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GH3 and RC-4BC cell lines are not suitable as *in vitro* models to study prolactin modulation and AHR responsiveness in rat pituitary

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ABSTRACT

Some environmental contaminants and pharmaceuticals increase the incidence of uterine tumors in toxicological studies with rats. These tumors can result from a hormonal imbalance due to rat-specific disrupted pituitary prolactin regulation, and are therefore of questionable relevance for humans. In this study we compared *in vitro* prolactin regulation in rat primary pituitary cells to that in pituitary cell lines, GH3 and RC-4BC. Moreover, we assessed the potential effects of aryl hydrocarbon receptor (AHR) activation on prolactin regulation by using two different AHR agonists, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and DELAQ, the N-deethylated minor metabolite of the pharmaceutical laquinimod.

In rat primary pituitary cells, known prolactin stimulant thyrotropin-releasing hormone (TRH) marginally increased prolactin secretion (1.2-fold) and gene expression (1.3-fold). In contrast, synthetic dopamine receptor agonist quinpirole, a known inhibitor of prolactin release, significantly inhibited prolactin secretion (2.6-fold) and gene expression (3.6-fold). In GH3 cells, TRH strongly increased prolactin secretion (6.8-fold) and gene expression (30.8-fold), whereas quinpirole did not affect prolactin secretion nor gene expression. In RC-4BC cells, both TRH and quinpirole did not modulate prolactin secretion nor gene expression. Prolactin secretion and gene expression did not significantly change upon exposure to TCDD or DELAQ. However, DELAQ, but not TCDD, attenuated quinpirole-inhibited prolactin gene expression by 51% in primary pituitary cells.

This study shows that pituitary prolactin regulation in rat primary pituitary cells *in vitro* is distinctly different from rat pituitary cell lines GH3 and RC-4BC. Therefore, effects on pituitary prolactin regulation *in vitro* should best be performed using rat primary pituitary cells. Additionally, AHR ligands may interact with rat pituitary prolactin regulation, but this appears to depend on the ligand and constitutive prolactin secretion. However, interpretation of the *in vitro* results with respect to occurrence of uterine tumors in rats should take the complex regulation of prolactin release in the pituitary into account as well as the *in vivo* hypothalamus-pituitary-gonadal (HPG) axis and its feedback loops.

1. Introduction

Uterine tumors are relatively common in carcinogenicity or toxicity studies using rats (Harleman et al., 2012). Although there are similarities in the pathogenesis of uterine tumors between humans and rats, it is especially the mechanism of prolactin regulation in the pituitary that fundamentally differs between rats and humans (Yoshida et al., 2015; Neumann, 1991). As a result, the occurrence of uterine tumors in toxicity and pre-clinical studies with the rat can be of questionable significance for the human situation. In contrast to humans, prolactin is luteotropic in rats, *i.e.* it promotes progesterone production in the corpus luteum after ovulation and maintains gestation (Neumann, 1991; Ben-Jonathan et al., 2008). Progesterone antagonizes the estrogenic stimulation of uterine growth by *e.g.* inhibiting estrogen receptor alpha (ERα) chromatin binding and non-transcriptional activity (Kim et al., 2013). Thus, any compound that decreases prolactin synthesis in the rat pituitary may change the uterine estradiol (E2)-to-progesterone (P4) ratio in favor of E2, stimulate uterine tissue growth and increase the risk of uterine tumors (Neumann, 1991).

Hypothalamic Thyrotropin-Releasing Hormone (TRH) is a direct stimulus for prolactin release via the TRH receptor in pituitary cells in both rats and humans (Fig. 1). However, TRH appears to stimulate prolactin release especially when dopaminergic input is low or absent (Ben-Jonathan et al., 2008). Rats exhibit a particularly sensitive

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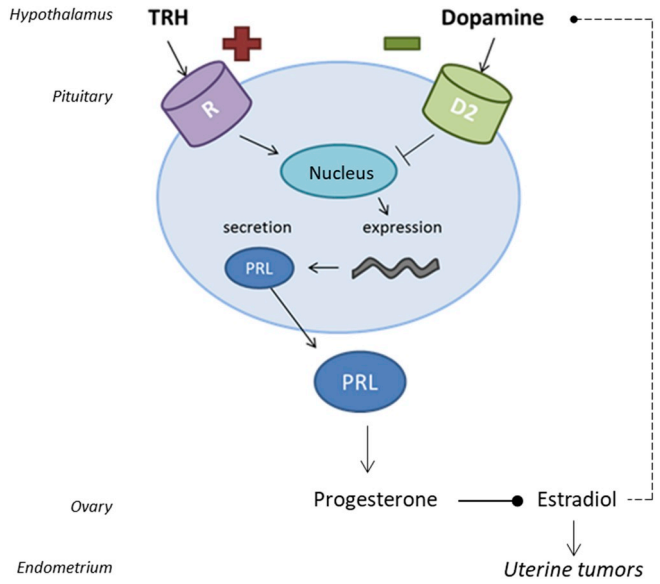

Fig. 1. Schematic overview of prolactin regulation in the pituitary. TRH; Thyrotropin-Releasing Hormone, D2; Dopamine receptor D2, PRL; prolactin, R; TRH receptor.

feedback mechanism between prolactin secretion and estrogens, while in humans, endogenous estrogens only have a modest stimulatory effect on pituitary prolactin release (Neumann, 1991; Ben-Jonathan et al., 2008). Rising estrogen levels drive the secretion of prolactin in rats by interacting with dopamine releasing systems in the hypothalamus (Freeman et al., 2000, Ben-Jonathan and Hnasko, 2001). Dopamine, secreted from the hypothalamus, inhibits prolactin release and gene transcription from the pituitary via the dopamine receptor D2 (D2R) (Ben-Jonathan et al., 2008, Beaulieu and Gainetdinov, 2011). Estrogens inhibit hypothalamic secretion of dopamine, and this subsequently results in a stimulated prolactin release from the pituitary (Ben-Jonathan and Hnasko, 2001; Mijiddorj et al., 2012). In view of the difference with humans, functional assays to screen for rat-specific effects on pituitary prolactin secretion is of high relevance in the pre-clinical testing phase of new pharmaceuticals as well as to support interpretation of uterine tumor incidences in carcinogenicity and toxicity rat studies with respect to potential human health risk.

Several rat studies have suggested a relationship between the development of uterine tumors and activation of the aryl hydrocarbon receptor (AHR). Yet, its specific role in prolactin-mediated tumorigenesis in the rat uterus is equivocal and under continuous debate. Yoshida et al. showed that the naturally occurring AHR agonist indole-3-carbinol (I3C) increased the incidence of uterine adenocarcinomas in rats (Yoshida et al., 2004). Additionally, a 2-year treatment with the AHR agonist laquinimod also increased the incidence of uterine tumors in rats (Sørensen et al., 2017). In contrast, other studies observed a decrease of uterine tumors in rats after treatment with the potent AHR agonist 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). This inhibitory effect on rat uterine tumors may have been caused by an inhibition of estrogen signaling via cross-talk between ER and AHR responsive elements in the DNA (Kociba et al., 1978; Romkes et al., 1987; Safe and Wormke, 2003). On the other hand, an increase in rat uterine tumors may have been caused, in part, by an AHR mediated mechanism involving dysregulation of prolactin synthesis in the pituitary (Moran et al., 2012). Until now, prolactin (dys)regulation in rat pituitary via an AHR mediated mechanism has not extensively been studied *in vitro*.

In this study, we evaluated prolactin regulation in two well-known rat pituitary cell lines, GH3 and RC-4BC, as well as in rat primary pituitary cells. Effects on prolactin regulation in these three rat *in vitro* systems were initially studied using TRH, a natural prolactin stimulus, and quinpirole, a synthetic D2R agonist and inhibitor of prolactin secretion. Next, the effect of AHR activation using TCDD and DELAQ, the active N-deethylated minor metabolite of the pharmaceutical laquinimod, was studied on prolactin secretion and gene expression in these rat *in vitro* systems. Our studies show clear differences in prolactin responses between the three rat *in vitro* systems used as well as between TCDD and DELAQ.

2. Methods

2.1. Primary tissue and isolation

Primary pituitary tissue was obtained from naïve Wistar and Sprague Dawley female rats (age 5 weeks to one year) offered as surplus animal at the animal facility at Utrecht University, the Netherlands. The animals did not receive any prior form of treatment or experimental handling. The pituitary cells were isolated from the tissue as described in Solak, 2015 with minor modifications (Solak, 2015). In short, the pituitaries were minced and incubated for 45 min at 37 °C in a mixture of 0.5% collagenase type I (Sigma) and 0.05% DNAse type I (Sigma). Supernatant was collected and the tissue was incubated for 15 min at 37 °C once more in a mixture of 0.25% trypsin (Life technologies). Supernatant was collected, pooled with the former supernatant fraction and flushed over a 70 µm filter. The filtrate was centrifuged and the pellet was resuspended in culture medium consisting of DMEM-F12 medium (Life technologies) with 10% dextran charcoal treated fetal bovine serum (FBS; GE Health Care HyClone) and 1% penicillin/streptomycin (Life technologies, 1000 U/ml). After isolation, the pituitary cells were directly seeded in a 12-well plate coated with 50 mg/l poly-l-lysine (Sigma, poly-l-lysine hydrobromide). Cells were maintained in an incubator at 37 °C in 5% CO_2. The isolated pituitary cells from 6 rats were used to seed one 12-well plate, typically at a density of $4*10^5$ cells/well. No differences in basal prolactin secretion or gene expression were observed between the two rat strains. As such, both Wistar and Sprague Dawley rats were used for individual experiments.

2.2. Cell line culture

The rat pituitary cell lines GH3 and RC-4BC used in this study were obtained from ATCC. GH3 cells were cultured on F–12K medium (Invitrogen) supplemented with 2.5% FBS (Life technologies), 15% horse serum (Sigma) and 1% penicillin/streptomycin. For the experiments, cells were grown on medium that was supplemented with 17.5% dextran charcoal treated FBS. RC-4BC cells were cultured in DMEM:αMEM 1:1 (Life technologies) with 10% heat inactivated and dialyzed FBS (HyClone), 15 mM HEPES (Life technologies), 0.01 mM nonessential amino acids (Life technologies), 0.2 mg/ml bovine serum albumin (Sigma), 2.5 ng/ml EGF (Sigma) and 1% penicillin/streptomycin. Cells were maintained in an incubator at 37 °C in 5% CO_2.

2.3. Experimental conditions

The pituitary cell lines GH3 and RC-4BC were grown on a 12-well plate at a cell density of $2.5*10^5$ cells/well . Primary pituitary cells were seeded at a cell density of $4*10^5$ cells/well. Optimal seeding density was determined for each cell line as well as the primary pituitary cells, and largely depended on the capacity of the cells to form a confluent monolayer 48 h after seeding. All experiments were performed in triplicate. After 48 h, the cells were exposed to TRH (Sigma), quinpirole (Sigma, quinpirole hydrochloride), TCDD (Wellington Laboratories Inc.), DELAQ (Teva Pharmaceutical Industries Ltd.), and AHR antagonist CH223191 (Merck, Calbiochem CAS 301326-22-7) either alone or in combination. After a 48h exposure, the culture media were collected and RNA Instapure (Eurogentec) was added to the cells to preserve the RNA. Both media and cells were stored at −80 °C until further processing.

2.4. Enzyme-linked immuno sorbent assay (ELISA)

Rat prolactin ELISA Kit (Sigma) was used to assess the concentration of prolactin in the supernatant. The appropriate dilutions of the experimental samples were assessed before analysis of the prolactin levels. The ELISAs were preformed according to the manufactures' instructions. These experiments were performed in duplicate.

2.5. Gene expression analysis

Total RNA was isolated using the phenol-chloroform extraction method (van Duursen et al., 2005). Purity and concentration of the isolated RNA were determined spectrophotometrically at an absorbance wavelength of 260 and 280 nm. Complementary DNA (cDNA) was synthesized using the Script cDNA Synthesis Kit (Bio-Rad), according to the manufacturer's instructions. The obtained cDNA was diluted 10 times and stored at 4 °C until further analysis. Quantitative reverse transcriptase-polymerase chain reaction (RT-qPCR) was performed in a blue 96-well hard-shell PCR plate using the CFX96 cycler (BioRad). A PCR mastermix was made containing IQ SYBR Green Supermix (BioRad), forward and reverse primers (concentration of 10 µM) and RNAse-free water per sample. Oligonucleotide sequences of the used primers are: Arbp 5′ CCTAGAGGGTGTCCGCAATGTG 3′ CAGTGGGAA GGTGTAGTCAGTCTC, β-actin 5′ AGCGTGGCTACAGCTTCACC 3′ AGT CTAGGGCAACATAGCACAGC, Prolactin 5′ CATCAATGACTGCCC CACTTC 3′ CCAAACTGAGGATCAGGTTCAAA, CYP1A1 5′ ATGTCCAG CTCTCAGATGATAAGGTC 3′ ATCCCTGCCAATCACTGTGTCTAAC. All primers had a primer-specific annealing temperature of 60 °C. Gene expression was determined using the ΔΔCq method of relative quantification where gene expression was normalized to housekeeping genes Arbp and β-actin. These experiments were performed in triplicate.

2.6. Data analysis

Gene expression experiments were performed in triplicate and prolactin ELISAs in duplicate. Fig. 2: Experiments were performed N = 5 (ELISA) and N = 6 (gene expression) for TRH treatment and N = 2 for quinpirole treatment in the GH3 cell line, N = 2 in the RC-4BC cell line. Fig. 3: N = 3 for all treatments in primary cells and N = 3 for 0.1 and 1 nM DELAQ, N = 2 for 10 nM DELAQ and N = 1 for 0.01 nM DELAQ in GH3 cells. Fig. 4: N = 2 for prolactin secretion and N = 3 for gene expression. Fig. 5: N = 3. Standard deviation (SD) values of the control groups were calculated by a group SD of the individual control values relative to their experimental group mean. Unpaired t-tests were used to analyze the statistical difference of the mean between the exposed and vehicle control treatments. Symbol meaning; * = P ≤ 0.05, ** = P ≤ 0.01, *** = P ≤ 0.001.

3. Results

3.1. Prolactin regulation in rat primary pituitary cells, GH3 and RC-4BC cell lines

Basal prolactin secretion and gene expression were assessed in primary pituitary cells obtained from female rats and in the commercially available rat pituitary cell lines, GH3 and RC-4BC. In addition, the cellular responses to the prolactin stimulant TRH and prolactin inhibitor quinpirole, a synthetic D2R agonist were assessed. Prolactin secretion can be modulated *in vitro* by TRH and quinpirole as described in the literature (Cheng et al., 1992; Niimi et al., 1993; Missale et al., 1996). In the primary pituitary cells, basal prolactin secretion was 454 ± 160 ng/ml/48 h. TRH increased prolactin secretion by 1.2-fold and statistically significantly increased prolactin gene expression by 1.3-fold in the primary pituitary cells compared to vehicle treated control cells (Fig. 2A). Quinpirole statistically significantly inhibited prolactin secretion by 2.6-fold and prolactin gene expression by 3.6-fold

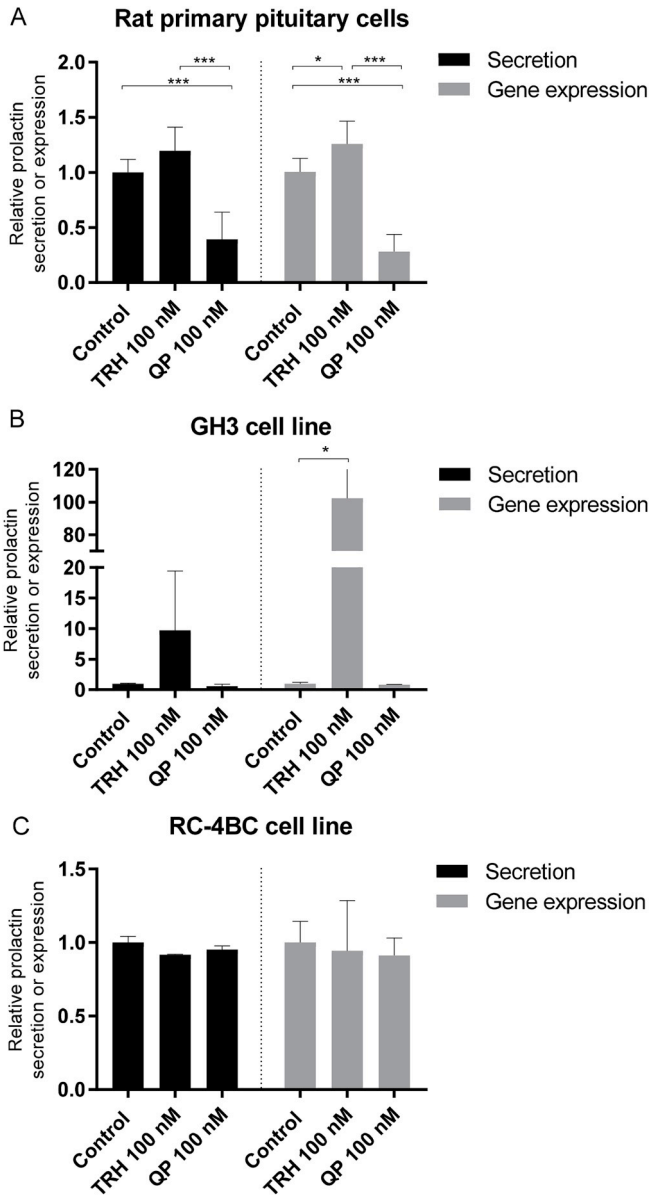

Fig. 2. Prolactin secretion and gene expression in rat (A) primary pituitary cells, (B) GH3 and (C) RC-4BC cell lines. The cells were exposed to a vehicle control (0.1% DMSO), 100 nM TRH, or 100 nM quinpirole (QP) for 48h. Data is expressed as mean fold change (with SD) relative to the vehicle-treated control cells for every individual experiment. *Statistically significantly different with (*) P ≤ 0.05 and (***) P ≤ 0.001.

in the primary pituitary cells compared to vehicle treated control cells (Fig. 2A).

Basal prolactin secretion was much lower in both the GH3 and RC-4BC cell lines compared with the primary rat pituitary cells. In GH3 cells, basal prolactin secretion was approximately 8 ± 8 ng/ml/48 h. TRH increased prolactin secretion on average by 9.7-fold and prolactin gene expression on average by 102-fold compared with vehicle treated control cells (Fig. 2B). In contrast to the primary pituitary cells, no inhibitory effects on prolactin secretion or gene expression were observed after treatment with quinpirole alone (Fig. 2B) or in combination with TRH after 24 or 48 h (data not shown). Basal prolactin secretion in RC-4BC cells was approximately 137 ± 27 ng/ml/48 h, significantly higher than prolactin secretion in GH3 cells, but lower than in primary pituitary cells. Furthermore, both TRH and quinpirole had no significant stimulatory or inhibitory effects on prolactin secretion or gene

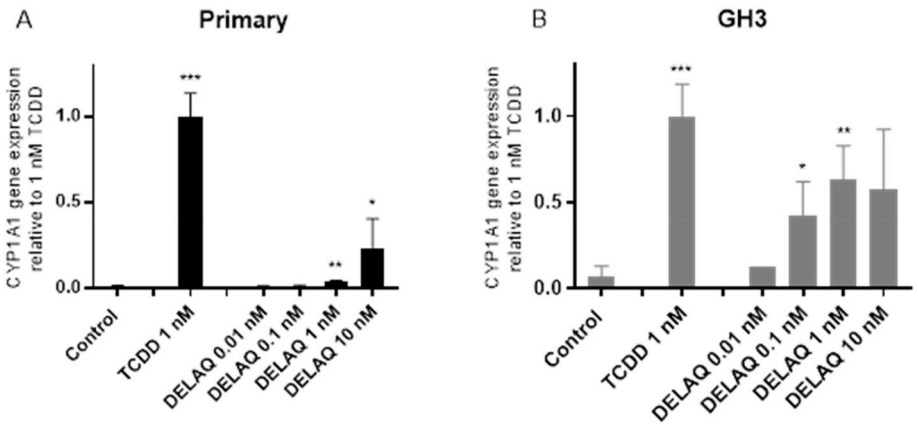

Fig. 3. CYP1A1 gene expression in rat (A) pituitary primary cells (B) GH3 cells. Data is expressed as mean fold change (with SD) relative to 1 nM TCDD treated cells. *Statistically significantly different from control with (*) P ≤ 0.05, (**) P ≤ 0.01 and (***) P ≤ 0.001.

expression in the RC-4BC cells (Fig. 2C).

3.2. AHR activation and prolactin regulation in rat pituitary cells

AHR-mediated dysregulation of pituitary prolactin secretion has been suggested to contribute to uterine tumorigenesis in the rat. Therefore, AHR activation by TCDD and DELAQ was determined in rat pituitary cells by assessing cytochrome P450 1A1 (CYP1A1) gene expression. No statistically significant effects of TCDD (1 nM) or DELAQ (10 nM) on CYP1A1 gene expression were observed in RC-4BC cells (data not shown). As these cells also did not respond to prolactin inhibition or stimulation, no further experiments were performed with this cell line. In contrast, TCDD (1 nM) caused an 81.8 (± 28.8)-fold and 22.7 (± 14.6)-fold induction of CYP1A1 gene expression in primary rat pituitary cells and GH3 cells, respectively. In both cell models, TCDD showed a higher efficacy than DELAQ in inducing CYP1A1 gene expression, but this was more apparent in the primary rat pituitary cells. The relative efficacy of 1 nM TCDD to induce CYP1A1 gene expression compared to DELAQ was approximately 25 and 1.7 in primary rat pituitary cells and GH3 cells, respectively (Fig. 3).

Next, the effects of AHR activation on pituitary prolactin were assessed. Up to 10 nM TCDD and DELAQ had no effect on basal prolactin secretion and gene expression in primary rat pituitary cells and the GH3 cells (data not shown). Constitutive prolactin secretion in primary

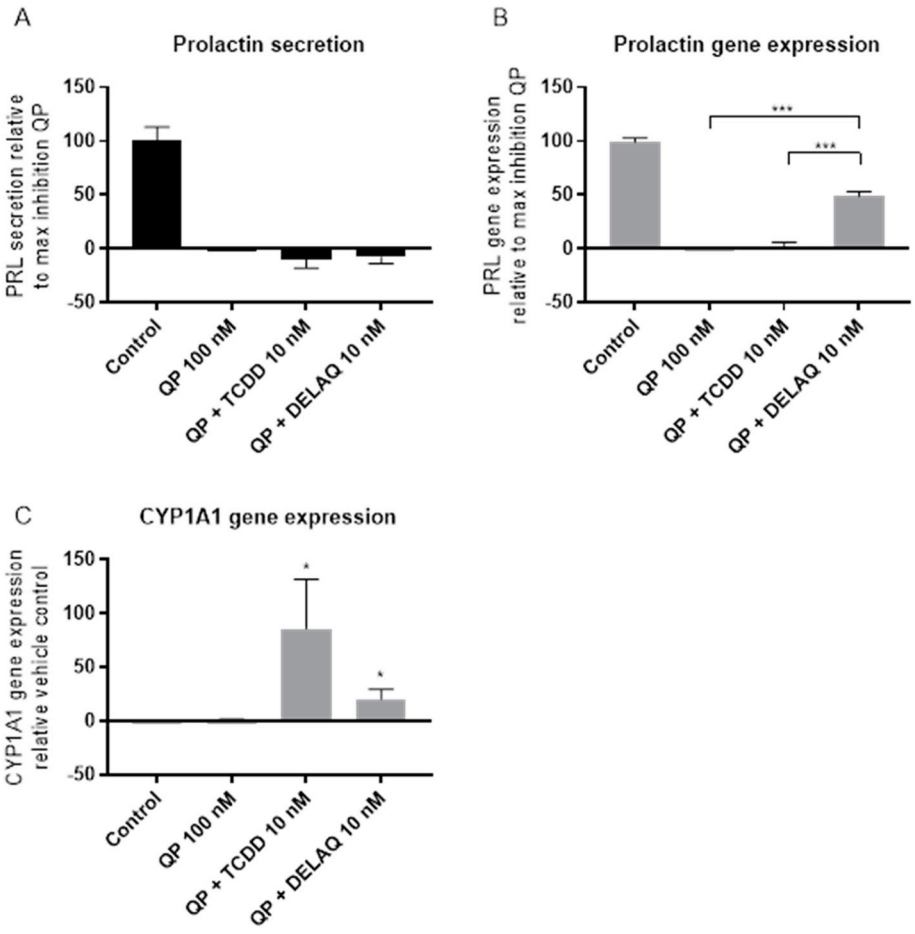

Fig. 4. (A) Prolactin secretion and (B) prolactin gene expression in primary rat pituitary cells after a 48h exposure to 100 nM quinpirole (QP) alone or in combination with 10 nM TCDD or 10 nM DELAQ. Data (A + B) is expressed as percentage secretion or expression, where vehicle-treated control cells (0.1% DMSO) is set to 100% and maximal inhibition by 100 nM quinpirole is set to 1%, or data (C) is expressed relative to vehicle-treated (0.1% DMSO) control cells. Statistically significantly different with (*) P ≤ 0.05 and (***) P ≤ 0.001.

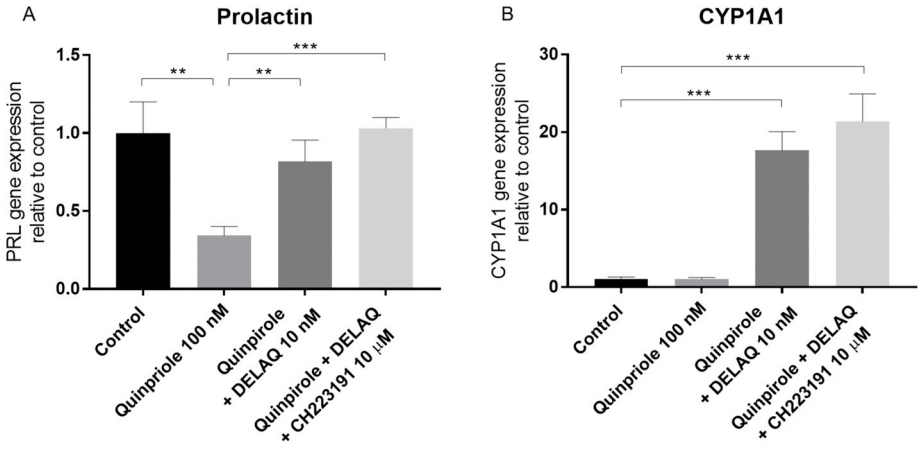

Fig. 5. Fold induction in prolactin and CYP1A1 gene expression in primary pituitary cells after a 48h treatment with 100 nM quinpirole, 100 nM quinpirole +10 nM DELAQ, 100 nM quinpirole +10 nM DELAQ +10 μM AHR antagonist CH223191. Data is expressed relative to the vehicle control (0.1% DMSO) treated cells. Statistically significantly different with (**) $P \leq 0.01$ or (***) $P \leq 0.001$.

pituitary cells is already high and could not be stimulated further with TRH (Fig. 2). As a consequence, possible stimulatory effects of AHR activation on prolactin secretion may not be detected. Therefore, the effect of AHR activation was further assessed in primary pituitary cells where basal prolactin secretion was inhibited with quinpirole. No difference in prolactin secretion was observed between primary pituitary cultures treated with quinpirole alone or in combination with 10 nM TCDD or DELAQ (Fig. 4A). However, inhibition of prolactin gene expression by quinpirole was partly negated when primary pituitary cells were co-exposed to 100 nM quinpirole and 10 nM DELAQ. Quinpirole alone inhibited prolactin gene expression by 76% compared with vehicle-treated control cells, whereas co-exposure of quinpirole and DELAQ caused a 39% inhibition of prolactin gene expression compared with vehicle-treated control cells. In other words, DELAQ attenuated the decrease in prolactin gene expression by quinpirole by 51% (Fig. 4B). In contrast to DELAQ, co-exposure with TCDD had no effect on quinpirole inhibition of prolactin gene expression.

Considering the difference between the effects of TCDD and DELAQ on quinpirole-mediated inhibition of prolactin gene expression, the role of AHR activation was studied further. CYP1A1 gene expression was determined to assess AHR activation under the experimental conditions in which inhibition of prolactin gene expression was observed in rat primary pituitary cells. 100 nM quinpirole alone did not statistically significantly affect CYP1A1 gene expression compared to the vehicle-treated control cells (Fig. 4C). The induction of CYP1A1 by TCDD and DELAQ was also not affected by co-exposure to quinpirole (data not shown). As the induction of CYP1A1 was higher by TCDD when compared to DELAQ, this suggests that the effect of DELAQ on quinpirole-inhibited prolactin gene expression (Fig. 4) may be AHR-independent. To study this further, the effect of DELAQ on the inhibitory properties of quinpirole was also assessed in combination with the AHR antagonist CH223191. Both prolactin and CYP1A1 gene expression did not statistically significantly change in the quinpirole + DELAQ + CH223191 treatment, compared to the quinpirole + DELAQ treatment (Fig. 5A and B).

4. Discussion

In rats, but not in humans, modulated pituitary prolactin levels can cause an altered estrogen:progesterone ratio and consequently lead to increased risk for uterine tumors. Our study shows that prolactin regulation in rat primary pituitary cells *in vitro* is distinctly different from that in the commercially available rat pituitary cell lines, GH3 and RC-4BC. In rat primary pituitary cells, the AHR ligands TCDD and DELAQ displayed ligand-specific effects on CYP1A1 gene expression, a marker for AHR activation. Moreover, prolactin modulation in primary pituitary cells was only observed for DELAQ and only when prolactin regulation was inhibited. This effect appears to be AHR-independent. Considering that uterine tumors are relatively common in carcinogenicity and toxicity studies using rats, these *in vitro* findings are important to correctly assess the potential of pharmaceuticals and chemical substances to modulate prolactin secretion and expression in rat pituitary and for translational risk assessment.

In order to evaluate which pituitary cells are an appropriate model to study prolactin modulation *in vitro*, responses in rat primary pituitary cells were compared with those in commercially available rat cell lines, GH3 and RC-4BC. Basal prolactin secretion was relatively high in primary pituitary cells, which is in line with the *in vivo* situation in rats where constitutive prolactin secretion is high and is predominantly modulated via the inhibitory dopamine regulating system (Stojilkovic et al., 2010). In line with this, TRH only moderately stimulated prolactin secretion and gene expression, while inhibiting the dopamine receptor D2R using the synthetic inhibitor quinpirole strongly inhibited prolactin secretion and gene expression in primary pituitary cells. On the contrary, basal prolactin secretion and gene expression was low in the GH3 cells and consequently was greatly induced by TRH. The GH3 cell line is derived from a pituitary tumor from a female rat and is often used in studies on rat pituitary cell function as it has been reported that these cells secrete prolactin (Gershengorn et al., 1981). Others also reported that TRH stimulates prolactin secretion and gene expression in GH3 cells, but only up to levels that are an order of magnitude lower than in our experiments (Kanasaki et al., 2002; Yajima and Saito, 1983). The serum-free experimental conditions in our study may have contributed to the high induction of TRH-stimulated prolactin release. Cheng et al. reported that GH3 cells cultured in serum-free media for 4 days produced less prolactin but are approximately 2.5-fold more stimulated by TRH to produce prolactin than GH3 cells that were cultured in serum-supplemented medium (Brunet et al., 1981). In addition, we observed that the TRH responsiveness of the GH3 cells decreased with increasing passaging of the cells, which has also been reported by Brunet et al. (1977). This accounts for the large standard deviation in the TRH treated cells in both the prolactin secretion and the gene expression experiments. Quinpirole did not inhibit prolactin secretion nor expression in GH3 cells in our experiments. This is in line with observations that GH3 cells lack a functional D2R and prolactin secretion is therefore resistant to the inhibition by quinpirole (Fischberg and Bancroft, 1995). A few studies reported that a functional D2R can be re-introduced in the GH3 cells by culturing the cells in the presence of EGF for several days (Missale et al., 1991). However, in our lab, culturing GH3 cells with EGF according to the methods used by Missale and colleagues did not result in an inhibition in prolactin secretion nor gene expression by quinpirole (data not shown). Basal prolactin secretion and gene expression in RC-4BC cells was lower than in the primary pituitary cells but higher than in GH3 cells. However, prolactin secretion and gene expression could not be stimulated nor inhibited in RC-4BC cells. The RC-4BC cell line is derived from a pituitary tumor from a

male rat and is known to secrete prolactin (Polkowska et al., 1991). However, the effects of TRH and D2R modulation on prolactin secretion in this cell line have not been described extensively in the literature. Based on these data, the use of GH3 and RC-4BC cell lines seem inappropriate to study pituitary prolactin modulation by exogenous compounds *in vitro*. This does however not exclude the applicability of these cell lines for other applications than those investigated in this study, for example oxytocin or growth hormone (GH) responsiveness.

Another difference between the primary pituitary cells and the cell lines is the potency of AHR ligands to activate the AHR. AHR activation was demonstrated in primary pituitary cells and GH3 cells by CYP1A1 gene expression, the hallmark for AHR activation, and using known AHR ligands TCDD and DELAQ. No statistically significant effects on AHR activation were observed in RC-4BC under the same experimental conditions. However, at 24 h after exposure, an equivocal induction of CYP1A1 gene expression was observed after stimulation by the highest concentration of DELAQ and TCDD (data not shown). The primary pituitary cells were more susceptible for TCDD-induced CYP1A1 gene expression than GH3 cells (and RC-4BC cells). Interestingly, a clear ligand-specific difference was found for AHR activation. At 1 nM, TCDD induced CYP1A1 mRNA 25-fold more than 1 nM DELAQ CYP1A1 in the primary cells, compared to only 1.7-fold in the GH3 cells. This may be attributed to the ligand promiscuity of the AHR, which has been discussed by several reviews. The AHR can be activated by an array of compounds like halogenated aromatic hydrocarbons, polyaromatic hydrocarbons, phytochemicals and endogenous compounds which can result in subsequent ligand-specific effects (Safe et al., 2017; Denison et al., 2011). It is believed that structurally different AHR activators and inhibitors (such as CH223191) can induce significantly different gene expression patterns due to *e.g.* flexible ligand binding domains (LBD) on the AHR, differential AHR heterodimerization complexes and recruitment of different co-factors (Denison and Faber, 2017).

AHR activation is often suggested to play a role in the occurrence of uterine tumors in rat studies via modulation of prolactin secretion. Despite the clear AHR activation by TCDD and DELAQ, no effect of these compounds on prolactin secretion or gene expression in the primary pituitary cells and GH3 cells was found in this study. In contrast, AHR activation by beta-napthoflavone reportedly suppressed prolactin gene expression in GH3 cells (Moran et al., 2012), but this difference with our findings may again be a result of ligand-specific actions on the AHR. Similarly to our findings, Cao and co-workers also reported no effect of AHR activation by TCDD on prolactin gene expression in GH3 cells (Cao et al., 2011). Yet, Russell et al. found that treatment with TCDD caused a significant decrease of serum prolactin concentrations in an *in vivo* study with rats (Russell et al., 1988). The researchers showed that the effect of TCDD could be reversed by pimozide, which is a serotonin receptor and dopamine receptor antagonist. This suggests that the decrease of prolactin levels by TCDD is the result of a direct effect on the dopamine receptor in the pituitary gland, or, an indirect effect on hypothalamic dopamine release via modulation of the negative feedback loop by estradiol. TCDD may have reduced estradiol levels via an inhibitory AHR:ER cross-talk in the ovaries (Safe and Wormke, 2003), leading to an increase in hypothalamic dopamine release and subsequent stronger inhibition and thus lower prolactin production in the pituitary (Fig. 1). The lack of TCDD effects in primary pituitary cells in this study support an indirect mechanism, rather than a direct effect of TCDD on pituitary prolactin. Interestingly, the AHR agonist DELAQ did not affect prolactin secretion or gene expression, but attenuated D2R-mediated inhibition of prolactin gene expression in the primary pituitary cells in our study. An attenuation of the inhibition on prolactin protein levels were however not observed. Possibly, a different experimental set-up would allow to pick this up at a different time point. These results appear to be AHR-independent, as such effect was not seen with TCDD despite the higher magnitude of AHR activation and the effect could not be antagonized by adding AHR antagonist CH223191. Zhao et al. reported on the selectively of this inhibitor to TCDD-specific action compared to other ligands, due to differential ligand binding (Zhao et al., 2010). This would explain the lack of effects of this inhibitor on CYP1A1 gene expression when cells were co-exposed to DELAQ. An interaction of DELAQ with dopamine signaling, possibly via a negative feedback through estradiol, and prolactin regulation cannot be excluded and may explain, in part, the mechanism underlying the development of uterine tumors that was observed in the 2-year rat study with laquinimod (Kaye et al., 2016). However, no decrease in implantation rate or impaired lactation, two sensitive hallmarks of hypoprolactinemia, were observed with laquinimod in rats. It is relevant to note that non-AHR ligands like the flame retardant tetrabromobisphenol A (TBBPA) and the dopamine agonist pharmaceutical bromocriptine are also associated with the development of uterine tumors in rats via prolactin modulation and disruption of the negative feedback loop in the hypothalamus-pituitary-gonadal (HPG) axis (Brott et al., 2014; Lai et al., 2015). In line with the species-specific role of prolactin in rats, there is no evidence of adverse effects, including carcinogenicity, of treatment with TBBPA and bromocriptine on the endometrium in humans (Lai et al., 2015, Well, 1986). Alternative hypotheses on pituitary prolactin regulation suggest that other factors, like glucocorticoids, are involved in the regulation of prolactin secretion as well (Brann et al., 1990, López-Fontana et al., 2011). In addition, in contrast to cell lines, primary pituitary cell cultures consist of several cell types from the anterior and posterior parts of the pituitary. It is suggested that there may be a coordinated mechanism for the different pituitary hormones as *e.g.* oxytocin can control prolactin as well as growth hormone (GH) and luteinizing hormone (LH) release (Gonzalez-Iglesias et al., 2015). We also observed an inhibitory effect of quinpirole on GH mRNA expression in primary pituitary cells in our experiments (data not shown). This would suggest that the dopamine receptor also controls the somatotropic pituitary cells. No evidence of direct cellular interaction between these cell types and prolactin modulation has been found in the literature, although it cannot be excluded.

5. Conclusion

Clear differences between rats and humans in the role of prolactin in the induction of uterine tumors warrant mechanistic studies to clarify the interactions between pituitary prolactin release in carcinogenicity and (pre-clinical) toxicity studies and uterine tumors. AHR ligands may interact with rat pituitary prolactin release, but this appears to depend on the ligand and constitutive prolactin secretion. Our data shows that studying effects on rat pituitary prolactin *in vitro* should best be performed using rat primary pituitary cells. However, interpretation of the *in vitro* results with respect to occurrence of uterine tumors in rats should take the complex regulation of prolactin release in the pituitary into account as well as the HPG axis and its feedback loops.

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Declaration of interest

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