kg, with euthanasia at 6 or 24 h following the second injection. Quantitative analysis of ARNT (A), TAT (B), and CYP3A23 (C) mRNA levels, relative to β-actin. Data represent the mean ± SD of determinations from four rats per group, expressed as a percentage of the mean for the 6 h vehicle control. The P values for the three-way ANOVA main effects were: *P < 0.0001 (dose), †P < 0.0001 (time), ‡P = 0.1482 (antagonist), P < 0.0001 (dose x time interaction), P < 0.0001 (dose x antagonist interaction), P = 0.9640 (time x antagonist interaction), P < 0.0001 (dose x time x antagonist interaction) for ARNT mRNA; P < 0.0001 (dose), P < 0.0001 (time), P = 0.0458 (antagonist), P < 0.0001 (dose x time interaction), P = 0.0058 (dose x antagonist interaction), P = 0.5922 (time x antagonist interaction), P = 0.0144 (dose x time x antagonist interaction) for TAT mRNA; P < 0.0001 (dose), P < 0.0001 (time), P = 0.0001 (antagonist), P < 0.0001 (dose x time interaction), P = 0.3807 (dose x antagonist interaction), P = 0.0338 (time x antagonist interaction), P = 0.7059 (dose x time x antagonist interaction) for CYP3A23 mRNA. Outcomes of Bonferroni-corrected post tests for the planned comparisons were: *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 time-matched, DEX dose-matched vehicle (no RU486) control.
3.4.3 CYP3A23 mRNA

There was an 8- and 45-fold induction of CYP3A23 mRNA levels when rats were treated with 50 mg/kg of DEX at 6 and 24 hours, respectively (Fig. 3.10C). Similarly, rats treated with RU486 and the high dose of DEX also showed an increase in CYP3A23 mRNA levels, with a 3-fold induction at 6 hours and an 11-fold induction at 24 hours post-dosing. The induction at 24 hours by high dose DEX was augmented slightly by RU486. There was no induction at 6 or 24 hours in rats treated with low dose DEX. The CY3A23 mRNA level in rats treated with low dose DEX at 24 hours was augmented by RU486. There was a tendency for RU486 alone to increase CYP3A23 mRNA levels, but this did not achieve statistical significance.

3.4.4 ARNT Protein

ARNT protein levels were upregulated 7-fold in rats treated with 50 mg/kg DEX, 24 hours post-dosing (Fig. 3.11 A and B). This effect was attenuated when rats were co-treated with RU486 and the same high DEX dose. The trend for increased ARNT protein levels at 24 hours in rats treated with low dose DEX did not achieve statistical significance. There was no induction of ARNT protein levels at 6 hours by either DEX dose in the absence or presence of RU486.

3.4.5 UAARP

UAARP levels were upregulated 16-fold and 12-fold at 6 hours when rats were treated with 0.5 mg/kg or 50 mg/kg DEX, respectively (Fig. 3.11A and C). The induction of UAARP levels at 6 hours following high dose DEX was not statistically significant in the presence of RU486, and the response to low dose DEX was
Figure 3.11. Immunoblot analysis of liver cytosolic ARNT and UAARP protein levels in rats treated with low- or high-dose DEX in the absence or presence of the GR antagonist RU486. Adult male Fischer 344 rats received an i.p. injection of corn oil vehicle or RU486 (50 mg/kg) followed 30 min later by an i.p. injection or corn oil vehicle or DEX at doses of 0.5 or 50 mg/kg, with euthanasia at 6 or 24 h following the second injection. (A) Immunoblot of cytosolic protein (30 µg) using a polyclonal antibody against ARNT and a monoclonal antibody against β-actin, showing results for one rat per treatment group. The main ARNT protein band migrates at approximately 88 kDa, with the secondary UAARP band at a slightly lower molecular mass; β-actin migrates at approximately 42 kDa. The positive control is cytosol from mouse Hepa-1 hepatoma cells and the internal control is the constant calibrator sample of cytosol from a 6 h vehicle-treated rat loaded on all gels. Quantitative analysis of ARNT (B) and UAARP (C) immunoreactivity levels, relative to β-actin. Data represent the mean ± SD of determinations from four rats per group, expressed as a percentage of the mean for the 6 h vehicle control. The P values for the three-way ANOVA main effects were: P = 0.0797 (dose), P < 0.0001 (time), P = 0.0054 (antagonist), P = 0.0364 (dose x time interaction), P = 0.0076 (dose x antagonist interaction), P = 0.0810 (time x antagonist interaction), P = 0.0684 (dose x time x antagonist interaction) for ARNT immunoreactivity; P = 0.0002 (dose), P < 0.0001 (time), P = 0.0001 (antagonist), P = 0.0043 (dose x time interaction), P = 0.0059 (dose x antagonist interaction), P = 0.0013 (time x antagonist interaction), P = 0.0044 (dose x time x antagonist interaction) for UAARP immunoreactivity. Outcomes of Bonferroni-corrected post tests for the planned comparisons were: *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 time-matched, DEX dose-matched vehicle (no RU486) control.
completely attenuated by RU486. DEX had no effect on UAARP levels at 24 hours with or without RU486.

3.4.6 Liver Weight to Body Weight Ratio

In rats treated with 50 mg/kg DEX, there was a 20 to 30% increase in the liver to body weight ratio at 6 and 24 hours post-dosing compared to time-matched, vehicle controls (Fig. 3.12). A similar increase was seen at 24 hours in rats co-treated with high dose DEX and RU486. The small increase in liver to body weight ratio caused by high dose DEX did not differ in the absence or presence of RU486.
Figure 3.12. Relative liver weight in rats treated with low- or high-dose DEX in the absence or presence of the GR antagonist RU486. Adult male Fischer 344 rats received an i.p. injection of corn oil vehicle or RU486 (50 mg/kg) followed 30 min later by an i.p. injection of corn oil vehicle or DEX at doses of 0.5 or 50 mg/kg, with euthanasia at 6 or 24 h following the second injection. The body weight was determined just prior to euthanasia and the wet liver weight was measured following in situ perfusion with HEGD buffer and excision. Quantitative analysis of the liver weight to body weight ratio. Data represent the mean ± SD of determinations from four rats per group, expressed as a percentage of the mean for the 6 h vehicle control. The P values for the three-way ANOVA main effects were: $P < 0.0001$ (dose), $P = 0.0002$ (time), $P = 0.9466$ (antagonist), $P = 0.2253$ (dose x time interaction), $P = 0.1838$ (dose x antagonist interaction), $P = 0.9903$ (time x antagonist interaction), $P = 0.9955$ (dose x time x antagonist interaction). Outcomes of Bonferroni-corrected post tests for the planned comparisons were: *significantly different ($P < 0.05$) from time-matched, antagonist-matched vehicle (no DEX) control.
SECTION 4: DISCUSSION

4.1 OBJECTIVE ONE: TO DETERMINE WHETHER RAT HEPATIC ARNT IS INDUCED BY DEX DOSES THAT ACTIVATE THE GR OR PXR

The results of the DEX Dose-Response Study suggest a GR-mediated induction of ARNT mRNA levels in rat liver, supporting my hypothesis. ARNT mRNA levels were significantly increased in rats treated with doses of DEX that activate the GR (1, 10, or 50 mg/kg) at the 6 hour time-point. The DEX dose-responses for induction of ARNT and TAT mRNA show some similarities. TAT mRNA levels were induced at 6 hours in response to 0.1, 1, 10, and 50 mg/kg of DEX. In contrast, the induction of CYP3A23 mRNA levels by DEX differs from the ARNT mRNA response. First of all, CYP3A23 mRNA levels were upregulated at 24 hours, but not 6 hours. Second, CYP3A23 mRNA levels were induced only at the highest DEX doses (10 or 50 mg/kg). These findings are consistent with a role for GR in the induction of ARNT mRNA levels by DEX.

There was a non-statistically significant trend for ARNT protein levels to increase at 24 hours post-dosing with the higher doses of DEX. This could indicate a GR- or PXR-mediated event. A lower molecular mass protein reacting with the ARNT antibody (UAARP) was upregulated at 6 hours in rats treated with 1 mg/kg of DEX. This is consistent with a GR-mediated effect. On a genome-wide scale, cellular abundance of protein is primarily controlled at the translational level (Schwanhäuser et al., 2011). It is unclear why a relatively large magnitude increase in ARNT mRNA levels results in a relatively modest elevation of ARNT protein. Although a GR-mediated increase in ARNT mRNA levels may drive increased synthesis of ARNT protein, a role for PXR
cannot be excluded since ARNT protein levels seem to be most affected at the highest DEX doses. Additional studies would be required to determine if ARNT mRNA levels are increased via transcription or mRNA stabilization and whether modulation of translation or protein stability impact ARNT protein levels.

4.2 OBJECTIVE TWO: TO DETERMINE WHETHER TA (GR AGONIST) OR PCN (PXR AGONIST) CAUSE INDUCTION OF RAT HEPATIC ARNT expression

ARNT mRNA levels were increased by the GR-selective agonist TA. This provides further evidence that the GR is involved in the modulation of ARNT expression. ARNT mRNA levels were significantly upregulated at the 6 hour time-point in rats treated with TA. No induction occurred when rats were treated with the PXR-selective agonist PCN. ARNT mRNA expression had a similar induction profile to TAT mRNA, and not CYP3A23 mRNA. Induction of TAT mRNA levels by TA occurred at 6 hours post-dosing. PCN had no effect on TAT mRNA levels. PCN induced CYP3A23 mRNA levels at 24 hours, while TA had no effect on CYP3A23 mRNA levels.

There was a non-statistically significant trend for ARNT protein levels to increase in response to TA at 24 hours. UAARP levels were induced at 6 hours in response to TA. That ARNT mRNA and protein levels were affected by the GR-selective agonist, and not the PXR-selective agonist, suggests that GR activation impacts ARNT expression. A GR-dependent increase in ARNT mRNA levels seems to be critical, but translational or post-translational events may also be modulated.
4.3 OBJECTIVE THREE: TO DETERMINE WHETHER HEPATIC \textit{ARNT} INDUCTION BY DEX IS ALTERED IN \textit{PXR}-KNOCKOUT RATS

The results from the \textit{PXR}-knockout rat study also suggest a role for GR in \textit{ARNT} induction by DEX. \textit{ARNT} mRNA levels were increased at 6 hours in wild-type Sprague-Dawley rats treated with 1 mg/kg DEX. This induced level of \textit{ARNT} mRNA was not significantly different from that found in the \textit{PXR}-knockout rat, although the apparent DEX induction response did not achieve statistical significance in the \textit{PXR}-knockout rats. The induction profile of \textit{ARNT} mRNA expression more closely resembles the TAT mRNA induction profile compared to the CYP3A23 mRNA induction profile. TAT mRNA levels were upregulated in rats treated with both doses of DEX (1 or 50 mg/kg) in wild-type and \textit{PXR}-knockout rats. CYP3A23 mRNA levels were induced at 24 hours in wild-type rats treated with 50 mg/kg DEX, but not in the \textit{PXR}-knockout rats.

Levels of UAARP were strongly increased by both DEX doses at 6 hours in wild-type and \textit{PXR}-knockout rats, suggesting a role for GR in this response. The findings with \textit{ARNT} protein levels were more equivocal. Although the induction of \textit{ARNT} protein level at 24 hours following high dose DEX treatment seemed to be partially attenuated in \textit{PXR}-knockout rats, the induced levels of \textit{ARNT} protein did not differentiate according to rat genotype.

The results from this study suggest a GR-mediated induction of \textit{ARNT} expression for two reasons. First, the \textit{ARNT} mRNA expression profile more closely resembles the expression profile for TAT mRNA compared to CYP3A23 mRNA.
Second, the induced ARNT mRNA and protein levels do not differ between wild-type and PXR-knockout rats. However, since the trends for increased ARNT mRNA and protein levels following DEX treatment in PXR-knockout rats do not achieve statistical significance, the involvement of the PXR cannot be ruled out.

4.4 OBJECTIVE FOUR: TO DETERMINE WHETHER RAT HEPATIC ARNT INDUCTION BY DEX IS ALTERED BY THE GR ANTAGONIST RU486

The involvement of the GR in the DEX-stimulated increase in rat hepatic ARNT expression was confirmed using the GR antagonist, RU486. The increase in ARNT mRNA levels in rats treated with 0.5 mg/kg of DEX was attenuated by the co-treatment with RU486. The induction of TAT mRNA levels by low dose DEX was also attenuated by RU486. CYP3A23 mRNA levels were induced by 50 mg/kg DEX and this response was potentiated at 24 hours by the co-treatment with RU486. This was not entirely surprising, as the activated GR induces transcription of PXR, and PXR can be activated by both DEX and RU486 at high doses (Kliewer et al., 2002; Pascussi et al., 2003).

The ARNT protein levels were increased at 24 hours in rats treated with 50 mg/kg DEX, and this effect was attenuated with concomitant treatment with RU486. UAARP levels were induced by DEX (0.5 or 50 mg/kg) at 6 hours, and this effect was abrogated by RU486 co-treatment in the rats receiving the lower dose of DEX. Since RU486 antagonizes the GR, these results suggest that GR activation by DEX is essential for the increase in ARNT mRNA levels and subsequent synthesis of ARNT protein.
The results of my four in vivo rat studies suggest a GR-mediated induction of hepatic ARNT expression with an apparent involvement of a pre-translational mechanism. The GR-dependent increase in ARNT mRNA levels may involve transcriptional or mRNA stabilization events. It is likely that the increased ARNT mRNA levels drive increased production of ARNT protein; a role for PXR cannot be excluded since ARNT protein levels are most affected at the highest DEX doses. Modulation of ARNT protein levels may also involve translational or protein stabilization mechanisms. The role of GR and PXR in the modulation of ARNT protein levels will be discussed in greater detail in Section 4.7.

4.5 LIMITATIONS OF THE PRESENT STUDY

There is diurnal variation in ARNT mRNA and protein expression (Richardson et al., 1998; Zhang et al., 2009). The GR expression also varies on a light/dark cycle (Atkinson et al., 1997; Shimmer and Funder, 2011). In all studies, this variation was an important factor in deciding the time of injection and euthanasia. The rats were injected in all studies at 10:00 am and euthanasia was 6 hours later (4:00pm) or 24 hours later (10:00 am the following day). Endogenous glucocorticoids increase in rodents at the beginning of the dark cycle and decrease during the light cycle. For the 6 hour time-point, the endogenous glucocorticoid levels would be relatively low, limiting their effect on this part of the study. However, glucocorticoid levels increase during the dark cycle, potentially influencing the results in rats euthanized at the 24 hour time-point. The average daily plasma corticosterone concentration is approximately 102 ng/ml in male rats with a peak of approximately 206 ng/ml (Atkinson et al., 1997). These levels are
much lower than the concentrations that would be achieved following treatment of rats with the pharmacological doses of glucocorticoids used in my study. A study measuring the diurnal rhythm of ARNT protein expression in rat liver found that the amount of protein peaks at 12:00 am and 12:00 pm (Richardson et al., 1998). The basal diurnal expression pattern from the Richardson study shows that the ARNT protein content has a trough-to-peak ratio of 0.5 at my 6 hour time-point (4:00 pm) and a trough-to-peak ratio of 0.8 at my 24 hour time-point (10:00 am the following day). As there is no peak protein concentrations at these time-points, the diurnal fluctuations of ARNT protein should have little effect on my results. In another study, the diurnal cycle of ARNT mRNA expression was measured in the liver of mice (Zhang et al., 2009). In this study, they found that ARNT mRNA expression peaked at 6:00 pm. At 6:00 pm the ARNT mRNA level was approximately 25% higher than that found at the lowest point in the day (10:00 am). The time at which ARNT mRNA expression is the lowest coincides with the 24 hour time-point for my studies, which showed no induction of ARNT mRNA in response to exogenous glucocorticoids. In a time-course study conducted previously in our lab, rats were treated at 4:00 am with 1.5 mg/kg DEX and then euthanized at 7:00 am the next day (27 hours); consistent with my work, induction of ARNT mRNA levels was seen at the 3 and 6 hour time-points (Mullen Grey and Riddick, 2009). Since both endogenous glucocorticoids and my main gene of interest (ARNT) show diurnal variation, shifts in injection times or euthanasia times could impact the results.

The dose of RU486 that was needed to properly antagonize GR also had the potential to activate the PXR (Teng and Piquette-Miller, 2005). A ratio of 100:1 (RU486: DEX) was necessary to achieve clear GR antagonism as assessed with the
TAT mRNA response. The results showed an induction of ARNT mRNA levels at 1 mg/kg DEX and not 0.1 mg/kg DEX, and, therefore, there was a limitation on how low the DEX dose could go for this study. This limitation dictated the dose of RU486 used in this study. A low dose of DEX (0.5 mg/kg) was sufficient to induce ARNT mRNA levels. This response was attenuated in the rats co-treated with DEX and RU486. I used only a single dose of RU486 (50 mg/kg) for rats treated with 0.5 and 50 mg/kg DEX. The high dose of DEX was enough to overcome the antagonistic effects of RU486, and therefore, I saw an increase in ARNT mRNA levels in rats treated with high-dose DEX and RU486. This is not surprising as RU486 may act as a partial GR agonist (Shulz et al., 2002; Peeters et al., 2008) and a PXR agonist at high doses (Teng et al, 2005). Also, the effects of RU486 on gene expression may depend on the dominant GR isoform that is activated (Weigel, 1993). GRβ acts to inhibit the effects of GRα-induced transcription and may complicate the effect on ARNT expression in response to DEX and RU486.

Optimizing the protocols for immunoblot analysis of ARNT protein was a challenge. Rat liver cytosolic ARNT protein levels are low relative to the endogenous internal control, β-actin protein. Relatively large amounts of cytosolic protein (30 µg) were required for reliable ARNT detection. Also, the polyclonal ARNT antibody selected was meticulously chosen, but not entirely specific to ARNT, as evidenced by the unexpected detection of UAARP on immunoblots using DEX-treated rats. The information given with the antibody did state that the antibody could detect ARNT2. Future work will investigate the identity of the unknown protein.
Finally, the use of PXR-knockout rats presented some limitations. These rats were bred on a Sprague-Dawley background and therefore this strain, and not the Fischer 344 rat, was used as a control. Interestingly, the Sprague-Dawley rats showed a greater magnitude induction of ARNT and TAT mRNA levels in response to DEX compared to the Fischer 344 rats. As described in Section 2.8, cost considerations limited us to just three rats per treatment group in the PXR-knockout rat study. For some end-points, this study may have had inadequate power to detect differences. This was apparent in comparing ARNT mRNA levels. ARNT mRNA levels were induced 17.5-fold in Sprague-Dawley rats treated with 1 mg/kg DEX, but the apparent 8-fold increase observed in PXR-knockout rats did not achieve statistical significance.

Boorman and colleagues found that inter-individual variation in basal gene expression in rats under controlled conditions is often in the range of 1.5-fold. This may have an impact when comparing vehicle- and drug-treated rats in studies with small n values (Boorman et al., 2005).

4.6 MECHANISM OF THE GR-MEDIATED INCREASE IN ARNT mRNA LEVELS

It is not surprising that ARNT mRNA levels increase in response to glucocorticoids. These results are consistent with a microarray analysis of rat liver showing an upregulation of ARNT mRNA levels following methylprednisolone treatment (Almon et al., 2005). Also, data from our lab showed a DEX-induced increase in rat hepatic ARNT mRNA, but not protein levels in an acute ADX and time-course study. In the time-course study, ARNT mRNA levels were upregulated at 3 and 6 hours after treatment with a DEX dose of 1.5 mg/kg. ARNT protein levels were not affected by low
dose DEX treatment in either the acute ADX or time-course study (Mullen Grey and Riddick, 2009).

mRNA levels are primarily modulated by events independent of those regulating protein levels. The rate of transcription and mRNA stability regulate the abundance of mRNA. The GR may directly upregulate ARNT mRNA levels or act through an intermediate to increase ARNT mRNA levels. Similarly, the rate of translation and the stability of the protein regulate the steady-state protein levels. The rate of translation may be controlled by translational initiation factors or other aspects of protein synthesis, while the post-translational modifications commonly modulate the stability of proteins.

The GR may be involved in the upregulation of mRNA levels through transcriptional mechanisms. A transcriptional mechanism could involve a direct effect, where the GR acts on response elements in a gene’s regulatory region to upregulate transcription. Use of the JASPAR core database suggests there are 8 putative GREs found in the proximal 10 kb of the 5'-flanking region of the rat ARNT gene (Mullen Grey, 2011). Whether or not the GR is recruited to these elements has not been examined and should be considered for future work.

An intermediate factor, upregulated by the activated GR, may regulate the expression of ARNT mRNA. Expression of PXR can be upregulated by the activated GR via a transcriptional mechanism and then PXR can be activated directly by DEX (Pascussi et al., 2003). The results in this study do not suggest that PXR is responsible for the observed changes in ARNT mRNA expression. First of all, ARNT
mRNA levels are upregulated by 1 mg/kg DEX, and the PXR is activated (at least according to CYP3A23 induction) at doses greater than 10 mg/kg of DEX. Second, the PXR agonist PCN does not upregulate ARNT mRNA or protein levels. Third, the profile of ARNT mRNA expression in PXR-knockout rats more closely resembles the expression of the GR target gene TAT and, not the prototypical PXR target gene CYP3A23. ARNT mRNA levels are upregulated at 6 hours after DEX dosing, whereas CYP3A23 mRNA levels are upregulated at 24 hours post-dosing. This upregulation at 6 hours mirrors TAT mRNA upregulation in this study and other studies (Almon et al., 2005; Mullen Grey and Riddick, 2009). ARNT mRNA levels are maximally induced by 1 mg/kg DEX, whereas CYP3A23 induction requires much higher DEX doses. CYP3A23 mRNA induction is clearly abrogated in the PXR-knockout rats, but the elevated ARNT mRNA levels following DEX treatment do not differ between wild-type and PXR-knockout rats. There are studies suggesting ARNT mRNA levels are upregulated under conditions of selective GR activation (Almon et al., 2005; Mullen Grey and Riddick, 2009). To my knowledge, there is no evidence to show an upregulation of ARNT mRNA levels by selective PXR activation.

NF-κB is another candidate for an intermediate factor that may be implicated in GR’s modulation of ARNT mRNA levels. NF-κB can act to increase ARNT mRNA and protein levels in Drosophila, and in mouse and human cell cultures (van Uden et al., 2011). Activated GR inhibits NF-κB signaling by tethering to this transcription factor and blocking transcription [reviewed in: (Smoak and Cidlowski, 2004)]. A study with glucocorticoid-treated rat hippocampal cells found a biphasic expression pattern, whereby genes that were initially downregulated at 1 hour were later upregulated at 3
and 5 hours (Morsink et al., 2006). It is possible that GR inhibits NF-κB, which is then later activated and able to exert its effects leading to elevated ARNT expression.

DBP is a third possible intermediate factor that may induce ARNT transcription. DBP is a transcription factor shown to upregulate certain P450s such as CYP3A4 (Ourlin et al., 1997), CYP2C6 (Yano et al., 1992), and CYP7A1 (Lee et al., 1994; Ogawa et al., 2003). DBP expression is controlled by circadian clock proteins. The diurnal rhythm of DBP expression may be regulated by the secretion of glucocorticoids. Its expression is low in the morning and increases throughout the day, peaking at 8:00 pm (Wuarin and Schibler, 1990). The promoter region of the DBP gene contains a GRE (Wuarin et al., 1992). However, glucocorticoids were shown to have an inhibitory effect on DBP mRNA expression in rat liver (Wuarin and Schibler, 1990). In a diabetic mouse model, DBP and ARNT mRNA levels were decreased in pancreatic islet cells (Nakabayashi et al., 2013). When DBP is overexpressed in cultured HEK293 cells, and mouse pancreatic MIN6 cells, ARNT expression is increased (Nakabayashi et al., 2013). Furthermore, using chromatin immunoprecipitation (ChIP) assay technology, DBP was shown to be recruited to the promoter region of the Arnt gene in vivo (Nakabayashi et al., 2013). The regulation of DBP by glucocorticoids may contribute to the decline in ARNT mRNA expression that I observed at the 24 hour time-point, and may also contribute to the upregulation of ARNT mRNA levels at the 6 hour time-point in my study.

The GR may modulate the expression of ARNT mRNA levels via message stabilization. Glucocorticoids increased the stability of PEPCK and TAT mRNAs (two
GR target genes) in rodent liver (Petersen et al., 1989; and Gadson and McCoy, 1993). Fatty-acid synthase expression is increased in fetal rat lung exposed to DEX due to both mRNA stabilization and an increase in transcription (Xu and Rooney, 1997). The stability of mRNAs may be enhanced through the actions of RNA-binding proteins. The activated GR acts as an RNA-binding protein interacting with the chemoattractant protein-1 (CCL2) mRNA in human airway epithelial cells and accelerating its decay (Ishmael et al., 2010). Glucocorticoids also stabilize growth hormone mRNA by increasing the length of the 3’ poly (A) tail (Paek and Axel, 1987).

Recent genome-wide studies have measured the contribution of transcriptional events to steady-state mRNA levels (Rabani et al., 2011) and the key determinants of steady state protein levels (Schwanhässer et al., 2011). Changes in transcription rates determine most temporal changes in RNA levels, but changes in mRNA degradation rates are important for rapid responses (Rabani et al., 2011). The correlation comparing mRNA levels to protein levels is poor (Schwanhässer et al., 2011). In mouse fibroblasts, translation rates are the dominant feature controlling protein levels, transcription is of secondary importance, and finally, the impact of degradation on protein levels has a small effect on the abundance of proteins (Schwanhässer et al., 2011). Thus, a single mechanistic explanation may not describe both the strongly increased abundance of ARNT mRNA at 6 hours and the trend for increased ARNT protein levels at 24 hours.
4.7 MECHANISMS EXPLAINING THE TREND FOR AN INCREASE IN ARNT PROTEIN LEVELS

The GR or the PXR (or both receptors) may modulate the ARNT protein levels. A consistent finding throughout my studies is that large magnitude increases in ARNT mRNA levels were associated with relatively small changes in ARNT protein levels. Given that ARNT is essential for survival (Maltepe et al., 1997), perhaps we should expect that ARNT protein levels would be under tight regulatory control. In this section, I discuss mechanisms by which glucocorticoids may act to increase ARNT protein levels and how the magnitude of this response may be held in check.

Post-translational mechanisms that depend on GR may explain the trend for increased ARNT protein levels in rats treated with high doses of DEX (50 mg/kg). Sumoylation increases in response to cellular stress (Hong et al., 2001). Cellular stress can signal the secretion of endogenous glucocorticoids. Sumoylation of ARNT increases the transactivation capacity of this protein (Tojo et al., 2002). When proteins are sumoylated there can be an effect on interaction with co-regulatory proteins and localization of the protein. Conjugation by Sumo-1 may block ubiquitination and therefore inhibit degradation of the protein product (Desterro et al., 1998). Phosphorylation is another post-translational modification that can influence protein stability (Andrew et al., 2002). In general, glucocorticoids decrease protein phosphorylation by inhibiting the AP-1 pathway (González et al., 2000). Low DEX doses may not increase ARNT protein levels because of inhibition of phosphorylation.
As mentioned above, ARNT protein levels may be under strict regulation because of its essential physiological functions. Protein synthesis has a much greater energetic cost compared to mRNA synthesis (Schwanhässter et al., 2011). Although the induction of ARNT mRNA levels by glucocorticoids may be part of a stress response, glucocorticoids themselves may also act post-transcriptionally to minimize the translation of mRNAs into protein. Glucocorticoids inhibit the expression of translational initiation factors and ribosomal proteins (Huang et al., 1989). Rats given a low dose of DEX (1 mg/kg) show decreased levels of protein synthesis through a reduced production of initiation factors (Shah et al., 2000). However, this was measured at 4 hours after the treatment, and I observed an increase in ARNT protein at 24 hours following high dose DEX treatment. The mechanism discussed here and other factors may contribute to the temporal delay in ARNT protein production. Abundant proteins are translated 100 times more efficiently than proteins with low abundance (Schwanhässter et al., 2011), and the abundance of ARNT protein in rat liver cytosol is relatively low.

Protein degradation increases in response to glucocorticoids (Gremlich et al., 1997; Brank et al., 1998). For example, glucocorticoids cause a decrease in acetylcholinesterase protein levels in rat skeletal muscle, while the mRNA levels are unchanged (Brank et al., 1998). This effect was also observed for the protein product of another gene, the glucose transporter 2 (Glut2), in rat pancreatic islet cells treated with DEX (Gremlich et al., 1997). The inhibition of translation and the stimulation of protein degradation by low glucocorticoid concentrations may be overwhelmed by GR
and/or PXR activation at high DEX doses, such that levels of proteins such as ARNT only increase under extreme conditions.

It is possible that PXR influences ARNT expression at the protein level but not the mRNA level. This is similar to the regulation of the multi-drug resistance-associated protein 2 (Mrp2) by PCN (50 mg/kg) and DEX (50 mg/kg) in male Sprague-Dawley rats (Johnson and Klassen, 2002). Mrp2 protein was induced by these PXR agonists, with no accompanying change in mRNA levels.

miRNAs are likely to be important factors in the post-transcriptional control of ARNT expression. miRNAs can impede the translation of target mRNAs and can stimulate deadenylation and subsequent degradation of their mRNA targets depending on the degree of sequence pairing (Smith et al., 2013). There are three known miRNA species that modulate ARNT expression: miR-24, miR-107, and miR-221. Overexpression of miR-24 repressed ARNT protein levels, but did not lower ARNT mRNA levels in HuH 7 or HepG2 human hepatocarcinoma cell lines (Oda et al., 2012). Interestingly, miR-24 levels are increased in response to reactive oxygen species (ROS) in human liver cells (reviewed in: Yokoi and Nakajima, 2013). ROS can also trigger a decrease in human ARNT protein levels (Choi et al., 2006, 2008). Interestingly, glucocorticoid treatment generates ROS in the rat hippocampus (Sato et al., 2010), creating a potential route from glucocorticoids to tempered ARNT protein production in the face of elevated ARNT mRNA levels. In primary murine hepatocytes, miR-221 decreased ARNT mRNA and protein levels (Yuan et al., 2013). The loss of ARNT protein by overexpression of miR-221 resulted in an increase in cell proliferation.
Finally, ARNT protein levels decreased in response to an overexpression of miR-107 in human colon cancer cells (Yamakuchi et al., 2010). The effect of these miRNAs on ARNT mRNA levels depends on the miRNA; however, all three miRNAs regulate ARNT protein expression.

miRNAs both regulate and are regulated by PXR. In a study in human hepatocytes treated with the PXR agonist rifampicin, 33 miRNAs were upregulated while 35 miRNAs were downregulated. The predicted interaction of some of these miRNAs with P450 mRNAs was confirmed in that changes in miRNA expression were associated with expression of P450 mRNAs (Ramamoorthy et al., 2013). Another study found a strong correlation between an increase in miR-155 expression and low CYP3A activity in cirrhotic human liver tissue (Vuppalanchi et al., 2013). In the LS180 human colon cancer cell line and the PANC1 human pancreatic cancer cell line, CYP3A4 protein levels were decreased by overexpression of miR-27b (Pan et al., 2009). In a related study in human liver cells, miR-148a bound to the 3'-UTR of the PXR mRNA, decreasing the amount of PXR protein while indirectly affecting the transcription of PXR target genes (Takagi et al., 2008).

ARNT mRNA and protein levels may be modulated by the activated GR acting via changes in miRNA levels. The GR regulates several miRNAs and is also regulated by miRNAs. Two miRNAs, miR-18 in rat and miR-124a in human, regulate the expression of the GR and subsequently may affect GR target genes (reviewed in: Yokoi and Nakajima, 2013). More research is needed in this area to determine the effect of miRNAs on GR target genes. A recent in silico study found over 200 miRNAs
that may be potentially regulated by glucocorticoids (Smith et al., 2013). The downregulation of miRNAs such as miR-166 and miR-44 in rat primary thymocytes could possibly increase the abundance of ARNT mRNA and ARNT protein (Smith et al., 2013). In murine T-cell lymphoma cells, glucocorticoids induced expression of *Bcl-2 interacting mediator of cell death (Bim)*, a pro-apoptotic protein, via a down-regulation of the miR-17~19 cluster (Molitoris et al., 2011). Similarly, there was an increase in glucocorticoid-induced myostatin mRNA levels when miR-27a and miR-27b were suppressed in murine skeletal muscle (Allen and Loh, 2011). New studies continue to find novel miRNAs regulated by the GR with possible consequences for control of ARNT expression.

**4.8 POSSIBLE IDENTITY OF UAARP**

I can only speculate on the identity of the UAARP without further experimentation. The protein appears as a lower molecular weight band on immunoblots compared to the main ARNT band. UAARP is detected at the earlier time-point (6 hours) in DEX- and TA-treated Fischer 344 rats, in DEX-treated Sprague-Dawley and *PXR*-knockout rats, but not in Fischer 344 rats co-treated with RU486 and low dose DEX. Also, the UAARP likely has some sequence similarity to ARNT at the C-terminal as the antibody was raised to this region.

One possibility, although unlikely in my view, is that UAARP is an ARNT protein degradation product. It is possible that ARNT is degraded at the earlier time-point, driving the increase in ARNT mRNA levels as a compensatory effect. Discussed in Section 4.7, glucocorticoids are implicated in both translational inhibition and
stimulation of protein degradation. However, if UAARP was a degradation product a smear would appear on the blot, not a discrete band. A typical protein smear is evident in a study showing the ubiquitination of HIF-1α in 293 cells transfected with HIF-1α and HA-tagged ubiquitin (Huang et al., 1998). Also, it would stand to reason that the main ARNT band would decrease in intensity as the degradation products increased, but this was not the case.

UAARP may be HIF-1α, although the antibody used was not advertized to cross-react with HIF-1α. The unknown protein appears on immunoblots at the earlier time-point. The early appearance of UAARP following DEX treatment suggests either a rapid de novo synthesis of protein and/or enhanced stabilization of the existing protein. HIF-1α levels are known to be controlled by protein stabilization. Under normoxia, HIF-1α is hydroxylated at key proline residues, and marked for degradation by the VHL protein. There is evidence that glucocorticoids can inhibit rat prolyl-hydroxylases (Benson and LuValle, 1981), thereby potentially preventing HIF-1α degradation under normoxia. However, the molecular mass of HIF-1α is higher than that of ARNT, diminishing its credibility as a UAARP candidate.

UAARP may be a post-translationally modified ARNT protein. Plausibly, a functional group is removed or prevented from being added to the ARNT protein in response to glucocorticoids. As mentioned in Section 4.7, glucocorticoids tend to decrease protein phosphorylation; UAARP could perhaps be a dephosphorylated form of ARNT, consistent with its lower molecular mass. Stimulated ARNT
dephosphorylation might be expected to decrease the intensity of the main ARNT band, but this was not observed.

Finally, UAARP may be an ARNT isoform. ARNT2 is a possible candidate for this protein. It has a molecular weight of 79 kDa, compared to 88 kDa for rat ARNT. It shares over 80% amino acid homology with ARNT in the HLH and PAS domains. Santa Cruz, the maker of the antibody used in all four studies, states that this antibody may interact with ARNT2. There are a few points that cast doubt upon this candidate. First of all, ARNT2 is predominantly expressed in the rodent central nervous system (CNS) and kidney (Hirose et al., 1996; Jain et al., 1998). Several studies suggest that it is not expressed in the rodent liver (Hirose et al., 1996; Jain et al., 1998; Aitola and Pelto-Huikko, 2003). However, as techniques are refined, ARNT2 has been detected in more rodent tissues. Second, the ARNT antibody was raised to the C-terminus, and this region of the protein is the most variable between ARNT and ARNT2.

ARNT3 and ARNT4 are possible but unlikely candidates. They do have lower molecular weights than ARNT. ARNT3 is approximately 69 kDa and ARNT4 is approximately 71 kDa. However, the amino acid sequence of the C-termini of ARNT3 and ARNT4 varies the most from ARNT (Dougherty and Pollenz, 2010). ARNT3 has a shorter, more acidic C-terminus, and ARNT4 has a significantly truncated C-terminal region (Dougherty and Pollenz, 2010).

UAARP may be another ARNT isoform, for example one of the ARNT splice variants mentioned in Section 1.2.4 that is missing exon 5 or other parts of the full ARNT gene. UAARP may be an uncharacterized ARNT relative. Future work is
necessary to identify this protein and to further characterize the different ARNT isoforms.

4.9 MECHANISMS EXPLAINING THE INCREASED LIVER WEIGHT TO BODY WEIGHT RATIO

The liver weight to body weight ratio increased slightly as a result of the drug treatments employed. First of all, in the DEX Dose-Response Study, the ratio increased in rats treated with high doses of DEX (10 mg/kg or 50 mg/kg), at 24 hours after administration. Interestingly, the high dose of DEX (50 mg/kg) increased the ratio in the GR Antagonist Study when rats were concomitantly treated with vehicle (at 6 and 24 hours) or RU486 (only at 24 hours). In both the wild-type Sprague-Dawley rats and PXR-knockout rats, the liver weight to body weight ratio increased at 24 hours in those rats treated with the low (1 mg/kg) and high (50 mg/kg) dose of DEX. The ratio was increased at 24 hours by the GR agonist TA, but not the PXR agonist PCN. Taken together, the liver to body weight ratio seems to be increased at 24 hours under conditions driving maximal GR activation.

The activated GR may be involved in the increase in the liver weight to body weight ratio. This suggestion is supported by my finding that the ratio is increased by TA and not PCN, and that the increase occurs in DEX-treated wild-type and PXR-knockout rats. In wild-type Sprague-Dawley rats, which seem to be quite sensitive to GR activation, the ratio was increased at low DEX dose (1 mg/kg) that activated GR, but not PXR. In Fischer 344 rats, only the highest DEX doses increased the ratio and the effect of 50 mg/kg DEX could not be blocked by RU486; although there could be a
role for PXR, it seems that high dose DEX driving maximal and sustained GR activation is key for this response.

An increase in liver weight may contribute to an increased liver to body weight ratio. Glucocorticoids increase liver weight via P450 and endoplasmic reticulum (ER) proliferation (Schulte-Hermann, 1974; Amacher et al., 1998) and by increasing glycogen deposition (Jerome and Cardell, 1983; Chatila and West 1996; Verrips et al., 1998, Man et al., 2002). DEX treatment can increase the liver size in children (Verrips et al., 1998). Also, Jackson and colleagues found that the liver weight and the liver to body weight ratio increased in rats treated with i.p. injections of 50 mg/kg DEX at 24 hours post-dosing (Jackson et al., 2008). In another experiment, 100 mg/kg of DEX was administered to 9-10 week-old Sprague-Dawley rats for 3 days. The rats were euthanized on day 4 (Man et al., 2002). In this experiment, the body weight decreased by 6% and the liver weight increased by 70% (Man et al., 2002). A single i.p. injection of 5 mg/kg TA increased the liver weight and decreased body weight in rats 24 hours after the injection (Weber and Singhal, 1964).

A decrease in body weight may contribute to an increased liver to body weight ratio. Glucocorticoids reduce food intake, stimulate protein catabolism, and cause osteoporosis, ultimately resulting in a reduction of weight. HC was found to decrease appetite and body weight in rats (Liu et al., 2011). In rats, low doses of DEX reduced food intake and body weight in two separate experiments (Zakrzewska et al., 1999; Konno et al., 2008). A previous study in our lab found a decrease in body weight in SHAM rats treated with DEX and a lack of body weight gain in adrenelectomized rats.
treated with DEX (Mullen Grey, 2011). This effect was observed as early as 24 hours after a low dose of DEX (1 mg/kg) (Mullen Grey, 2011). Bone loss due to osteoporosis is usually a side effect after long-term glucocorticoid treatment and may not be a contributor to the rapid weight loss.

PXR activation may cause an increase in liver weight via P450 induction or by causing an increase in hepatic triglycerides. As mentioned above, P450 induction and ER proliferation is associated with an increase in liver weight (Schulte-Hermann, 1974; Amacher et al., 1998). Steatosis is a condition characterized by an abnormal increase in triglycerides in a cell. Several studies have found that PXR agonists increase hepatic triglycerides in rodents (Zhou et al., 2006; Nakamura et al., 2007; Cheng et al., 2012). Additionally, steatosis is suppressed in mice lacking PXR (Dai et al., 2008). Conversely, PXR may play a hepatoprotective role. In PXR \( ^{+/+} \) mice, cholic acid-induced hepatotoxicity was attenuated by treatment with PCN, possibly due to a decrease in serum liver enzymes, bilirubin, and bile acid (Teng and Piquette-Miller, 2007). There was no change in body weight in these mice (Teng and Piquette-Miller, 2007). In fact, PXR activation probably does not affect body weight. After 19 weeks of feeding PCN to rats, there was no change in body weight (Vansell et al., 2004). This was also the result in mice fed PCN for 2 weeks (Zhou et al., 2009). If PXR is involved in the increase in liver to body weight ratio, then it is likely due to an increase in liver weight and not a change in body weight. It is most probable that the increase in liver to body weight is due to maximal and sustained GR activation and not PXR activation, considering the findings in this study and the studies mentioned above.
4.10 FUTURE RESEARCH DIRECTIONS

4.10.1 The Modulation of ARNT Expression and Function by Glucocorticoids

Future work pertaining to the modulation of ARNT expression and function would entail \textit{in vivo} and \textit{in vitro} approaches. It is important to note that the upregulation of ARNT mRNA levels in response to DEX was not observed in the rat hepatoma cell line H4IIE (Mullen Grey, 2011). For \textit{in vitro} approaches, it would be pertinent to find another rat liver-derived cell line where ARNT mRNA levels are upregulated by glucocorticoids. This would include primary rat hepatocytes and rat hepatoma cell lines. Also, it would be important to determine whether ARNT is induced by glucocorticoids in cell lines or primary cultures from other tissues and species. In order to gain insight into the human relevance of my findings, it would be informative to determine whether ARNT is upregulated in response to DEX in animals that are phylogenetically similar to humans. This could be done in cell culture (e.g. primary human hepatocytes) or in intact animals such as a nonhuman primate.

ARNT mRNA levels are upregulated by glucocorticoids via the activated GR. The GR may accomplish this via a direct transcriptional upregulation, through an intermediate factor, or by altering mRNA stability. An \textit{in vitro} approach could assess the direct transcriptional upregulation of ARNT by DEX. First, a plasmid containing the rat \textit{ARNT} 5'-regulatory and promotor regions attached to a luciferase reporter gene would be transfected into the glucocorticoid-responsive cells, most likely primary rat hepatocytes. An ARNT luciferase reporter construct has already been successfully generated by the insertion of approximately 2 kb of the mouse \textit{Arnt} promoter region.
into a pGL3-Basic Vector and transfecting the construct into MIN6 cells (Nakabayashi et al., 2013). Following treatment of transfected hepatocytes with various concentrations of DEX (1 nM to 100 μM) for defined times (6, 12, and 24 hours), I would assess the induction of endogenous ARNT mRNA and protein as well as luciferase activity. As usual, I would assess TAT and CYP3A23 mRNA levels as indicators of GR and PXR activation, respectively. GR involvement would be assessed using RU486 as a GR antagonist and the roles of GR and PXR would be deciphered by siRNA-mediated knock-down.

mRNA stability could be ascertained by the rate of ARNT mRNA decay in cells treated with the transcription inhibitor actinomycin D or 5,6-dichlorobenzimidazole riboside (DRB). The cells would be co-treated with transcriptional inhibitor and either vehicle or DEX to determine whether the ARNT mRNA half-life is altered by DEX. If this analysis suggests post-transcriptional regulation, then I would design and test luciferase constructs containing sections of the ARNT 3’-UTR as potential determinants of mRNA stability.

Another possibility is that ARNT mRNA levels are increased through the action of an intermediate factor that is induced by the activated GR. The identity of such an intermediate factor is unknown, but I previously discussed PXR, NF-κB, or DBP. Without knowing the intermediate factor, a direct approach cannot be pursued by any test for the intermediate transcription factor that regulates ARNT mRNA expression via the GR. If there were a tenable candidate, then this candidate could be overexpressed in primary rat hepatocytes to determine whether endogenous ARNT mRNA levels are
upregulated. Use of a protein synthesis inhibitor (e.g. cycloheximide) could indicate whether de novo synthesis of a protein is required for induction of ARNT mRNA levels by DEX. Deletion analysis of the ARNT-luciferase construct could also help to identify potential intermediate factors by revealing response elements involved in ARNT regulation.

Future work may assess whether the GR binds to putative GREs found in the proximal promoter region of the ARNT gene. The rat ARNT gene contains 8 putative GREs in the proximal 10-kb of the 5′-flanking region detected by bioinformatics (Mullen Grey, 2011). Results from a ChIP assay could determine whether the GR is recruited to any of these GREs; DEX treatments could be given to rats in vivo or in primary rat hepatocytes. Different concentrations of DEX should be used as the GR binds to certain GREs at low concentrations of DEX and other GREs at higher concentrations of DEX (Oakley and Cidlowski, 2013). A previous study in our lab, using DEX-treated male Fischer 344 rats, showed the recruitment of the GR to a known GRE upstream of the TAT transcription start site (Mullen Grey, 2011). This information provides a positive control gene and a sufficient rat model for a future experiment. Such ChIP assays, in conjunction with site-directed mutagenesis of GREs in the ARNT-luciferase constructs, would be instrumental in establishing a role for a GR•GRE interaction in the direct transcriptional control of ARNT expression.

According to my results, it is uncertain whether the GR or PXR mediates the increase in ARNT protein levels. ARNT protein levels increase in response to higher doses of DEX and to 5 mg/kg TA at the 24 hour time-point. When rats are treated with
DEX there is a non-significant increase in ARNT protein levels in PXR-knockout rats, and an attenuation of induction of ARNT protein with RU486. Because of the increase at higher doses of DEX and a lack of significant increase in ARNT protein levels in PXR-knockout rats, it is possible that PXR is mediating this effect. However, the GR may mediate this effect based on the TA response, the attenuation of ARNT protein induction by RU486, and the fact that the high DEX doses activate both GR and PXR. To determine whether the increase in ARNT protein levels is due to enhanced protein stability, it is necessary to administer a protein synthesis inhibitor such as cycloheximide to vehicle-treated, TA-treated (to indicate GR mediation), and PCN-treated (to indicate PXR mediation) primary rat hepatocytes. In these cycloheximide-treated cells, stability of the protein will be indicated by comparing the ARNT protein degradation rate in treated to untreated cells. Because it is uncertain which receptor is affecting ARNT protein levels, PXR ligands and GR ligands should be used in separate experiments when investigating the upregulation of ARNT protein levels.

Certain miRNAs affect the level of ARNT protein and therefore miRNAs regulated by the GR or the PXR should be examined. miR-24, miR-107, and miR-221 decrease ARNT protein levels. In vivo studies in rats would further characterize the role each receptor plays in the regulation of ARNT protein levels by measuring the expression of these specific miRNAs in response to the GR agonist TA or the PXR agonist PCN. In vitro experiments would examine an overexpression or knockdown of these miRNAs in cells treated with TA or with PCN to ascertain whether ARNT protein levels were affected. Luciferase reporter constructs containing sections of the ARNT
3'-UTR and site directed mutagenesis could help to reveal functional effects of the binding of candidate miRNAs.

It would be complicated, yet essential, to determine whether the DEX-mediated increase in ARNT expression had an effect on the function of ARNT. Measuring ARNT function in terms of gene transcription is difficult, as it is a heterodimeric partner of other transcription factors which may confound results. It would be necessary to ensure that protein levels of AHR or HIF1-α (the main ARNT dimerization partners) remained unchanged under test conditions. Under either in vitro or in vivo conditions, it will be important to determine whether the elevation of ARNT protein levels by DEX impacts functional responses to AHR ligands or hypoxia. CYP1A1 induction is a sensitive indicator of AHR activation, but we know that the activated GR binds to a GRE in the first intron of CYP1A1, thereby potentiating AHR responsiveness. Thus, I would instead transfect primary rat hepatocytes with an AHR-activated luciferase reporter construct, treat with an AHR agonist, and assess the effects by varying ARNT protein levels. Similarly, I would use a hypoxia-activated luciferase reporter construct, treat the cells with hypoxia or a hypoxia-mimetic, cobalt II chloride (CoCl₂), and assess the effects of varying ARNT protein levels. The variation in ARNT protein levels could be achieved by treating with different DEX concentrations, or through siRNA knock-down and transfection-based overexpression approaches.

4.10.2 Identifying UAARP

UAARP needs to be isolated and sequenced to properly identify this protein. A repetition of any of the experiments where UAARP appeared on the immunoblot would
be necessary. The protein would be purified using affinity chromatography. A similar method for protein detection would be replicated, including separating the proteins by electrophoresis and probing with the ARNT antibody used in these experiments. Colorimetric detection should be used instead of chemiluminescent detection so that UAARP can be directly visualized on the nitrocellulose, and cut out of the membrane for subsequent isolation. It would be eluted from the nitrocellulose and eventually sequenced by mass spectrometry.

4.11 SUMMARY AND SIGNIFICANCE OF FINDINGS

ARNT is a master regulatory partner in a number of significant signaling pathways, and as such it is essential in mammalian survival. It is involved in xenobiotic metabolism through the dioxin-responsive AHR signaling pathway. In the hypoxia signaling pathway, it plays a role in hypoxic response, angiogenesis, and vasculogenesis. ARNT is necessary for development; when it is knocked out, mice display an embryonically lethal phenotype. The limited pool of ARNT has been vigorously investigated, as it is involved in crosstalk between a number of signaling pathways including NF-κB signaling, ER signaling, GR signaling, and those pathways in which it acts as a heterodimeric partner to induce transcription. ARNT has been implicated in diabetes, cancer, and inflammation. It is ubiquitously expressed throughout the body, and, should the body’s levels of ARNT fluctuate, there could be a number of consequences depending on the context.

Glucocorticoids have proven to be profoundly effective therapeutic agents and are used to treat a number of diverse ailments. They are used as anti-inflammatory
and immunosuppressant agents. Endogenously, they are involved in anti-inflammatory/immunosuppressive responses and energy homeostasis. An imbalance in endogenous glucocorticoids may result in conditions of excess, such as Cushing's syndrome, or conditions of deficiency, such as Addison's disease. Glucocorticoid levels fluctuate in response to food intake, physical activity, stress, and in a circadian rhythm. A surge of glucocorticoids under many of these circumstances could influence the level of ARNT expression and potentially its functional role as a transcription factor.

Are there circumstances in which modulated ARNT expression by glucocorticoids could be important for human health and disease? Humans are exposed to PAHs through cigarette smoke, vehicle exhaust, wood smoke, and through the consumption of some foods. As well, we are all exposed to persistent and lipophilic HAHs through dietary sources. Hypoxia occurs in preterm birth, at high altitudes, and under conditions of ischemia. The hypoxic response is activated in asthma and in chronic obstructive pulmonary disorder. Notably, glucocorticoids are used to treat these two disorders. In addition to its roles in AHR and hypoxia signaling pathways, ARNT plays a role in ER signaling. ER signaling is activated in hormone replacement therapy, cancer treatment, and cyclically by endogenous hormones. Humans are also exposed to estrogen-like chemicals in the environment. Finally, ARNT is involved in NF-κB signaling, which is activated by various stimuli in response to stress or injury. Considering the numerous factors that cause fluctuations in glucocorticoid levels, and the diverse functions of ARNT, there would seem to be multiple opportunities for glucocorticoid regulation of ARNT expression and function to take on physiological, pharmacological, or pathological importance.
The level of ARNT mRNA, and potentially protein, can be impacted by a change in glucocorticoid levels. This may affect the body’s response to toxic chemical insult or low oxygen. The role that GR plays in the upregulation of ARNT has not been fully elucidated. Previous studies showed an increase in ARNT mRNA levels in response to glucocorticoids, but this study shows that the GR is involved in this increase. If these responses are also important in humans, then the new insights into GR/ARNT crosstalk from this thesis may help us to understand how sensitivity to hypoxia or pollutant exposure can be modulated by stress, steroidal therapies and conditions of glucocorticoid excess or deficiency.
SECTION 5: REFERENCES


Kumar, M.B., and Perdew, G.H. (1999). Nuclear receptor coactivator SRC-1 interacts with the Q-rich subdomain of the AHR and modulates its transactivation potential. Gene Expression, 8(5-6), 273–286.


Ma, Q., and Baldwin, K.T. (2000a). 2,3,7,8-tetrachlorodibenzo-p-dioxin-induced degradation of aryl hydrocarbon receptor (AHR) by the ubiquitin-proteasome pathway. Role of the


heterologous basic helix–loop-helix proteins generate complexes that bind specifically to a common DNA sequence. Cell. 58(3), 537-44.


hydrocarbon receptor repressor gene: implication for the susceptibility to dioxins. *Fertility and Sterility, 82* (Suppl 3), 1067–1071.


