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Aryl hydrocarbon receptor activity modulates prolactin expression in the pituitary

Tyler B. Moran[1], **Katherine E. Brannick**[1], and **Lori T. Raetzman**[1,2]

[1]Department of Molecular and Integrative Physiology, University of Illinois at Urbana-Champaign, Urbana, IL, USA

Abstract

Pituitary tumors account for 15% of intracranial neoplasms, however the extent to which environmental toxicants contribute to the proliferation and hormone expression of pituitary cells is unknown. Aryl-hydrocarbon receptor (AhR) interacting protein (AIP) loss of function mutations cause somatotroph and lactotroph adenomas in humans. AIP sequesters AhR and inhibits its transcriptional function. Because of the link between AIP and pituitary tumors, we hypothesize that exposure to dioxins, potent exogenous ligands for AhR that are persistent in the environment, may predispose to pituitary dysfunction through activation of AhR. In the present study, we examined the effect of AhR activation on proliferation and endogenous pituitary hormone expression in the GH3 rat somato-lactotrope tumor cell line and the effect of loss of AhR action in knockout mice. GH3 cells respond to nM doses of the reversible AhR agonist β-naphthoflavone with a robust induction of *Cyp1a1*. Although mRNA levels of the anti-proliferative signaling cytokine TGFbeta1 are suppressed upon β-naphthoflavone treatment, we did not observe an alteration in cell proliferation. AhR activation with β-naphthoflavone suppresses *Ahr* expression and impairs expression of prolactin (PRL), but not growth hormone (GH) mRNA in GH3 cells. In mice, loss of *Ahr* similarly leads to a reduction in *Prl* mRNA at P3, while *Gh* is unaffected. Additionally, there is a significant reduction pituitary hormones *Lhb* and *Fshb* in the absence of *Ahr*. Overall, these results demonstrate that AhR is important for pituitary hormone expression and suggests environmental dioxins can exert endocrine disrupting effects at the pituitary.

Keywords

pituitary; prolactin; gonadotrope; dioxin; AhR

Introduction

The aryl hydrocarbon receptor (AhR) is a basic-helix-loop-helix ligand activated transcription factor of the Per-ARNT-Sim (PAS) family, which regulates the response of many toxic aromatic hydrocarbons (Burbach, et al. 1992, Ema, et al. 1992). AhR ligands occur in common sources including pesticides and tobacco smoke (Baglole, et al. 2008). There is strong evidence to support a role in AhR influencing endocrine function,

[2]Corresponding author: University of Illinois at Urbana-Champaign, 524 Burrill Hall, 407 South Goodwin Avenue, Urbana, IL 61801, USA. Fax: +1 217 333 1133; Phone: +1 217 244 6233. raetzman@life.illinois.edu.

particularly with respect to gonad development and fertility (Matikainen, et al. 2001, Miller, et al. 2004, Sharara, et al. 1998). Female mice exposed to AhR ligands such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) have a depletion of primordial and primary oocytes (Mattison, et al. 1989), reduced fecundity (Gray and Ostby 1995), cleft clitoris, and vaginal mesenchymal threads (Flaws, et al. 1997). Also, $Ahr^{-/-}$ female mice have reduced conception, litter size, pup survival (Abbott, et al. 1999, Baba, et al. 2005), and have impaired development of mature follicles (Baba, et al. 2005, Benedict, et al. 2000, Benedict, et al. 2003). Data suggests disruptions in ovarian follicle development is an intrinsic effect, and mediated to some extent though interactions with estrogen, and exogenous estradiol administration can partially rescue the knockout phenotype (Baba, et al. 2005). AhR can influence estrogen actions by directly binding to estrogen receptors and activate or inhibit transcription of estrogen downstream target genes in a ligand and concentration-dependent manner. Specifically in MCF-7 breast cancer cells, AhR-ARNT complexes associate with both ERα and ERβ in the absence of estrogen and activate transcription at estrogen response elements (ERE) with co-activator p300 (Ohtake, et al. 2003). However when estrogen is bound to ER, AhR can inhibit ER-mediated DNA binding. AhR can also affect androgen and progesterone receptor actions (Barnes-Ellerbe, et al. 2004, Gregoraszczuk, et al. 2000), further expanding its role as an endocrine disruptor. Collectively, AhR is important for reproduction and additionally has a vast array of interactions to facilitate or inhibit steroid hormone actions.

While AhR can dramatically affect reproductive endocrine function, the role of AhR signaling in pituitary hormone synthesis and secretion is less understood. The pituitary possesses AhR and responds to TCDD treatment with induction of *Cyp1a1*, *Cyp1a2* and *Cyp1b1* expression (Cao, et al. 2011). Environmentally relevant concentrations of TCDD cause reductions in serum luteinizing hormone (LH) and follicle stimulating hormone (FSH) levels in preovulatory rats (Gao, et al. 1999). However, mRNA levels of the subunit constituents of LH, *Cga* and *Lhb*, are increased in the pituitary in response to TCDD (Cao, et al. 2011). Dioxin effects on the pituitary appear not limited to gonadotropins, as TCDD-treated mice display reduced basal and estradiol stimulated prolactin (*Prl)* expression (Cao, et al. 2011). Studies *in vitro* have similarly shown important endocrine modulating effects of AhR activity in the pituitary, albeit with mixed results. In rainbow trout pituitaries, TCDD in nM concentrations increases growth hormone (*Gh)* and *Prl* mRNA *in vitro* (Elango, et al. 2006). However, TCDD treatment in the somatolactrope cell line GH3 did not affect basal *Prl* mRNA levels, although it did blunt the effect of estradiol (Cao, et al. 2011). Flavonoids also can act at AhR and have been examined in the context of altering pituitary hormone expression. Although less potent than TCDD, the AhR agonist β-naphthoflavone decreased *Gh* and pro-opiomelanocortin (*Pomc)* in mouse pituitaries (Aluru and Vijayan 2008). α-naphthoflavone has been characterized as an antagonist of AhR activity, however it can also have no activity or even partial agonist activity, depending on the dose and cell system (Smith, et al. 2011). In GH3 cells, α-naphthoflavone at a concentration of 1×10^{-6} M has been shown to inhibit the ability of polychlorinated biphenyl (PCB) 126 to induce CYP1A1 and CYP1B1, but it's effect on β-naphthoflavone treatment has not been explored (Gauger, et al. 2007). These results show AhR can alter transcription of many pituitary hormones, and differences in magnitude and direction of gene changes may be due to ligand or concentration effects.

Additional evidence potentially linking the importance of AhR in normal pituitary function came from the identification of germline mutations in aryl hydrocarbon interacting protein (AIP), as a cause of familial growth hormone secreting pituitary adenomas (Vierimaa, et al. 2006). Two AIP mutations accounted for 16% of all patients with growth hormone secreting tumors, and remarkably the age of diagnosis was significantly lower for patients with AIP mutations and 40% were under age 35. Moreover, AIP expression is reduced in invasive

versus non invasive somatotrophinomas (Jaffrain-Rea, et al. 2009), and AIP silencing in the somatolactotroph cell line GH3 causes increased proliferation (Heliovaara, et al. 2009). While not common in sporadic pituitary adenomas (Buchbinder, et al. 2008, Yu, et al. 2006) the aggressive nature and early onset of pituitary adenomas with AIP mutations clearly warrant further investigation on how AhR affects normal pituitary function and proliferation.

Alterations in AhR activity can cause serious health concerns, particularly in endocrine and reproductive tissues. Studies on the direct actions of AhR in the pituitary are limited, however evidence suggests AhR ligands can alter normal pituitary hormone production, and that increased AhR activity, presumably though mutations in AIP, may be able to cause adenomas. The present study examines AhR signaling using the agonist β-naphthoflavone and α-naphthoflavone in culture as well as loss of function in *Ahr*$^{-/-}$ mice, to investigate if AhR can alter endocrine function in hormone producing pituitary cells as well as promote proliferation.

Materials and Methods

Cell culture

GH3 cells were obtained from American Type Culture Collection (ATCC; Manassas, Virginia). Cells for RNA isolation were plated at 600,000 per well in 12-well plates and incubated in DMEM/F12 (1:1) media without phenol red (Hyclone) with 10% charcoal dextran treated fetal calf serum and 10 nM, 100 nM, or 1 μM β-naphthoflavone with or without 100nM α-naphthoflavone for 20 hours. Each condition was plated in triplicate and each experiment was repeated three times. β-naphthoflavone (Sigma) and α-naphthoflavone (Sigma) were dissolved in DMSO and controls were treated in DMSO alone. The final concentration of DMSO in media in all conditions was 0.07%. For cell cycle analysis, cells were also grown in standard DMEM/F12 (1:1) media with phenol red and 10% calf serum.

Mice

Ahr$^{-/-}$ mice were generously provided and genotyped by B. Karman and M. I. Hernandez from the laboratory of Jodi Flaws, University of Illinois. Mice were originally obtained from C. Bradfield's laboratory at the University of Wisconsin, but bred and housed for these experiments at the University of Illinois. Pituitaries from mice at postnatal day 3 and 90-100 were dissected and stored in RNAlater (Ambion) at −20°C prior to RNA isolation. All mice were maintained according to the University of Illinois Institutional Animal Care and Use Committee.

RNA isolation and qRT-PCR

RNA isolation from cultured cells was performed by standard Trizol (Invitrogen) extraction. Pituitary RNA was isolated with an RNaqeous kit (Ambion) after tissue disruption in lysis solution with a homogenizer. cDNA synthesis with 0.5 μg of RNA was performed with Superscript II kit (Invitrogen). Samples were run in triplicate and analyzed using real time quantitative reverse transcriptase PCR (qRT-PCR) on a Biorad iCycler iQ5. Primer sequences were developed on Beacon Designer 7.0.

Primer sequences for rat GH3 cells: *Gh* Forward 5′-AGG GCA TCC AGG CTC TGA T-3′; *Gh* Reverse 3′-GCA TGT TGG CGT CAA ACT TG-3′; *Prl* Forward 5′-CAT CAA TGA CTG CCC CAC TTC-3′; *Prl* Reverse 5′-CCA AAC TGA GGA TCA GGT TCA AA-3′; *Actb* Forward 5′-AAC CCT AAG GCC AAC CGT GAA AAG-3′; *Actb* Reverse 5′-CGA CCA GAG GCA TAC AGG GAC AAC-3′; *Tgfb1* Forward 5′-TCC AAA CGT CGA GGT GAC-3′; *Tgfb1* Reverse 5′-CAG GTG TTG AGC CCT TTC CA-3′; *Ahr* Forward 5′-TCA

CTG CGC AGA ATC CCA CAT CC-3′; *Ahr* Reverse 5′-TCG CGT CCT TCT TCA TCC GTT AGC-3′; *Esr1* Forward 5′-CCA AAG CCT CGG GAA TGG-5′; *Esr1* Reverse 5′-AGC TGC GGG CGA TTG AG-3′; *Esr2* Forward 5′-TTG GTG TGA AGC AAG ATC ACT AGA G-3′; *Esr2* Reverse 5′-AAC AGG GCA GGC ACA ACT G-3′; *Cyp1a1* Forward 5′-GTC CCG GAT GTG GCC CTT CTC AAA-3′; *Cyp1a1* Reverse 5′-TAA CTC TTC CCT GGA TGC CTT CAA-3′; The conditions for all primer sets used were 95 °C for 20 sec., 55 °C for 30 sec., 72 °C for 30 sec. (45 cycles).

Primer sequences for $Ahr^{-/-}$ mice: *Gh* Forward 5′-AGG GCA TCC AGG CTC TGA T-3′ *Gh* Reverse 5′-GCA TGT TGG CGT CAA ACT TG -3′; *Prl* Forward 5′-TCA GCC CAG AAA GCA GGG ACA-3′; *Prl* Reverse 5′-GGC AGT CAC CAG CGG AAC AGA-3′; *Cga* Forward 5′-GTA TGG GCT GTT GCT TCT CC-3′ *Cga* Reverse 5′-GTG GCC TTA GTA AAT GCT TTG G; *Lhb* Forward 5′-CCC AGT CTG CAT CAC CTT CAC-3′ *Lhb* Reverse 5′-GAG GCA CAG GAG GCA AAG C-3′; *Fshb* Forward 5′-TGG TGT GCG GGC TAC TGC TAC-3′ *Fshb* Reverse 5′-ACA GCC AGG CAA TCT TAC GGT CTC-3′; *Gapdh* Forward 5′-GGT GAG GCC GGT GCT GAG TAT G-3′ *Gapdh* Reverse 5′-GAC CCG TTT GGC TCC ACC CTT C-3′; The conditions for all primer sets used were 95 °C for 20 sec., 55 °C for 30 sec., 72 °C for 30 sec., 40 cycles except *Cga* (95 °C for 20 sec., 62 °C for 30 sec., 72 °C for 30 sec.).

Data were analyzed according to standard delta delta CT method with three (P3) or five to six (P90-100) biological replicates for each experimental condition. Error bars show standard error of mean. Statistical analysis for *in vitro* experiments was done using analysis of variance (ANOVA) in SAS. *In vivo* results were analyzed using a two tailed t-test in Microsoft Excel.

Cell culture for cell cycle analysis

GH3 cells were plated at 250,000 cells/well in 12-well plates and serum starved for 3 days in 0.1% serum to synchronize cell cycles prior to 3 day β naphthoflavone treatment in either standard media (DMEM/F12 (1:1) with phenol red) or with charcoal dextran stripped serum and phenol red free media. Cells were stained in propidium iodide after 3 days of β naphthoflavone treatment and analyzed by flow cytometry. Cell cycle analysis was performed by FCS Express. Graphs represent n=3.

Results

β-naphthoflavone, and when combined with α-naphthoflavone, activates *Cyp1a1*, suppresses *Ahr*, and does not dramatically affect Esr1 expression

Expression of CYP1A1 (cytochrome P4501A1) is the most well known and commonly used indicator of AhR activation *in vivo* and in culture (Spink, et al. 1998, Stephen, et al. 1997, Vanden Heuvel, et al. 1993). In fact, adult mouse pituitaries exhibit a robust induction of *Cyp1a1* following TCDD treatment (Huang, et al. 2002). To determine if the somatolactotrope GH3 cells also respond to AhR activation, cells were treated with β-naphthoflavone and α-naphthoflavone and the levels of *Cyp1a1* were determined by real time qRT-PCR. To reduce the actions of steroid hormones, which might alter AhR-mediated effects, all culture experiments for qRT-PCR used charcoal dextran treated serum (Figures 1-3). Compared to vehicle treatment, β-naphthoflavone significantly increased *Cyp1a1* expression to levels approximately 6-fold higher than controls at the highest dose (Figure 1A). Interestingly, 100 nM of the partial antagonist α-naphthoflavone had no effect alone, but significantly enhanced the β-naphthoflavone-induced activation of CYP1A1 to levels approximately 14 fold higher than control samples. This suggests that α-naphthoflavone acts as an agonist in this context at a concentration of 100 nM.

It has been reported AhR can upregulate its own expression in the pituitary in response to TCDD and β-naphthoflavone treatments (Aluru and Vijayan 2008, Huang, et al. 2002), and altered levels of AhR are important to consider when interpreting gene expression changes. In contrast to previously reported effects on AhR expression in the whole pituitary (Huang, et al. 2002), we find that AhR activation caused suppression of *Ahr* mRNA levels in GH3 cells (Figure 1B). Strong suppression of *AhR* was evident in β-naphthoflavone plus α-naphthoflavone conditions, which correlate with the highest *Cyp1a1* induction.

Estrogen signaling can have a profound effect on the expression of GH and PRL, as well as proliferation of pituitary cells (Liu, et al. 1987, Shull, et al. 1987). AhR has been reported to interact with both ERα and ERβ, recruit co-activator p300 to estrogen response elements and initiate transcription in human breast cancer-derived MCF-7 cells (Ohtake, et al. 2003). Therefore, it is useful to determine if AhR is altering the expression levels of *Esr1* (ERα) and *Esr2* (ERβ), and subsequently causing effects due to altered ER-dependent transcriptional events. We found *Esr1* expression levels in GH3 cells were significantly reduced at the two higher doses of β-naphthoflavone, however only in the presence of α-naphthoflavone treatment. *Esr2*, although expressed at much lower levels than *Esr1*, was also reduced under the same conditions as *Esr1* (Figure 1C).

Growth hormone and prolactin are differentially affected by AhR activation

We examined whether β-naphthoflavone with or without α-naphthoflavone, alters *Gh* or *Prl* mRNA expression in GH3 cells. Treatment of GH3 cells with β-naphthoflavone had no significant effect on *Gh* expression (Figure 2). *Prl*, however, was significantly suppressed at all concentrations of β-naphthoflavone tested, with the greatest effects at 100 nM. Adding α-naphthoflavone enhanced the suppression of *Prl* to approximately half the expression of DMSO and DMSO plus α-naphthoflavone controls. This shows AhR activation can significantly reduce *Prl* but not *Gh* expression.

AhR activation suppresses TGFβ1

AhR activation is correlated with decreased expression levels of extracellular cytokine transforming growth factor β (TGFβ), and this may be important for AhR-mediated pathologies. For example, TCDD treatment causes reduced TGFβ1 expression in epithelial and mesenchymal cells during palate formation (Abbott and Birnbaum 1990). Additionally, the absence of *Ahr*, in $Ahr^{-/-}$ mice leads to increased TGFβ levels in fibrotic livers (Gonzalez and Fernandez-Salguero 1998). We show β-naphthoflavone at 100 nM can significantly reduce *Tgfb1* expression, and β-naphthoflavone plus α-naphthoflavone significantly reduced *Tgfb1* to a greater extent (Figure 3).

Ahr knockout mice have reduced hormone mRNA levels at P3

As a step toward understanding the physiological role of AhR in the pituitary *in vivo*, we examined mRNA levels of *Gh* and *Prl*, at postnatal day 3 (P3), several days after the cells are specified, in mice lacking *Ahr*. In P3 female $Ahr^{-/-}$ pituitaries there was no change in *Gh* and a significant reduction in *Prl* (p=0.0002) mRNA levels, compared to wildtype littermates (Figure 4A). At P90-100, an age where mice have reached full maturity, there was no difference observed for *Gh* or *Prl* mRNA levels. (Figure 4B).

Because gonadotropes have recently been shown to be targets of AhR activation through TCDD *in vivo* (Cao, et al. 2011), *Cga, Lhb* and *Fshb* mRNA levels were examined at P3 in the pituitaries of mice lacking *Ahr*. A significant reduction was found in *Cga* (p=0.0321), *Lhb* (p=0.0035), *and Fshb* (p=0.0200) mRNA levels (Figure 4C) in female pituitaries lacking *Ahr*, compared to wildtype littermates.

AhR activation by β-naphthoflavone does not alter cell cycle progression in GH3 cells

Mutations in AIP are associated with pituitary adenomas (Vierimaa, et al. 2006), where presumably AIP no longer sequesters AhR to the cytoplasm and potentially allows enhanced AhR-mediated transcription. Further, AIP knockdown in GH3 cells enhances proliferation (Heliovaara, et al. 2009) and over expression of AhR enhances proliferation in carcinomic human alveolar basal epithelial A549 cells and mouse mammary fibroblasts (Mulero-Navarro, et al. 2005, Shimba, et al. 2002). Based on this potential proliferative effect of AhR activation, we investigated if three days of treatment with β-naphthoflavone enhanced cell cycle progression, using propidium iodide staining and flow cytometric analysis. Our data showed that β-naphthoflavone at 1 and 10 μM did not alter the percentage of cells in G1, S, or G2 phases in media with untreated serum (Figure 5A) or in media containing charcoal dextran stripped serum (Figure 5B). It is interesting to note that there appears to be a small difference between growth medias, such that cells grown in the standard media tended to have fewer cells in G1 and more in S phase, which would be expected given the additional presence of steroid hormones. Overall, this suggests AhR activation at these levels is insufficient to alter proliferation in either media growth condition.

Discussion

The work presented here demonstrates that AhR activity affects pituitary hormone mRNA levels *in vitro* as well as *in vivo.* This was first shown by β-naphthoflavone effectively inducing AhR activation though *Cyp1a1* induction in GH3 cells, which was expected based on previous studies (Aluru and Vijayan 2008, Nannelli, et al. 2009). CYP1A1 is normally expressed at very low level in the pituitary (Huang, et al. 2003). It is likely induced by AhR activation to aid in metabolism of xenobiotics, although CYP1A1 can also act on endogenous compounds such as hormones and fatty acids (Whitlock Jr., 1999). Unexpectedly, the AhR antagonist α-naphthoflavone had a synergistic effect with β-naphthoflavone in inducing *Cyp1a1*, yet had no effect on *Cyp1a1* alone with the same concentration. The actions of α-naphthoflavone here is contradictory to previous reports of *Cyp1a1* induction in GH3 cells (Gauger, et al. 2007), however there are several reasons that could account for this. The study from Gauger et al. (2007) used PCB congeners for AhR induction and was antagonized with α-naphthoflavone, so the differences observed here from β-naphthoflavone could be due to different effects between the AhR agonists and α-naphthoflavone. It is possible α-naphthoflavone in the presence of different agonists may alter AhR conformation and subsequent transcriptional activity. Further more, the concentrations of α-naphthoflavone used in our study of 100 nM was lower than the 1 μM levels used by Gauger et al. (2007), therefore in our experimental conditions and drug concentrations, α-naphthoflavone exhibited agonist actions. The GH3 cells described here for qRT-PCR data were cultured in media with serum that had been treated with charcoal dextran, which depletes the media of steroid hormones. Other groups have used alternate methods of removing steroid hormones including an anion exchange column (AG 1-X8 resin), which reduces T_3 and T_4 levels (Gauger, et al. 2007). Therefore it is possible different ligands and concentrations of steroid hormones were present during culturing conditions between studies. Last, treatments in this study examining mRNA levels were acute and consisted of 20 hours, which could have very different effects than studies with several day treatments.

Robust AhR activation, identified by greatest *Cyp1a1* induction, occurred in the cells treated with 1 μM β-naphthoflavone and 100 nM α-naphthoflavone. These same conditions caused the most *Prl* gene suppression, yet had little effect on *Gh gene* expression. These results appear to contrast with the reported findings of Elango et al. (2006) in which TCDD increases both *Gh* and *Prl* in trout pituitaries *in vitro*, however at very low doses in the pM range, TCDD may suppress both *Gh* and *Prl* (Elango, et al. 2006). Also TCDD is a much

more potent ligand than β-naphthoflavone, and therefore, these differences may be dose and ligand specific and highlights the importance of understanding "mixture effects." This concept of multiple ligands causing an outcome, which is different than the sum of each of the independent actions, was identified as an important consideration for future studies in a recent statement from the Endocrine Society (Diamanti-Kandarakis, et al. 2009). Each study, however, provides important insight that AhR can disrupt pituitary hormone synthesis, and the differences we observe in *Prl* but not *Gh* expression may be due to variants in xenobiotic response elements for each gene.

These data identifying a pituitary hormone suppressing effect of AhR agonist treatment is complemented by the results of our knockout study. There is a significant reduction of *Prl,* but not *Gh*, in $Ahr^{-/-}$ animals at P3. This suggests AhR may be necessary for timely initiation of *Prl* expression, maximal transcription, or mRNA stability. It also suggests that the reduction of *Prl* we observed in GH3 cells treated with β-naphthoflavone may be due to the reduction in *Ahr* mRNA and not necessarily due to activation of AhR signaling. By the third month of life, however, *Prl* expression is independent of *Ahr*, indicating that potentially other factors compensate for its loss.

Gonadotropes also appear to be a target of AhR signaling. At P3, $Ahr^{-/-}$ females have reduced *Cga*, *Fshb*, *Lhb* mRNA levels, suggesting the AhR activity can modulate pituitary gonadotropin levels. These studies highlight that AhR has an important role in normal reproductive endocrine function, but may also partially explain the phenotype of impaired antral follicle development and reduced corpora lutea in $Ahr^{-/-}$ females (Benedict, et al. 2000, Benedict, et al. 2003). Despite the fact that these mice have normal LH serum levels between P25 and P28 (Barnett, et al. 2007), there may be a pre-pubertal reduction of LH which alters ovarian follicle development, before LH levels can be later restored. Additional studies to follow *Lhb* mRNA levels in $Ahr^{-/-}$ females during puberty and into adulthood could be illuminating on understanding this ovarian phenotype. Further evidence to support a role in AhR directing *Lhb* transcription comes from studies showing fetal rats had reduced *Lhb* mRNA as well as LH serum protein levels following maternal administration of TCDD (Mutoh, et al. 2006, Takeda, et al. 2009). However, treatment with TCDD in adulthood increases *Lhb* expression (Cao, et al. 2011). These studies highlight that AhR can affect gonadotropin expression both normally and in the case of exposure to environmental toxins and there may be differences in effect based on species and timing of exposure.

The more surprising findings of this study were demonstrated by the lack of effects on GH3 cell proliferation following β-naphthoflavone treatment. There is considerable evidence showing that AhR activation can promote proliferation. $Ahr^{+/+}$ mouse mammary fibroblasts are more tumorigenic than $Ahr^{-/-}$ cells (Mulero-Navarro, et al. 2005), and AIP knockdown in GH3 cells, which presumably allows enhanced AhR transcription, promotes enhanced proliferation three days after transfection (Heliovaara, et al. 2009). Also, mutations in AIP have been identified as a cause of familial growth hormone secreting pituitary adenomas (Vierimaa, et al. 2006). Further relevant to the data presented here, there is an interesting inverse relationship with respect to AhR and TGFβ levels on proliferation. Experiments using primary hepatocytes found increased TGFβ secretions from $Ahr^{-/-}$ cells and also had lower proliferation rates than wild type (Zaher, et al. 1998). Additionally, adding a TGFβ neutralizing antibody to $Ahr^{-/-}$ fibroblasts restores proliferation close to wild type levels (Mulero-Navarro, et al. 2005). It has been known for some time that TGFβ can suppress estrogen-induced lactrotrope proliferation (Sarkar, et al. 1992), and these actions are modulated by dopaminergic hypothalamic input (Ben-Jonathan and Hnasko 2001). It is tempting to speculate lactrotrope hyperplasia responsive to TGFβ administration, may also involve altered AhR or AIP activity. In fact, it is possible the proliferative effects of AIP silencing occurs in an AhR-independent manner as others have shown AIP interacts with

phosphodiesterase4A5 and may affect proliferation by altering cyclic adenosine monophosphate (cAMP) levels (Heliovaara, et al. 2009, Leontiou, et al. 2008). The results described here demonstrating a strong suppression of *TGF*β, concomitant with a lack of proliferation changes following AhR activation are unexpected given the previous data presented above. However, it is possible more efficacious AhR ligands can induce cell cycle progression in GH3 cells, or much higher doses of β-naphthoflavone would be required than what was used in this study.

Many questions remain to be answered, however the present study demonstrates ligand-induced AhR can alter transcription of many pituitary hormones and has little effect on pituitary cell proliferation in culture. Future studies examining different AhR ligand effects on pituitary hormone response as well as further gene transcription changes in $Ahr^{-/-}$ mouse pituitaries will be highly beneficial to the endocrine toxicology field.

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Highlights

AhR signaling suppresses *Prl* mRNA expression

AhR signaling does not influence pituitary proliferation in culture

AhR is necessary for *Prl, Lhb* and *Fshb* expression at postnatal day 3

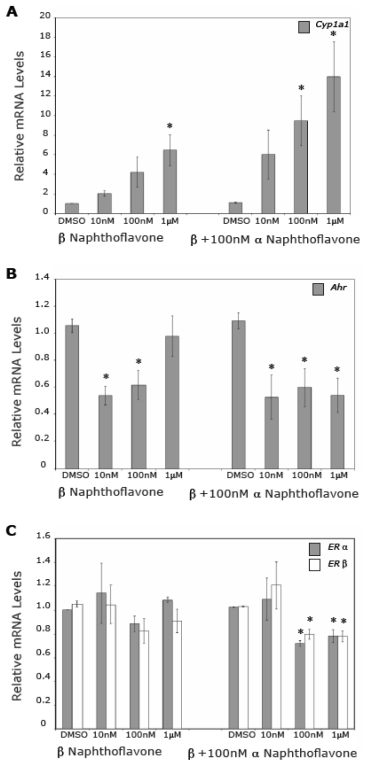

Figure 1. β-naphthoflavone alone and with α-naphthoflavone activated *Cyp1a1* and suppressed *AhR* expression

Real time quantitative reverse transcriptase PCR (qRT-PCR) showed significantly higher levels of *Cyp1a1* at 1 μM β-naphthoflavone and both 100 nM and 1 μM β-naphthoflavone plus 100 nM α-naphthoflavone (1A). *AhR* was significantly reduced at 10 nM and 100 nM β-naphthoflavone and at all doses of β-naphthoflavone plus α-naphthoflavone in cultured GH3 cells (1B). Levels of ERα *(Esr1)* and ERβ *(Esr2)* mRNA were also mildly affected, based on treatment (1C). mRNA values normalized to *beta-actin*, n=3.

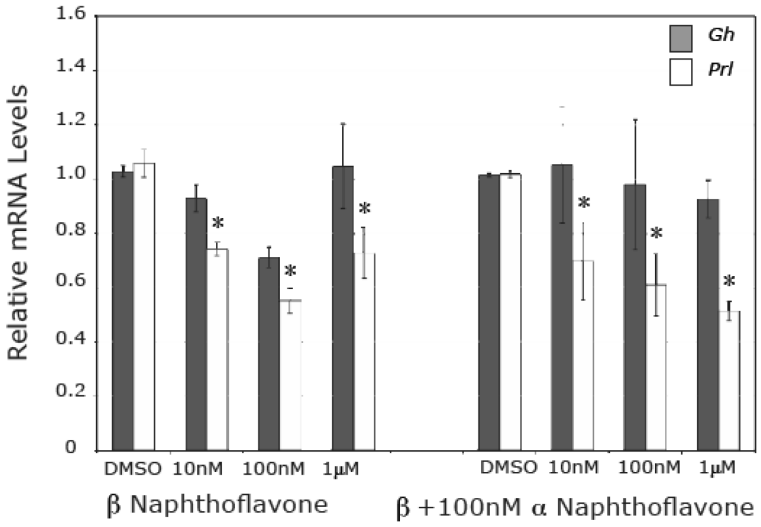

Figure 2. Growth hormone is unaffected but prolactin is suppressed by β-naphthoflavone

Growth hormone mRNA levels remained largely unchanged, however *Prl* was significantly reduced with all doses of β-naphthoflavone and of β-naphthoflavone plus α-naphthoflavone in cultured GH3 cells (2A). mRNA values normalized to *beta-actin*, n=3.

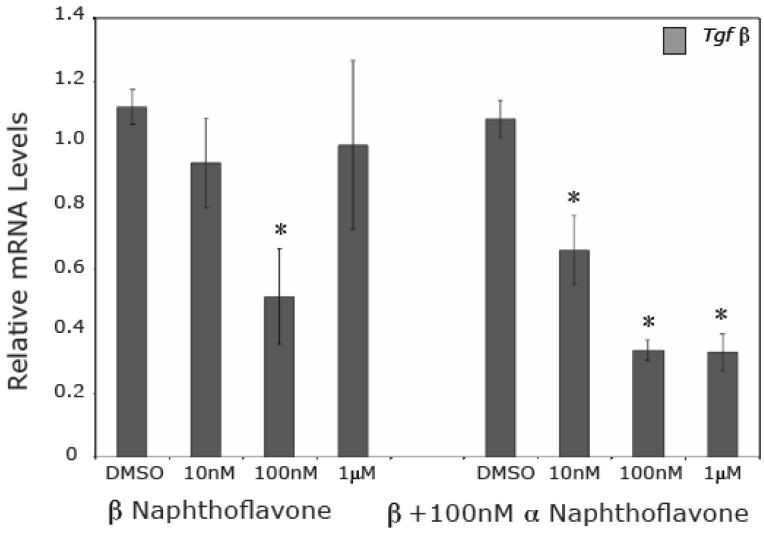

Figure 3. AhR suppressed TGFβ mRNA expression

A significant reduction in TGFβ mRNA occurred at 10 nM and 100 nM β-naphthoflavone and all doses of β-naphthoflavone plus α-naphthoflavone in cultured GH3 cells. mRNA values normalized to *beta-actin*, n=3.

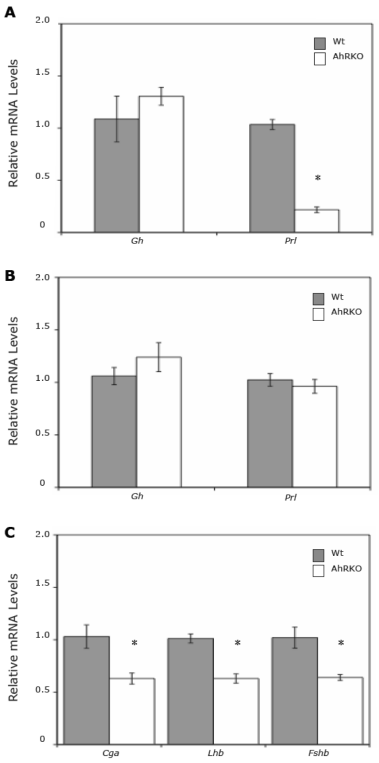

Figure 4. Prolactin and gonadotropin subunit mRNA expression were reduced in *Ahr*$^{-/-}$ females

qRT-PCR performed on isolated female P3 pituitaries from *Ahr*$^{-/-}$ and wildtype littermates shows a significant decrease in *Prl* (p=0.0002) (A), as well as *Cga* (p=0.0321), *Lhb* (p=0.0035), and *Fshb* (p=0.02) (C). *Gh* levels remained unchanged (A), n=3 individual pituitaries of each genotype. qRT-PCR performed on isolated female P90-100 pituitaries from *Ahr*$^{-/-}$ and wildtype littermates shows no change in *Gh* or *Prl* at this age. (B) n≥5 individual pituitaries for each genotype. All mRNA values were normalized to *Gapdh*.

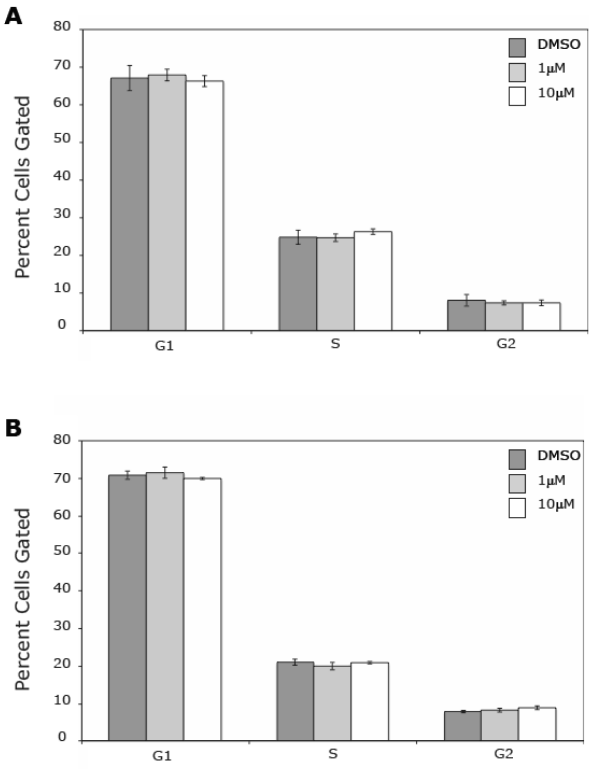

Figure 5. AhR activation with β-naphthoflavone did not affect the proliferative capacity of GH3 pituitary cells

β-naphthoflavone at 1 and 10 μM did not alter the percentage of GH3 cells in G1, S, or G2 phases of the cell cycle in either standard (5A) or charcoal dextran treated serum, phenol red free medias (5B). Staining occurred by propidium iodide, and cell cycle analyzed by FACS, n=3.